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MECHANISMS OF PATHOGENESIS

The presence of a galactosamine substituent on the arabinogalactan of *Mycobacterium tuberculosis* abrogates full maturation of human peripheral blood monocyte-derived dendritic cells and increases secretion of IL-10

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SUMMARY

Slow-growing and pathogenic *Mycobacterium* spp. are characterized by the presence of galactosamine (GalN) that modifies the interior branched arabinosyl residues of the arabinogalactan (AG) that is a major heteropolysaccharide cell wall component. The availability of null mutants of the polyprenyl-phospho-*N*-acetylgalactosaminyl synthase (Rv3631, PpgS) and the (*N*-acetyl-) galactosaminyl transferase (Rv3779) of *Mycobacterium tuberculosis* (*Mtb*) has provided a means to elucidate the role of the GalN substituent of AG in terms of host–pathogen interactions. Comparisons of treating human peripheral blood monocyte-derived dendritic cells (hPMC-DCs) with wild-type, *Rv3631* and *Rv3779* mutant strains of *Mtb* revealed increased expression of DC maturation markers, decreased affinity for a soluble DC-SIGN probe, reduced IL-10 secretion and increased TLR-2-mediated NF-κB activation among GalN-deficient *Mtb* strains compared to GalN-producing strains. Analysis of surface expression of a panel of defined or putative DC-SIGN ligands on both WT strains or either *Rv3631* or *Rv3779* mutant did not show significant differences suggesting that the role of the GalN substituent of AG may be to modulate access of the bacilli to immunologically-relevant receptor domains on DCs or contribute to higher ordered pathogen associated molecular pattern (PAMP)/pattern recognition receptor (PRR) interactions rather than the GalN-AG components having a direct immunological effect *per se*.

1. Introduction

Tuberculosis is a major global cause of death. It is estimated that one third of the world's population is infected with *Mtb* causing approximately 1.4 million deaths per year. The primary host target for *Mtb* is the human macrophage with survival of the bacterium within phagosomes. Innate immunity is implemented by the activated macrophage and is the initial and primary response against newly acquired *Mtb*. However it is the subsequent recruitment of T cells by host dendritic cells (DCs) that determines quality and extent of cell-mediated adaptive immunity. This development of an adaptive immune response ultimately provides protection against mycobacterial infections and/or restricts its dissemination within the host. Immature DCs are present throughout peripheral tissue in effect acting a sentinels guarding against pathogens that bode harmful intent to the host. DCs are among the first host cells that are encountered by *Mtb* and their precise activation and differentiation occurs through a vast network of receptors (termed pattern recognition receptors (PRR)) that engage with cognate ligands

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expressed on *Mtb* and other potential pathogens known collectively as pathogen-associated molecular patterns (PAMPs). The outcome of DC activation determines the nature of the adaptive immune responses (Th1, Th2, T_{reg}, Th17 etc.) and is determined by signaling events arising from PRR/PAMP interactions. The crosstalk between these numerous signaling pathways ultimately determines the type of host immune response that is mounted against the pathogen [1]. The activated DCs resulting from a signature encounter with PAMPs translocate cytoplasmic MHC class II molecules to the cell surface for antigen presentation and up-regulate co-stimulatory molecules such as CD80, CD86 and CD40 ensuring activation of naïve T cells through antigen presentation and co-stimulation. Suboptimal DC activation results in lower co-stimulatory and MHC surface expression and can ultimately result in antigen-specific T cell anergy. Pathogens have evolved numerous mechanisms to thwart PRR/PAMP interactions in order to evade host immunity and allow for establishment of infection.

The cell envelope of *Mtb* is a complex structure comprising an inner membrane, a cell wall core composed of three-covalently-bound macromolecules (peptidoglycan, arabinogalactan (AG) and mycolic acids), an outer membrane (or mycomembrane) and a loosely attached polysaccharide and protein-containing capsule-like structure [2]. Various non-covalently bound lipids, glycolipids and lipoglycans, in particular phosphatidyl-*myo*-inositol mannosides (PIMs), lipomannan (LM) and lipoarabinomannan (LAM) populate the inner and outer membranes [2]. This collective assemblage of glycolipids, polysaccharides and lipoglycans exhibit a broad range of immunomodulatory activities that are implicated in the pathogenesis of tuberculosis [3–6].

Galactosamine (GalN) is a minor covalently bound amino sugar component of *Mtb* AG that substitutes the C2 position of a portion of the internal 3, 5-branched D-Ara*f* residues in this molecule [7,8]. The GalN residue has been estimated to occur at approximately one residue per entire AG molecule [7–9]. Most intriguingly, a similar sugar residue has been observed to modify the AG of slow-growing pathogenic/opportunistic mycobacteria such as *Mycobacterium avium*, *Mycobacterium kansasii*, *Mycobacterium bovis*, and *Mycobacterium leprae* [10] but not that of *Mycobacterium smegmatis*, *Mycobacterium neoaurum* and *Mycobacterium phlei* [9] [7,8] suggestive of the notion that the AG of opportunistic fast-growing *Mycobacterium* spp. may be devoid of the GalN substituent. This begs several questions as to the significance of the GalN motif on AG of slow growing mycobacteria: Does the GalN motif provide any adaptive advantage to the mycobacteria? Does the GalN motif contribute to the tendency toward pathogenicity? If so, does the GalN substituent in the cell wall of slow-growing pathogenic mycobacteria confer any immune evasive tactics?

We recently reported on the discovery of the biosynthetic pathway for the galactosaminylation of AG [11]. Disruption of the genes encoding either the polyprenyl-phospho-*N*-acetylgalactosaminyl synthase, Rv3631 or the polyprenyl-phospho-(*N*-acetyl) galactosaminyl-dependent glycosyltransferase Rv3779 which ultimately transfers the GalN (or *N*-acetyl-GalN) residue to AG resulted in *Mtb* H37Rv mutants devoid of GalN substituent on AG. The availability of *Mtb Rv3631* and *Rv3779* knockout mutants has provided the unique opportunity to explore and elucidate the function(s) of the GalN substituent of AG as it pertains to host immune responses. Specifically, we sought to test the idea that this motif may, in some manner, either directly or indirectly (e.g., by altering the topology and/or composition of the bacterial surface) provide some sort of immune evasive tactic to *Mtb*. In this study, we compared the ability of wild-type (WT) vs. GalN-deficient *Mtb* strains to activate human peripheral blood-derived dendritic cells (hPBM-DCs) by measuring their ability to induce DC maturation markers. Since we were working in a human system, we chose to compare expression of maturation markers from hPBM-DCs derived from several blood donors in order to address heterogeneity issues that arise when examining out-bred human populations. In all donors screened, we show that when the GalN motif is present on the AG of *Mtb*, hPBM-DCs consistently fail to achieve full maturation over 36 h when compared to DCs treated with GalN-deficient strains. In addition, when analyzing patterns of cytokine secretion, DCs treated with WT *Mtb* strains producing galactosaminylated AG were stimulated to secrete significantly more IL-10 than their corresponding mutants. When probing intact bacilli with the soluble human chimeric PRR, hDC-SIGN, we observed that the WT strains bound more of the soluble probe than their GalN-deficient counterparts suggestive of a profound difference in either the amounts or accessibility of surface ligands to the DC-SIGN probe. Significant differences were also observed in the ability of WT vs. GalN-deficient strains to activate TLR2-mediated NF-κB translocation with the knock-out mutants showing significantly more activation than their WT counterparts. Collectively, these data suggest that the GalN motif on the AG of *Mtb* endows the bacillus with an immune evasive tactic and may contribute to virulence.

2. Material and methods

2.1. Bacterial strains and growth conditions

Mtb H37Rv ATCC 25618 (hereafter renamed H37Rv(a)), *Mtb* H37Rv TMC102 (hereafter renamed H37Rv(b)), their corresponding isogenic *Rv3136* and *Rv3779* knock-out mutants and complemented mutant strains were described elsewhere [11,12]. All *Mtb* strains were grown in either Sauton's, Middlebrook 7H9 supplemented with 10% OADC and 0.05% Tween 80 or Glycerol-Alanine-Salts (GAS) medium. *M. smegmatis* strain mc^2155 was grown in 7H9-OADC-Tween 80 or LB medium. When required, kanamycin (kan) and hygromycin (hyg) were added to final concentrations of 20 μg/ml and 50 μg/ml, respectively. Cultures were harvested at mid log phase ($O.D._{600nm}$ = 0.6–0.8) and washed 3 times with PBS. Cell pellets were suspended in PBS at 10^9 CFU/ml. To safely remove the bacteria from BSL3 containment in order to perform all of the experiments described herein, the suspended bacteria were gamma-irradiated in a JL Shepherd 31-14 machine using a 6000 Ci ^{137}Cs source for 1620 min (27 h) at 1543 rad/minute for a total dosage of 2.5 Mrad. Loss of viability was confirmed by AlamarBlue® testing. In some experiments, *Mtb* bacilli were killed by heat treatment at 90 °C for 40 min. *M. leprae* was harvested from either 9-banded armadillo tissues (liver or spleen or from *nu*/*nu* mouse foot pads and characterized and counted by the method of Shepard et al. [13]. Both the *M. smegmatis* and *M. leprae* were irradiated identically to the *Mtb* strains.

2.2. Antibodies

All monoclonal antibodies were obtained through BEI Resources, NIAID, NIH and were produced in cell culture using B cell hybridomas generated by fusion of myeloma cells with immunized mouse splenocytes. The following mouse monoclonal antibodies were used: anti-*Mtb* LAM, clone CS-35; Anti-*Mtb* 19 kDa (LpqH), clone IT-19; anti-*Mtb* DnaK, clone IT-40 (HAT1), clone α-Rv1411c and Anti-*Mtb* Mpt32 (45/47-kDa antigen or Apa), clone CS-93.

2.3. Procurement of human blood

Human whole blood was obtained from the Garth Englund Blood Center at Poudre Valley Hospital which is part of the University of Colorado Health System, Fort Collins, CO. Whole blood

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