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Communication

Lyme Disease, *Borrelia burgdorferi*, and Lipid Immunogens

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ABSTRACT: The human immune system detects potentially pathogenic microbes with receptors that respond to microbial metabolites. While the overall immune signaling pathway is known in considerable detail, the initial molecular signals, the microbially produced immunogens, for important diseases like Lyme disease (LD) are often not well-defined. The immunogens for LD are produced by the spirochete *Borrelia burgdorferi*, and a galactoglycerolipid (**1**) has been identified as a key trigger for the inflammatory immune response that characterizes LD. This report corrects the original structural assignment of **1** to **3**, a change of an α-galactopyranose to an α-galactofuranose headgroup. The seemingly small change has important implications for the diagnosis, prevention, and treatment of LD.

Addressing the diagnostic and therapeutic challenges of Lyme disease (LD), the most common vector-borne disease in the US, requires a better understanding of its etiology.[1−3] LD is characterized by dysregulated immune responses to bacteria introduced by tick vectors. Typically, a female tick (*Ixodes scapularis*) releases bacteria (*Borrelia burgdorferi*) from her saliva into the human host during a blood meal. LD initially presents as an inflammatory response, characteristically as a red ring (*erythema migrans*) around the meal site about a week after feeding. Other symptoms can include fever, headache, and fatigue.[3] Diagnosis is largely through symptoms, and while most cases respond favorably to antibiotic treatment, long-term autoimmune-like symptoms persist in a significant minority.[1,3]

LD's link to inflammatory immune responses led to a search for the responsible immunogen(s) from *B. burgdorferi* (Bb). Over the past several decades, two different classes of immunogens have been investigated: (1) lipid-bearing small proteins from the bacterial membrane called outer surface proteins (Osp) with OspA as the primary suspect, and (2) glycoglycerolipids with no associated protein and with BbGL-II (Bb glycolipid II, **1**) as the main suspect. The Osp family has been the basis of several vaccine efforts, and Lymerix, a recombinant OspA vaccine, was approved for humans in 1998 but withdrawn in 2002.[1,4] There are no human LD vaccines currently available, but efforts based on lipoproteins—mixtures of naturally occurring ones as well as engineered ones—are continuing.[1,4−6]

Studies on lipid immunogens from Bb have led to confusing results. A German lab specializing in diagnostic analysis of spirochete infections (LD and syphilis) regularly noted a low molecular weight antigen (<10 kDa) that reacted with sera from LD but not syphilis patients.[7] Further studies identified the antigen as a glycoglycerolipid with a galactose headgroup and two acyl chains.[7] This initial study was followed up by a more detailed study, which resulted in a complete structural characterization of the antigenic glycolipid as 1-oleoyl-2-palmitoyl-3-(α-D-galactosyl)-*sn*-glycerol (**1**), which entered the literature as BbGL-II (Figure 1).[8] For the past 20 years,

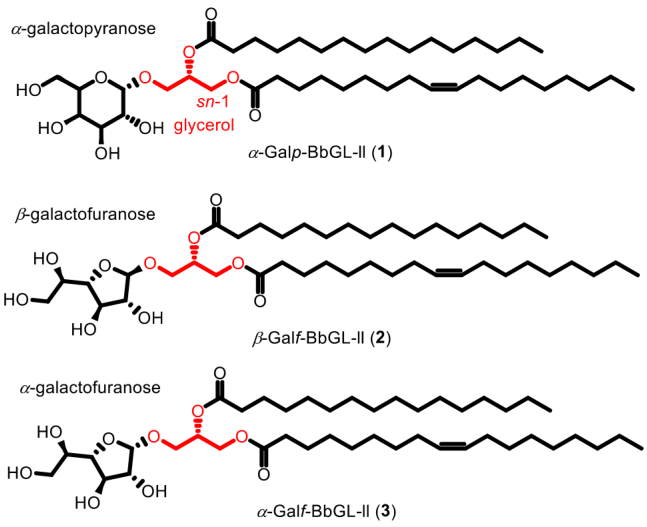

Figure 1. Structures of galactoglycerolipids (**1**−**3**).

BbGL-II has dominated research on lipid antigens for LD. Three studies are especially informative.[9−11] Kinjo et al. convincingly demonstrated the ability of glycolipids, including isolates from Bb to induce natural killer T (NKT) cell proliferation and cytokine production.[9] However, *in vivo* studies showed that synthetic **1** was no better than media

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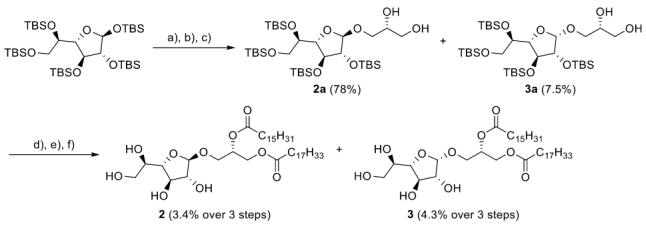
control at activating NKT cells.[9] Pozsgay et al. explored the synthesis of analogs of **1** and noted that synthetic **1** induced IL-2 production at levels "barely above baseline"—significantly less than some analogs.[11] Reinink et al. investigated the possibility that **1** needs an auxiliary protein (Cd1b, cluster of differentiation 1b) to present **1** to immune cells.[10] This study showed that while Cd1b did present **1** to human T cells and that the recognition required an alpha beta T cell receptor, **1** was not essential—immune responses with and without it were the same.[10] The authors concluded with the important insight that Cd1b's effects were likely due to its presentation of diacylglycerols that were self-antigens.[10]

These, and other, observations indicate that **1** is not the relevant Bb immunogen. Reviewing the original analysis suggested that the incorrect form had been assigned to the galactose headgroup. While human glycolipids typically feature galactopyranose, bacteria typically incorporate galactofuranose.[11−14] The synthase in Bb that adds galactose to a diacylglyceride, bbMGS, has been identified, but the analysis does not distinguish the form added.[15] We pursued two independent but complementary paths: synthesizing candidate galactoglycerolipids and identifying the immunogenic lipid from Bb cultures.

Our model could be tested by assaying three molecules: the original α-pyranose structure, α-Gal*p*-BbGL-II (**1**), along with two galactofuranoses, β-Gal*f*-BbGL-II (**2**) and α-Gal*f*-BbGL-II (**3**) (Figure 1). Compound **1** is commercially available, and the synthesis of **2** and **3** followed the standard route outlined in Scheme 1. To produce both anomers of the desired

Scheme 1. Synthesis of 2 and 3[a]



[a]Reagents and conditions: (a) TMSI, 4 Å mol. sieve, 15 min, 0 °C; (b) DIPEA, (*S*)-(+)-solketal, 3h, rt; (c) TFA/water/DCM, 10 min, rt (for β-anomer (**2a**): 78%, for α-anomer (**3a**): 7.5%); (d) Oleoyl chloride, 2,4,6-Collidine, DCM, 1h, −78 °C (for β-anomer (**2b**): 47%, for α-anomer (**3b**): 28%); (e) Palmitic acid, DCC, DMAP, DCM, rt, o/n (for β-anomer (**2c**): 52%, for α-anomer (**3c**): 85%); (f) TBAF, THF, rt, o/n, (for β-anomer (**2**): 14%, for α-anomer (**3**): 18%).

galactofuranoses in one reaction, we used the nonstereospecific TMSI promoted glycosylation of penta-TBS protected β-D-galactofuranose with (*S*)-(+)-solketal, followed by selective deprotection of the ketal, and chromatographic separation of the anomers to obtain compounds **2a** and **3a** (Scheme 1).[16,17] Regiospecific esterification at the *sn*-1 position of **2a** and **3a** with oleoyl chloride gave compounds **2b** and **3b** and an additional esterification at their *sn*-2 positions with palmitic acid via the Steglich procedure yielded compounds **2c** and **3c** (Scheme 1). Deprotection of the TBS groups with TBAF and extensive purification by NP- and RP-chromatography gave the final compounds β-Gal*f*-BbGL-II (**2**) and α-Gal*f*-BbGL-II (**3**) in four steps (Scheme 1). Distinguishing galactofuranose anomers **2** and **3** is accomplished by comparing the ^3J-coupling constant and shift of the anomeric H-1′ protons (Figure S19). For β-Gal*f*-BbGL-II (**2**), H-1′ appears at 5.02 ppm as a singlet[16] whereas H-1′ of α-Gal*f*-BbGL-II (**3**) appears at 4.93 ppm as a doublet (^3J = 4.8 Hz). Identification of the ring forms can be made, most reliably, by analyzing the ^{13}C-shift values of C-2′, C-3′, and C-4′ (Figure S20). In the galactopyranose form of α-Gal*p*-BbGL-II (**1**), all of these signals appear between 69.5 and 71.0 ppm; the same carbons resonate at significantly higher values of 76.2 ppm (C-3′), 78.1 ppm (C-2′), and 83.4 ppm (C-4′) for the galactofuranose α-Gal*f*-BbGL-II (**3**).

Compounds **1**−**3** were tested in an inflammatory assay: TNF-α release from murine bone marrow dendritic cells (mBMDC).[18] Results are shown in Figure 2a and 2b. Compounds **1** and **2** have no detectable activity while compound **3** with its α-galactofuranose headgroup had an EC_{50} of 15 μM.

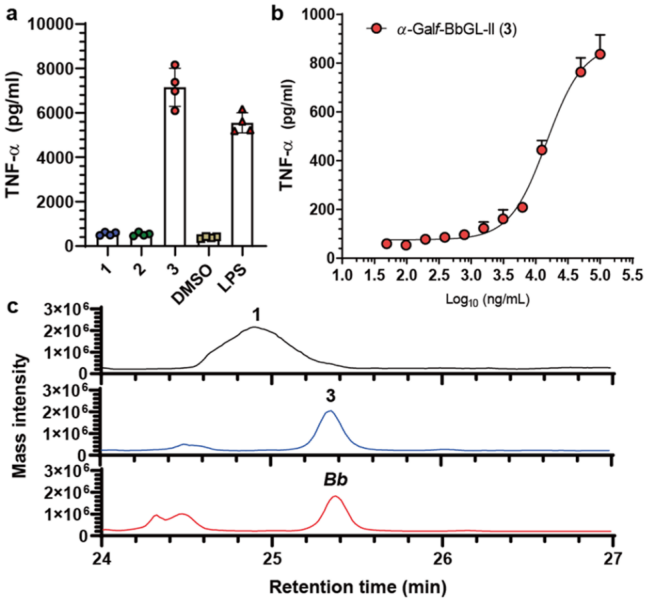


Figure 2. Proinflammatory activities and HPLC-MS chromatogram of **1**−**3**. (a) TNF-α induction activities of **1**−**3** (66 μM) in mBMDCs and (b) dose−response curve of **3**. No detectable activity from **1** and **2**. (c) HPLC-MS chromatogram of **1**, **3**, and active lipid fraction of *B. burgdorferi* (**Bb**). Extracted ion value was *m*/*z* 757.

As an independent check, we compared compounds **1**, **2**, and **3** with the active lipid fraction from Bb cultures. *Borrelia burgdorferi* B31 (ATCC 35210), a fastidious microaerobic Gram-negative, was cultured in BSK-H media, and the cell pellet extract ($CHCl_3$/MeOH) was partially purified. The active fraction (TNF-α production) was analyzed by HPLC-MS (Figure 2c). The Bb active extract had a large peak corresponding to **3**, while **1** and **2** were clearly different. The stark differences between **1**, **2**, and **3** in functional analyses contrast with the miniscule variations seen in spectroscopic analyses. For example, the ^{1}H NMR spectra of **1**, **2**, and **3** are similar enough that a *de novo* structure determination of any one of them would be challenging in the absence of authentic synthetic samples (Table 1, Supporting Information).[8]

While a plausible retaining galactosyl transferase by which Bb could install an α-galactose has been identified, no obvious candidates for the mutase needed to convert the thermodynamically favored galactopyranose to galactofuranose could be identified in the published Bb genome. A variation on the S_Ni transferase mechanism could provide a solution.[19,20] The

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mutase and transferase functions could be combined in a single enzyme using a bridged intermediate from a first inversion at C1 via attack by the C4 hydroxyl to displace the UDP leaving group, and a second inversion in which the *sn*-3 hydroxyl of a diacylglycerol displaces the C4 hydroxyl attached to C1 (Figure S30). The combination would convert the pyranose to a furanose with a retaining transfer.

The identification and synthesis of Bb's immunogenic lipid prompted an investigation into its receptor target. The toll-like receptors TLR2 and TLR4, members of the innate immune system, were the most likely.[21] These receptors are widely distributed on sentinel cells like macrophages and dendritic cells and recognize structurally conserved molecular features associated with microbes.[21] They link the innate immune system to the adaptive immune system through the release of cytokines like TNF-α. We distinguished which TLR was responsible by using mBMDCs from mice with genetic knockouts, *tlr*2$^{-/-}$ and *tlr*4$^{-/-}$ mice, in the cytokine induction assay (Figure 3).[18] Cells lacking TLR2 did not respond to **3**,

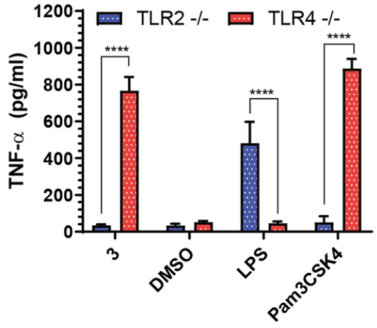

Figure 3. TNF-α production of **3** in mBMDC assay from *tlr*2$^{-/-}$ and *tlr*4$^{-/-}$ mice.

while those lacking TLR4 showed a robust response. TLR2 is the canonical receptor for di- and triacylpeptide ligands, so this is the expected result.[21−24]

The results presented here make a convincing case that α-Gal*f*-BbGL-II (**3**), not α-Gal*p*-BbGL-II (**1**), is the innate immunogen reported by earlier researchers. While the structural correction is minor, immune receptors like TLR2 are exquisitely sensitive to molecular structure. Bacterial lipids, especially membrane lipids, provide highly informative molecular ID tags of their producers.[25,26] Their distinctive structures and compositions reflect both the evolutionary history and current lifestyle of their producer.[24] In addition to their distinguishing characteristics, membrane lipids are both essential and readily accessible, especially in the case of Gram-negative bacteria like Bb that release outer membrane vesicles (OMV).[27,28] While their potential as immunogens is known, it is often overlooked.[22,29] The tendency to downgrade the importance of lipid immunogens, coupled with the mis-identification of **1** some 20 years ago, has led to a garbled understanding of LD etiology and misguided vaccine development efforts. Identifying **3** as the relevant immunogen could redirect prevention and therapeutic efforts for LD and possibly guide the search for self-antigens that might be responsible for post-treatment LD syndrome (PTLDS) and other tick-borne diseases.[2,4,6,10,30] Finally, this correction has potential implications for other systems in which bacterially produced galactolipids activate human immune responses.[31,32]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c12202.

Supplementary figures, NMR spectra for synthetic compounds, and detailed experimental method (PDF)

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Notes

The authors declare no competing financial interest.

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