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Communication

Revisiting Coley's Toxins: Immunogenic Cardiolipins from *Streptococcus pyogenes*

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ABSTRACT: Coley's toxins, an early and enigmatic form of cancer (immuno)therapy, were based on preparations of *Streptococcus pyogenes*. As part of a program to explore bacterial metabolites with immunomodulatory potential, *S. pyogenes* metabolites were assayed in a cell-based immune assay, and a single membrane lipid, 18:1/18:0/18:1/18:0 cardiolipin, was identified. Its activity was profiled in additional cellular assays, which showed it to be an agonist of a TLR2−TLR1 signaling pathway with a 6 μM EC_{50} and robust TNF-α induction. A synthetic analog with switched acyl chains had no measurable activity in immune assays. The identification of a single immunogenic cardiolipin with a restricted structure−activity profile has implications for immune regulation, cancer immunotherapy, and poststreptococcal autoimmune diseases.

Spontaneous regressions of cancer tumors, rare and seemingly miraculous events, have been known since ancient times.[1−4] They were often preceded by bacterial infections, especially erysipelas, a raised, tender, and bright red skin rash. This association led a German surgeon, Friedrich Fehleisen, to identify *Streptococcus pyogenes* as the causative agent of erysipelas in 1883.[1,2] A decade later, William Coley, a young New York surgeon, began a sustained, partially successful, and controversial program of using live and later dead bacteria to treat cancer patients. Coley originally injected live *S. pyogenes* into tumors, but he soon switched to killed bacteria and later added additional strains. His approach went from a promising treatment in the early 1900s to being doubted, dismissed, and ridiculed by the time of his death in 1936. Ultimately, the treatment was effectively banned by the FDA in 1963.[5] The development of cancer immunotherapy has resurrected both Coley's reputation (he has become "The Father of Cancer Immunotherapy") and interest in bacterial metabolites as therapeutically useful immunomodulators.[6−8] Efforts to recreate the original toxins have met with limited success.[5,9] Rather than trying to reconstruct Coley's toxins, we deconstructed them by identifying immunogens produced by *S. pyogenes* that could form the basis of a more informative approach to discovering the relation, if any, of *S. pyogenes* metabolites to immunoregulation.[10]

We used an assay that we had previously used to identify immunogens from *Ruminococcus gnavus* associated with Crohn's disease and from *Akkermansia muciniphila* associated with cancer immunotherapy, metabolic disease, and homeostatic immunity.[6,11−13] The assay measures induction of proinflammatory cytokines, typically TNF-α or IL-6, from murine bone-marrow-derived dendritic cells (mBMDCs) to identify immunogenic bacterial metabolites. The cell pellet and supernatant fractions enriched in extracellular vesicles from cultures of *Streptococcus pyogenes* (ATCC 700294) contained a single lipid component with significant immunomodulatory activity that is the subject of this paper.

A small culture of *S. pyogenes* was separated into a cell pellet and supernatant extracts (1:1 chloroform/methanol and ethyl acetate, respectively) with the activity overwhelmingly in the cell pellet (Figures 1a and S1). A larger (160 L) culture provided 4.4 g of cell pellet extract, which was subsequently fractionated with normal/reverse phase and size-exclusion chromatography to yield single active compound SpCL-1 (**1**) with robust TNF-α induction activity (Figure 1b and Figure S2). A dose−response curve for SpCL-1 indicated an EC_{50} of ∼6 μM, comparable to the values of other immunogenic metabolites in our assay (Figure 1c).[13,14]

High-resolution electrospray ionization mass spectrometry provided the molecular formula $C_{81}H_{154}O_{17}P_2$ ($[M - H]^-$ m/z 1460.0583, calcd 1460.0589). Initial ^1H and ^{13}C NMR analysis showed 3 glycerol fragments and 4 acyl chains, which combined with the molecular formula indicated a cardiolipin. Partial structures of SpCL-1 (**1**) based on 1D ^1H and ^{13}C NMR augmented with 2D (gCOSY, gHSQC, and gHMBC) NMR spectra (Table S1 and Figures S3−S8) revealed 4 carbonyl carbons, 9 oxygenated methine/methylene groups, 4 olefinic methine groups, 4 methyl groups, and multiple overlapped aliphatic methine groups along with all of the $^1H-^{13}C$ one bond correlations (Table S1). These moieties and the virtual symmetry seen in the spectra fit the canonical cardiolipin pattern (Figure 2).[15] The identities of the four acyl chains were determined by methanolysis-esterification, fol-

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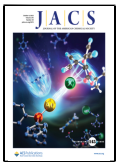

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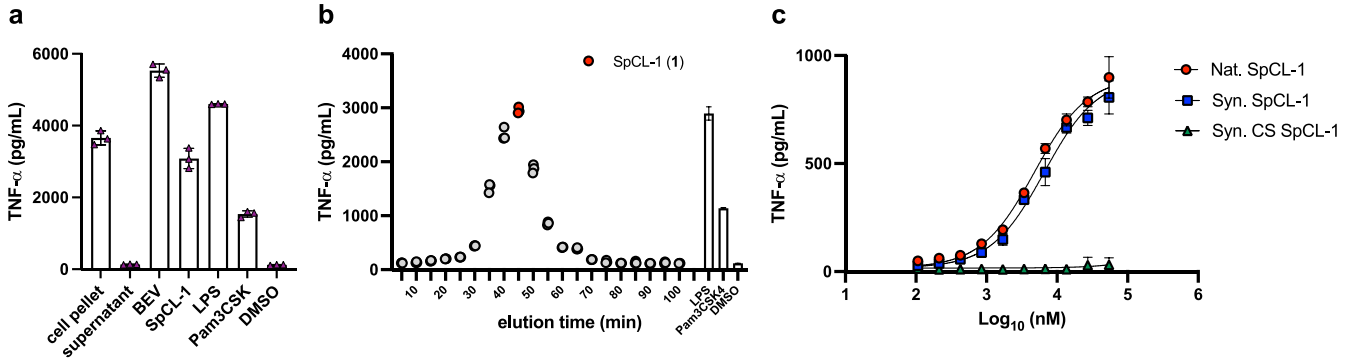

Figure 1. (a) TNF-α inducing activity of the cell pellet, supernatant, bacterial extracellular vesicles (BEV), and SpCL-1 from *S. pyogenes* ATCC 700294 in mBMDCs. (b) Induced TNF-α production of mBMDCs treated with *S. pyogenes* size-exclusion chromatography fractions. (c) Dose–response curves of TNF-α inducing activities of natural SpCL-1 (Nat. SpCL-1), synthetic SpCL-1 (Syn. SpCL-1), and synthetic chain-switched SpCL-1 (Syn. CS SpCL-1). Error bars = SD of technical replicates (n = 3 or 4). LPS, lipopolysaccharide (TLR4 ligand); Pam3CSK4, a synthetic triacylated lipopeptide (TLR2/TLR1 ligand).

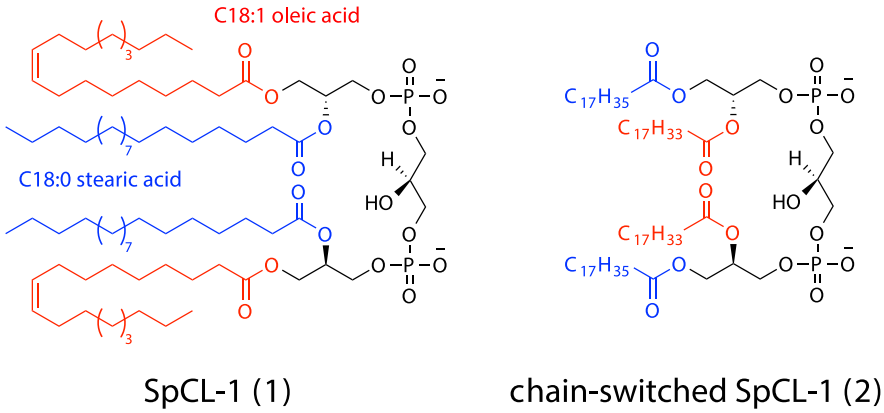

Figure 2. Structures of SpCL-1 (**1**) and its chain-switched analog (**2**).

lowed by gas chromatography–mass spectrometry (GC–MS) analysis (Figure S9). SpCL-1 (**1**) has two C18:0 and two C18:1 acyl chains, stearic and oleic acids, respectively (Figure 2). Acyl chain positions were originally determined by preferential *O*-deacylation of the *sn*-2 and *sn*-2′ positions followed by high resolution MS/MS analysis of the product (Figure S10). Based on these analyses, SpCL-1 is an 18:1/18:0/18:1/18:0 cardiolipin (Figure 2).

Cardiolipins (CL) like SpCL-1 (**1**) are found in the lipid membranes of both human and bacterial cells.[15] They are dimers in which phosphatidic acids are joined by a glycerol fragment, and typically the diacylglycerol (DAG) moieties are identical. CLs are pseudo symmetric dimers as their potential 2-fold symmetry is frustrated by the stereogenic center at the *sn*-2 position of the central glycerol.[16] Their V-shaped structure with the compact anionic phospholipid head group at the narrow end and the four acyl chains fanning out makes cardiolipins important contributors to concave surfaces like the inner leaflet of the inner mitochondrial membrane in human cells. In bacteria, they are similarly located in the inner leaflets of cell membranes and other specialized structures. Cardiolipins are formed by the Kennedy pathway using similar, but not identical, enzymatic steps in humans and bacteria.[15] Cardiolipins from *S. pyogenes* have been previously reported and are formed by the lone cardiolipin synthase gene, *cls* (Spy1212).[17−19]

Both **1** and **2** were synthesized to confirm the acyl chain order, to rule out a confounding contaminant, and to establish an initial structure–activity relationship (Scheme 1 and Figures S11–S38; see Supporting Information). The syntheses, which assumed the typical stereochemistry at the *sn*-2 and *sn*-2′ positions, began by converting commercially available (*S*)-(+)-1,2-isopropylideneglycerol to PMB-protected glycerol (**3**), which was then esterified sequentially with oleoyl chloride and stearic acid to give the PMB-protected diacylglycerol (**5a**) for **1**. Reversing the esterification order yielded **5b** for **2**. After PMB deprotection, the intermediate (**6a** or **6b**) was treated with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl-phosphordiamidite to generate the diacylglycerol-phosphoramidite (**7a** or **7b**). A PMB-protected glycerol linker (**8**) prepared in the same way from **3** was mixed with **7a** (or **7b**) and 1*H*-tetrazole to yield the protected cardiolipin **9a** (or **9b**). The final products **1** and **2** were obtained by deprotecting the central hydroxyl and cyanoethyl protecting groups. Synthetic SpCL-1 (**1**) is fully active in the TNF-α assay, and **2** has no detectable activity (Figure 1c).

The striking activity differential between **1** and **2** indicates that SpCL-1 has a selective receptor. Given that TLR2 and TLR4 are the primary microbe sensors in the mammalian innate immune system, we tested mBMDCs with cells derived from *tlr2*$^{-/-}$ or *tlr4*$^{-/-}$ mice in our assay. The results clearly indicate that **1** requires a functional TLR2 receptor for TNF-α induction (Figure 3). TLR2 typically responds to lipid-containing immunogens, while TLR4 typically responds to carbohydrate-derived immunogens. This is further supported

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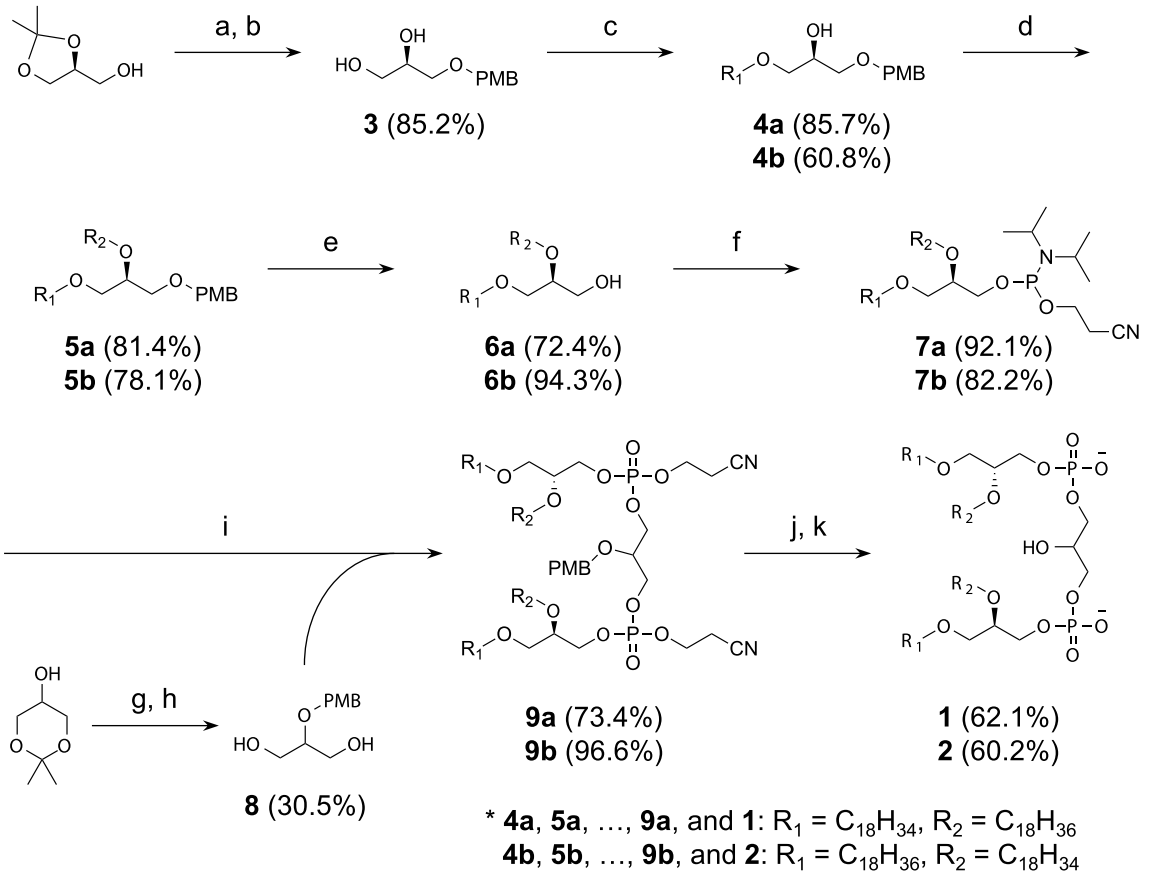
Scheme 1. Outline for Synthesis of SpCL-1 (1) and Its Chain-Switched Analog (2)[a]



[a]Reagents and conditions: (a) (*S*)-(+)-1,2-Isopropylideneglycerol, PMB-Cl, NaH, dry DMF, 0 °C to rt, overnight; (b) PTSA, MeOH, rt, 3 h (85%); (c) oleoyl-Cl or stearoyl-Cl, 2,4,6-trimethylpyridine, dry DCM, −78 °C, 2 h (86% and 61%); (d) stearic acid or oleic acid, DMAP, EDC−HCl, dry DCM, rt, overnight (81% and 78%); (e) DDQ, DCM, rt, overnight (72% and 94%); (f) 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite, 1*H*-tetrazole, DCM/MeCN (2:3), rt, 3 h (62% and 60%); (g) 2,2-dimethyl-1,3-dioxan-5-ol, PMB-Cl, NaH, dry DMF, 0 °C to rt, overnight; (h) PTSA, MeOH, rt, 3 h (30%); (i) 1*H*-tetrazole, DCM/MeCN (2:1), rt, 2 h/H_2O_2 (30%), rt, 15 min (73% and 97%); (j) DDQ, MeCN/H_2O (10:1), rt, overnight; (k) DBU, DCM, rt, 15 min (62% and 60%).

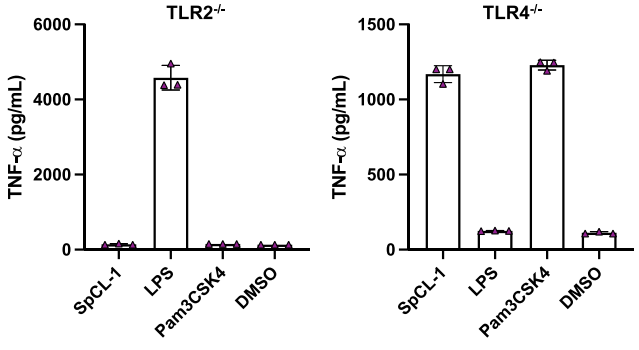


Figure 3. TNF-α inducing activity of SpCL-1 in TLR2/4$^{-/-}$ mBMDCs. Error bars = SD of technical replicates (n = 3 or 4).

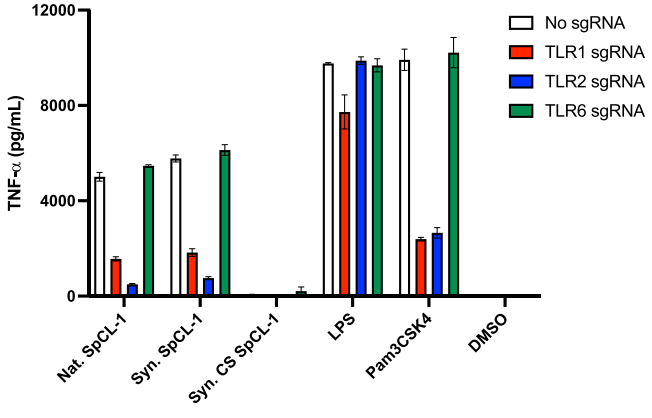


Figure 4. TNF-α inducing activities of natural SpCL-1 (Nat. SpCL-1), synthetic SpCL-1 (Syn. SpCL-1), and synthetic chain-switched SpCL-1 (Syn. CS SpCL-1) in wild type (WT) and nucleofected human MDDCs. Error bars = SD of technical replicates (n = 3).

by previous reports that TLR2 can respond to cardiolipin self-immunogens released by damaged mitochondria.[20,21]

Signaling through the TLR2 receptor typically requires a heterodimer of TLR2−TLR6 or TLR2−TLR1.[22−25] We used CRISPR/C as knockdowns in human monocytes to distinguish these possibilities (Figure 4). This analysis established a requirement for TLR1 and TLR2 but not TLR6 for TNF-α induction. TLR2−TLR6 heterodimers usually have diacyl glycerolipids-based agonists, and TLR2−TLR1 usually have triacyl glycerolipid-based agonists. Cardiolipins, with their four acyl chains, have not been characterized in this regard. Having established TLR2−TLR1 as an initiator of the cellular response, we surveyed the cytokine output using human monocytes and CRISPR/Cas gene knockdowns shown in

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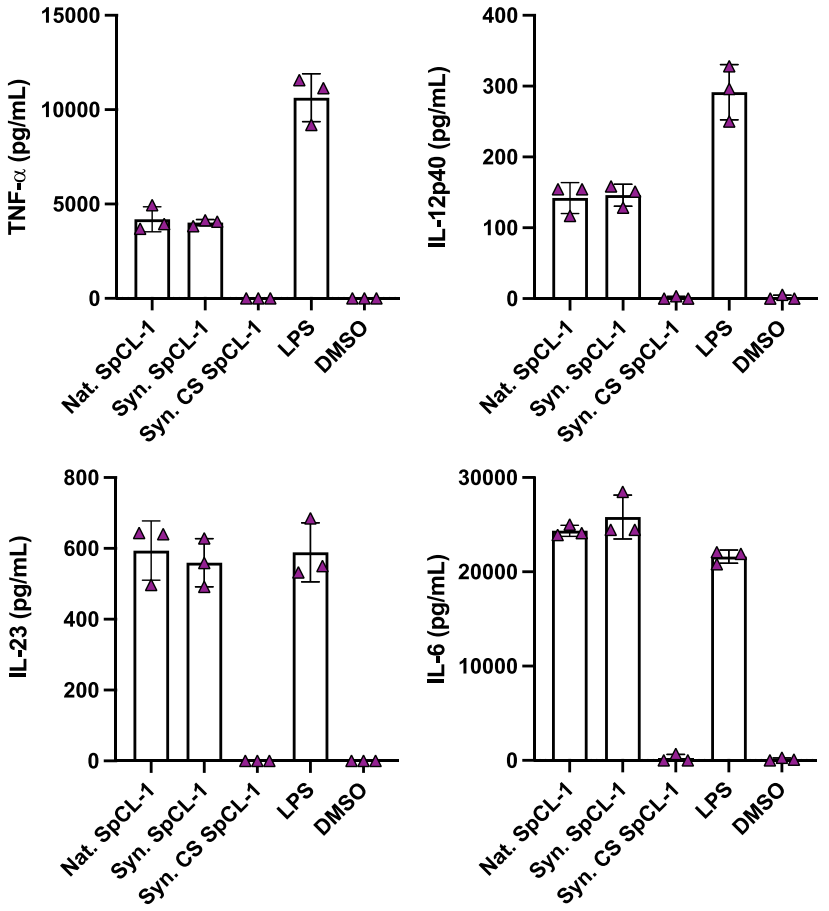

Figure 5. Proinflammatory activities of natural SpCL-1 (Nat. SpCL-1), synthetic SpCL-1 (Syn. SpCL-1), and synthetic chain-switched SpCL-1 (Syn. CS SpCL-1) in human MDDCs. Error bars = SD of technical replicates (n = 3).

Figure 5. SpCL-1 robustly triggers release of proinflammatory cytokines TNF-α, IL-6, and significantly both IL-23 and IL-12p40.[26]

A functional assay of *Streptococcus pyogenes* for immunogenic metabolites led unexpectedly to a lone cardiolipin SpCL-1 (**1**) that signals through TLR1 and TLR2. Its singular nature is highlighted both generally by the absence of bacterial cardiolipins in the catalog of canonical activators of TLR2 and specifically by the inability of its switched-chain analog (**2**) to activate immune responses.[27] Cardiolipins are associated with immune responses with the anticardiolipin antibodies associated with some autoimmune diseases like rheumatic fever and lupus.[28,29] However, these are responses to cardiolipin self-immunogens, not to bacterial cardiolipins.[27,28,30] Autoimmune diseases can begin with the activation of autoreactive T-cells by cross-reactive microbial immunogens in genetically susceptible individuals, and SpCL-1 could be a bacterial immunogens linking Strep infections to rheumatic fever and other poststreptococcal autoimmune disorders.[29,31,32]

The utility, if any, of SpCL-1 in cancer immunotherapy needs to be established by additional studies, but the historical record provides some reason for optimism. It is important to note that SpCL-1's cytokine selectivity and activation differ from those of another simple lipid immunogen, a15:0-i15:0 PE from *A. muciniphila*, which is also associated with cancer immunotherapy.[6] Even if SpCL-1 never becomes therapeutically useful, it identifies a plausible molecular mechanism for a historically prominent cancer treatment and some poorly understood autoimmune diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c07727.

Supplementary figures, NMR spectral data, and detailed experimental methods (PDF)

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Author Contributions

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Notes

The authors declare the following competing financial interest(s): Some of the authors have filed patent applications related to the research reported in this article.

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