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Aryl Trehalose Derivatives as Vaccine Adjuvants for *Mycobacterium tuberculosis*

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Abstract

Mycobacterium tuberculosis (*Mtb*) continues to be a major health threat worldwide and the development of *Mtb* vaccines could play a pivotal role in the prevention and control of this devastating epidemic. Th17 mediated immunity has been implicated in disease protection correlates of immune protection against *Mtb*. Currently there are no approved adjuvants capable of driving a Th17 response in a vaccine setting. Recent clinical trial results using trehalose dibehenate have demonstrated a formulation dependant proof of concept adjuvant system CAF01 capable of inducing long lived protection. We have discovered a new class of Th17-inducing vaccine adjuvants based on the natural product Brartermicin. We synthesized and evaluated the capacity of a library aryl trehalose derivatives to drive immunostimulatory responses and evaluated the structure activity relationships in terms of the ability to engage the Mincle receptor, induce production of innate cytokines from human and murine cells. We elaborated on the structure activity relationship of the new scaffold and demonstrated the ability of the lead entity to induce a pro-Th17 cytokine profile from primary human peripheral blood mononuclear cells and demonstrated efficacy in generating antibodies in combination with tuberculosis antigen M72 in a mouse model.

Graphical Abstract

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Supporting Information.

The following files are available free of charge:

NMR spectroscopic data (PDF) and Molecular Formula Strings

Keywords

Mincle; trehalose; brartermicin; Th17; vaccine; tuberculosis; adjuvant

INTRODUCTION

The World Health Organization (WHO) reported 6.3 million new cases of *Mtb* in 2016 (up from 6.1 million in 2015), including 480,000 cases of multi-drug resistant *Mtb*.¹ In addition to new *Mtb* cases, an estimated 1.7 billion people carry the latent disease.² The currently available bacille Calmette-Guérin (BCG) vaccine does not prevent primary infection or reactivation of latent *Mtb* infection in adults.^{3–4} Subunit vaccines have been developed to address these limitations but are limited by their lack of immunogenicity.⁵ Adjuvants can address this inadequacy, but most approved vaccine adjuvants such as MPL, Alum, and combinations with QS-21 drive a Th2 or Th1 mediated immune response, which have not proven effective against *Mtb*.⁴ Evidence has suggested a Th17 or Th1/Th17 combination response may be beneficial for protection against *Mtb*,^{6–7} but no Th17-inducing adjuvants are approved for human use. C-type lectin receptors (CLRs) are pattern recognition receptors (PRRs) that show promise as targets able to drive a Th17-response upon stimulation. The CLR/PRR Mincle is essential for recognition and adjuvant activity of the mycobacterial cord factor trehalose 6,6'-dimycolate (TDM) the chief immunostimulatory component of the *Mtb* cell wall^{8–9} and its synthetic analog trehalose-dibehenate (TDB)^{10–11} (Figure 1). While TDM and an achiral synthetic derivative, sTDCM, are potent agonists they are far too reactogenic for human vaccine applications¹² qualifying a need for adjuvants with improved therapeutic windows. The development of TDB^{10, 12–15} and other trehalose diesters (TDEs) such as Vizantin,¹⁶ have partially met this need although the physiochemical properties of these adjuvants, being very lipophilic with limited aqueous solubility, limits their application to complex liposomal formulations.¹⁷ Thus new small molecule Th17 inducing adjuvants with improved formulation, safety, efficacy and physiochemical properties are highly desirable.

Unique to this family of compounds is the natural product brartermicin,¹⁸ a high affinity ligand for the carbohydrate-recognition domain of Mincle demonstrating inhibition of mannoseconjugated serum albumin binding to the extracellular carbohydrate recognition domain (CRD) of Mincle (Figure 2).^{19–20} An *in silico* docking study of brartermicin with bovine Mincle positions an aryl substituent of the ligand in close proximity to Phe198 in the hydrophobic groove adjacent to the CRD and the other aryl group interacts with Arg182.¹⁹

This interaction was confirmed by Stoker and co-workers through the synthesis of the lipidated analogue C18dMeBr and subsequent mutation studies demonstrating an important pi-pi interaction of the aryl group and ARG183 of human Mincle.²¹

Several derivatives of brartermicin have been prepared and tested in cancer invasion models providing some very interesting structure activity relationships (SAR) related to this model.²² To our knowledge, these derivatives have not been investigated for Mincle immunostimulatory capacity or adjuvant activity. Most recently a library of lipidated diaryl trehalose derivatives has been described to elicit Th17 cytokines in a Mincle-dependent fashion with improved adjuvant activity over TDB,²¹ demonstrating the apparent necessity for a high degree of lipidation for effective Mincle activation.^{16, 21, 23–24} Interestingly all of the non-lipidated analogs tested to date fail to induce bone marrow derived macrophages (BMDM) to produce inflammatory cytokines.²¹

We hypothesized that a high affinity brartermicin based scaffold could potentially be used to generate new libraries of Mincle ligands with improved physicochemical properties for application as a vaccine adjuvant. We focused on the combination of the commercial availability and diversity of aryl carboxylic acids and our efficient and robust processes for the preparation of TDE's to allow for the rapid investigation of the unique SAR of aryl functionalized trehalose derivatives. Specific structural motifs required for Mincle activation, including aryl substitution, functionality and spacing of the aryl substituent in the CRD, were initial targets.

RESULTS AND DISCUSSION

Design of Diaryl Trehalose Analogues.—Crystal structures and systematic molecular dynamics studies have demonstrated complexation of trehalose with a calcium ion adjacent to hydrophobic grooves on the protein surface and subsequent site mutation revealed the importance of Arg183 that may interact with the hydroxyl groups of trehalose.^{25–28} Analysis of the crystal structure of trehalose bound to bovine Mincle (bMincle) and comparison of this structure to the human model suggest that structural features adjacent to the central calcium ion of the proposed active site, in particular the surface accessible Phe198, can be utilized in the design of novel ligands (Figure 2). The very strong interactions of lipids in the hydrophobic groove adjacent to the calcium ion in the CRD could be complemented through strategic positioning of an aryl functional group to optimize aryl pi-pi interactions of the ligand and Phe198. An initial library of aryl TDE compounds was designed to evaluate aryl substitution patterns in combination with slight increases in steric hindrance at various positions of the ring and to evaluate incremental increases in alkyl ether chain length. Previous studies had determined a chain length of 7 carbons was optimal for human Mincle activity^{16, 23} and we anticipated that incorporation of the aryl group may improve interactions and effectively reduce the requirement for longer alkyl chains. In addition, we were interested in the potential effect of the position of substituents on the aryl ring.

Synthesis of 6,6'-diaryl trehalose derivatives.—A pilot library of 6,6'-diaryl trehalose derivatives was prepared from the readily accessible 6,6'-hydroxy hexasilyl trehalose **1**²⁹ and commercially available aryl acids, taking advantage of easily prepared and

versatile 6,6'-hydroxy hexasilyl trehalose and the compatibility of this intermediate with various ester forming reactions (Scheme 1). The appropriate aryl acid was coupled using dicyclohexyl carbodiimide (DCC) in the presence of *N,N*-Dimethyl-4-pyridinium 4-methylbenzenesulfonate (DPTS), a neutral pyridine reagent. DPTS is particularly useful for coupling reactions in the presence of the sensitive trimethylsilyl (TMS) protecting groups.

Aryl acids containing a 2-hydroxyl functionality could be coupled through an SN2 displacement of the 6,6'-trifluoromethanesulfonyl activated hexasilyl trehalose using the potassium salt of the aryl acids in excellent yields (Scheme 2).³⁰ Other coupling conditions, including various carbodiimide and Mitsunobu conditions failed to provide the desired products.

The caffeic acid derived trehalose diester, UM1021, could not be accessed directly without first protecting the phenols (Scheme 3). *t*-Butyldimethylsilyl (TBS) protection allowed for efficient synthesis of the ester with subsequent deprotection of the TBS ethers with 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU)³¹ followed by removal of the remaining silyl protecting groups with Dowex 50W X8 resin to provide the target UM1021.

Activation and specificity of diarytrehalose compounds in murine cell lines and human PBMCs.—Mincle agonist have been shown to induce inflammatory cytokine production, such as Tumor necrosis factor alpha (TNF α), and promote the development of a Th1/Th17 immune response that is associated with and dependent on the induction of innate cytokines such as IL-6, IL-1 β , and IL-23.³² Due to species-specific activity observed with a series of alpha-branched trehalose diester Mincle agonists,²³ we routinely test new compounds for cytokine induction in both mouse monocytic cell line RAW 264.7 and primary human peripheral blood mononuclear cells (PBMC). To assess the immunostimulatory activity of the initial library in both Murine and human cell lines and to investigate the potential of the compounds to act as Mincle ligands we compared them head to head plate coated or diluted in from a stock DMSO solution (Figure 3).

Some very interesting structural features correlating with activity were immediately obvious. Activity of the 3,5-diether was slightly improved in the murine assay (Figure 3A) and very pronounced in hPBMC's (Figure 3C and D) through extension of the hydrocarbon chain from UM1014 to UM1015 and similarly with UM1016 to UM1017, though less drastic for the shorter overall chain length. These compounds are intriguing based on the observed activity and novelty of a short alkyl chain structure and the observed increase in activity and potency when compounds were solubilized in DMSO prior to addition to media. Of note, the compounds solubilized in DMSO form aggregates or crystals upon addition to cell culture media and macromolecular aggregates (plate coat or aqueous dispersions) are critically important for compound activity and are currently under investigation. While we continue to explore the structure activity relationships of this scaffold through extension of the carbon chains and aryl substitution patterns, we were intrigued by the observed activity of UM1024, demonstrating significant potency (low ED50) in human PBMCs and UM1015 with high maximum response across a wide dose range (Figure 3C and D). The addition of steric bulk at the 3 and 5 positions in this compound drove drastically improved cytokine production, exemplified by the huge increase in potency for cytokine induction that is not evident with

the less bulky isopropyl derivative UM1023. While Brartemicin has not been reported to activate Mincle and was not analyzed in this preliminary screen, the dimethyl ether Brartemicin derivative UM1022 demonstrated activity in the plate coated murine system (Figure 3A) and slight activity in the plate coated hPBMc assay suggesting hydrophobicity and possibly not ring substitution regiochemistry is a requirement for Mincle activation in this class of molecules. The interesting reduced activity of many of the compounds at higher concentrations was not associated with toxicity or cell death (data not shown) and will be a subject of future investigations.

Compounds that induced the highest levels of TNF α secretion from PBMCs, UM1015, 1019, 1020, 1022, and 1024 (Figure 3C and D) were further investigated as potential lead adjuvants able to induce a Th17 response. The innate cytokines required to elicit a Th17 response (IL-6, IL-1 β , and IL-23) were measured in a Luminex multiplex assay (Figure 4), along with TNF α to confirm the activity shown in Figure 3. Each compound elicited secretion of similar levels of all cytokines measured although there were differences in the potency to do so between compounds. Of particular note is UM1024. This derivative demonstrated a varied response in its absolute cytokine response but has a much greater potency with a maximum response achieved at 1 μ M for all cytokines investigated here. Interestingly UM1024 also demonstrated an unusual bi-phasic dose response curve for IL-6 and IL-1 β , which remains unexplained.

SAR of diaryl trehalose scaffold.—In order to evaluate the structural features responsible for the remarkable activity of UM1024, we systematically removed and substituted functionality of the aryl group (Scheme 4). The derivatives were prepared in similar fashion to the previous library compounds and the results confirmed the *t*-butyl substituent at the 3-position of UM1024 to be critical to in vitro activity (Figure 5), as demonstrated by the retained activity of compounds UM1046, UM1047 and loss of activity with UM1048. Additionally, it was found that moving the hydroxyl to the 4 position, in UM1087, resulted in decreased human cytokine production. In addition to production of TNF from RAW murine cells as this cell line does not produce IL6, the compounds were assayed for IL-6 production in human PMC's as this is key cytokine leading to an IL-17 phenotype.³³ Interestingly, low-level activity of plate coated brartemicin at high concentrations in murine cells and human PBMCs was observed (Figure 5, left plots, black circles). To our knowledge, this is the first demonstration of biological activity of brartemicin in a Mincle specific assay. This activity was not drastically attenuated through introduction of steric bulk at the 6 position of the aromatic ring (UM1068).

Mincle receptor specificity of UM1024.—Despite the modifications made to the parental core structure, UM1024 remained the most potent (lowest ED50) compound at inducing activation of immune cells so more in depth analysis of its activity was undertaken. The Mincle-selective activity of UM1024 was evaluated using human embryonic kidney (HEK) cells expressing the human or mouse Mincle receptor along with an NF- κ B-driven secreted embryonic alkaline phosphatase (SEAP) reporter. UM1024 induced production of SEAP in a dose dependent manner (Figure 6). HEK null cells (HEK cells containing the NF- κ B reporter without Mincle receptor) were used as negative controls to confirm the receptor

specificity of the compounds. The reference compounds, TDM and TDB, all demonstrated the ability to signal through both mouse and human Mincle though with less potency to activate these receptors than UM1024. The relative activity of TDM in the mouse and human HEK cells is consistent with the results reported by others.³⁴

Structural evaluation of UM1024 in the CRD of bMincle.—The current models for Mincle activation through binding of trehalose diesters suggest that there is a major hydrophobic requirement for efficient receptor activation.^{21, 25} Our findings suggest activation of Mincle is possible through an alternate CRD binding mode. To explore this hypothesis, we used computational docking to probe potential interactions between UM1024 and bovine and human Mincle crystal structures by using the bound trehalose from the 4KZV crystal structure to define the binding site in both human and bovine Mincle. Figure 7 depicts a high-ranking docked pose of UM1024 in the bovine Mincle structure that maintains both the core conformation of bound trehalose and the trehalose Ca²⁺ interactions. Surprisingly, when all docked poses in human and bovine Mincle were filtered for conformations and interactions similar to the bound trehalose, none of the filtered poses yielded interactions of a pendant aryl group with Agr182 which was found to be critical to binding of other Mincle agonists.²¹ Instead, the poses interact with a small hydrophobic pocket adjacent to Phe198, shifting Phe198 towards significant pi-pi interactions with the UM1024 aryl side chain.

Adjuvant activity of UM1024.—The established in vitro activity and potency of UM1024 prompted the evaluation of this compound for adjuvant activity in combination with a clinical-stage *Mycobacteria tuberculosis* antigen M72.³⁵ M72 is a fusion protein consisting of two mycobacterial proteins Mtb32a and Mtb39a which are expressed in both BCG and *M. tuberculosis*.³⁶ For this study mice were administered 1 µg of M72 antigen intramuscularly (IM) in combination with DDA/DSPC liposome formulations of 10 nmol TDB (as a positive control), the α-branched TDE compound B16²³ or UM1024 on days 0 and 14. Mice were analyzed 14 days post- post-secondary immunization and M72-specific total IgG, IgG1, and IgG2 antibody titers in serum were measured by ELISA (Figure 8). Liposomal B16 significantly increased total IgG and IgG1 M72-specific antibody titers compared to antigen and blank liposome as reported previously (Figure 8, left and center).²³ Conversely, liposomal UM1024 significantly increased IgG2a M72-specific antibody titers compared to blank liposomes, TDB and B16 (Figure 8, right). M72-specific total IgG and IgG1 were not increased significantly by the addition of UM1024 compared to blank liposomes, although the titer of both total IgG and IgG1 do trend higher (Figure 8, left and center). Taken together, these data indicate the liposomal UM1024 is able to drive a strong IgG2a/ humoral Th1-biased response against the M72 antigen, making it a strong candidate as a tuberculosis vaccine adjuvant.

In a followup study to measure T cell responses, mice were vaccinated twice, IM with 0.1 µg antigen plus 2, 10, or 50 nmol of liposomal TDB or liposomal 1024. 5 days after the second vaccination, spleen cells were re-stimulated with M72 antigen to measure antigen-specific CD4⁺ T cell responses. Both TDB and UM1024, in DDA:DSPC liposomes induce high levels of antigen-specific CD4⁺ IL17A producing cells by intracellular cytokine staining

(Figure 9), indicating a Th17 polarized T cell response. Re-stimulation of spleen cells from mice that were vaccinated with B42 at the highest and lowest dose, as well as the highest dose of TDB, induced some IL-17A, although it is difficult to say if these responses are significant due to a small sample size (n=3). The induction of antigen-specific Th17 cells was encouraging as Th17 cells have been shown to provide protection against *Mtb* after immunization in mice and validates our adjuvant screening approach. Production of IFN γ (indicative of a Th1 response) or IL-5 (indicative of a Th2 response) was not found (data not shown).

Conclusion.—Herein we report synthesis and evaluation of a focused library of aryl trehalose derivatives as a new class of relatively small and potent Mincle receptor selective agonists. These compounds displayed significantly improved pharmacology over existing Mincle ligands with improved *in vitro* and *in vivo* adjuvant activity. The compounds are easily prepared from readily available materials in high yield. Initial docking studies have identified a possible novel receptor binding motif involving a small hydrophobic pocket adjacent to the Ca²⁺ binding site that we will attempt to validate in future work through CRD mutation studies and crystal structure determination of bMincle with UM1024. When UM1024 was combined with an *Mtb* antigen *in vivo*, IgG2a titers were significantly boosted over liposomes alone control as well as the currently used TDB DDA:DSPC liposomes. This result suggests compounds of this class will be able to generate an antigen specific immune response. The lead ligand identified, UM1024, has great potential, through ease of preparation, formulation and activity, in future efforts to advance vaccine discovery and development in the fight to circumvent tuberculosis infection and morbidity.

Experimental Section

General Experimental.—All reagents and solvents were used as received. Reactions were monitored by TLC-analysis on Merck Silica gel 60 F254 plates and visualized by UV at 254 nm and dipping in vanillin (vanillin/water/ethanol/sulfuric acid, 0.2 g:5 mL:5 mL:1 mL) or phosphomolybdic acid in ethanol (PMA) and developed with heat. All compounds were confirmed to be >95% pure by TLC, NMR and HPLC-MS analysis. ¹H and ¹³C NMR spectra were recorded on an Agilent or Bruker 400 MHz or a Bruker 500 MHz instrument and were referenced to TMS or a solvent peak. High resolution HPLC-MS analysis was obtained on an Agilent 6520 Q-TOF mass spectrometer utilizing electrospray ionization source in positive or negative mode. Chromatography was performed on Grace or Biotage automated medium pressure chromatography instruments with preloaded Buchi silica gel cartridges. Brartemicin was synthesized according to literature procedures.²¹ 2,6-hydroxy-3,5-ditertbutylbenzoic acid was prepared by hydrolysis of methyl 2,6-hydroxy-3,5-ditertbutylbenzoate.³⁷ Spectral data for UM1013,¹⁹ 1014, 1022 and 1019^{38–39} were comparable to those reported in the literature. M72 antigen was generously supplied GlaxoSmithKline.

Synthesis of 6,6'-bis(2,3-dimethoxybenzoyl)- α,α -D-trehalose (UM1013)

General procedure for esterification via carbodiimide mediated coupling and silyl deprotection.: 2,2',3,3',4,4'-Hexa-trimethylsilyl- α,α -D-trehalose²⁹ (528.3 mg, 682 μ mol), 2,3-dimethoxybenzoic acid (306.1 mg, 1.68 mmol), and 1,4-Dimethylpyridinium *p*-

toluenesulfonate (105.0 mg, 357 μ mol) were combined. The materials were dissolved in anhydrous tetrahydrofuran (4 mL), placed on an ice bath under nitrogen, and DCC (487.3 mg, 2.37 mmol) was added. The reaction was allowed warm to room temperature and stirred for three days at which point it was filtered, reduced in vacuo and subjected to chromatography using the Biotage system with a 12 g silica column and a zero to 15% ethyl acetate in heptane gradient with the product eluting around 15% ethyl acetate. This yielded 2,2',3,3',4,4'-hexa-trimethylsilyl-6,6'-Bis(2,3-dimethoxybenzoyl)- α,α -D-trehalose (384.9 mg, 51%) after reducing product fractions in vacuo and drying overnight under high vacuum. The silyl intermediate (367.6 mg, 333 μ mol) was dissolved in equal amount of methylene chloride and methanol (8 mL) treated with Dowex 50WX8 resin (668.4 mg) with magnetic stirring. Upon consumption of the starting material as determined by TLC (20% methanol in methylene chloride and charring with vanillin stain) the reaction was filtered, concentrated and chromatographed on a silica column eluting with a 40% to 80% methylene chloride to methanol gradient (Biotage system using a 12 g pre-packed column) to provided 6,6'-bis(2,3-dimethoxybenzoyl)- α,α -D-trehalose (39.2 mg, 18%) as a white solid. 500 MHz ^1H -NMR DATA (ppm) DMSO 7.24 (d, J = 7.5 Hz, 2H), 7.20 (d, J = 8.0 Hz, 2H), 7.14 (t, J = 8.1 Hz, 2H), 5.17 (d, J = 5.4 Hz, 2H), 4.95 (d, J = 2.9 Hz, 2H), 4.89 (d, J = 3.5 Hz, 2H), 4.86 (d, J = 5.8 Hz, 2H), 4.39 (d, J = 10.2 Hz, 2H), 4.29 (dd, J = 11.6, 5.4 Hz, 2H), 4.03 (m, 2H), 3.83 (s, 6H), 3.74 (s, 6H), 3.60 (t, J = 8.2 Hz, 2H), 3.27 (m, 4H); 125 MHz ^{13}C -NMR DATA (ppm) DMSO 165.74, 153.19, 147.96, 126.06, 124.13, 121.41, 116.26, 93.64, 72.76, 71.48, 70.8, 69.76, 64.06, 61.01, 55.97; HRMS: calcd: 670.2109 (688.2453M+NH₄) found: 688.2459 m/z.

6,6'-Bis(3,5-dimethoxybenzoyl)- α,α -D-trehalose (UM1014).—The diaryl ester was prepared according to the general procedure for esterification via carbodiimide mediated coupling and silyl deprotection to obtain 6,6'-bis(3,5-dimethoxybenzoyl)- α,α -D-trehalose (72.7 mg, 42% for 2 steps) as a white solid. ^1H -NMR (500 MHz, DMSO- D_6) δ 7.07 (s, 4H), 6.77 (s, 2H), 5.22 (br, 2H), 4.94 (br, 4H), 4.90 (d, J = 3.2 Hz, 2H), 4.45 (d, J = 10.2 Hz, 2H), 4.28 (m, 2H), 4.08 (d, J = 3.8 Hz, 2H), 3.78 (s, 12H), 3.61 (q, J = 7.8, 4.6 Hz, 2H), 3.30 (d, J = 3.4 Hz, 2H), 3.23 (t, J = 3.8 Hz, 2H); ^{13}C -NMR (125 MHz, DMSO- D_6) δ 165.30, 160.48, 131.77, 106.78, 105.29, 93.53, 72.81, 71.64, 70.27, 69.80, 64.36, 55.45; HRMS (ESI+, m/z) calcd for (M+NH₄)⁺: 688.2453; found: 688.2478.

6,6'-Bis(3,5-diethoxybenzoyl)- α,α -D-trehalose (UM1015).—The diaryl ester was prepared according to the general procedure for esterification via carbodiimide mediated coupling and silyl deprotection to obtain provided 6,6'-Bis(3,5-diethoxybenzoyl)- α,α -D-trehalose (93.5 mg, 49%). ^1H -NMR (500 MHz, DMSO- D_6) δ 7.03 (s, 4H), 6.72 (s, 2H), 5.23 (d, J = 5.2 Hz, 2H), 4.97 (d, J = 4.8 Hz, 2H), 4.94 (d, J = 3.0 Hz, 2H), 4.89 (d, J = 5.8 Hz, 2H), 4.44 (d, J = 11.3 Hz, 2H), 4.26 (dd, J = 11.6, 5.3 Hz, 2H), 4.08 (br, 2H), 4.03 (q, J = 2.9 Hz, 8H), 3.62 (m, 2H), 3.03 (m, 2H), 3.25 (m, 2H) 1.31 (t, J = 6.9 Hz, 12H); ^{13}C -NMR (125 MHz, DMSO- D_6) δ 165.32, 159.69, 131.69, 107.13, 106.05, 93.47, 72.79, 71.65, 70.24, 70.23, 69.75, 64.26, 63.44, 14.51; HRMS (ESI+, m/z) calcd for (M+NH₄)⁺: 726.2735 (744.3079 M+NH₄); found: 744.3061.

6,6'-Bis(3,5-dimethylbenzoyl)- α,α -D-trehalose (UM1016).—The diaryl ester was prepared according to the general procedure for esterification via carbodiimide mediated coupling and silyl deprotection to obtain 6,6'-Bis(3,5-dimethylbenzoyl)- α,α -D-trehalose (45.6mg, 17% for two steps). $^1\text{H-NMR}$ (500 MHz, DMSO-D_6) δ 7.56 (s, 4H), 7.27 (s, 2H), 5.21 (d, $J=5.3$ Hz, 2H), 4.99 (d, $J=4.8$ Hz, 2H), 4.92 (m, 4H), 4.41 (d, $J=10.6$ Hz, 2H), 4.30 (dd, $J=11.8, 5.9$ Hz, 2H), 4.09 (m, 2H), 3.62 (td, $J=8.6, 4.6$ Hz, 2H), 3.31 (m, 2H), 3.25 (m, 2H), 2.31 (s, 12H); $^{13}\text{C-NMR}$ (125 MHz, DMSO-D_6) δ 165.85, 137.94, 134.61, 129.79, 126.87, 93.55, 72.93, 71.64, 70.20, 69.88, 63.97, 20.73; HRMS (ESI+, m/z) calcd for $(\text{M}+\text{NH}_4)^+$: 624.2656; found: 624.2648.

6,6'-bis(3,5-diethylbenzoyl)- α,α -D-trehalose (UM1017).—The diaryl ester was prepared according to the general procedure for esterification via carbodiimide mediated coupling and silyl deprotection to obtain 6,6'-bis(3,5-diethylbenzoyl)- α,α -D-trehalose (172.5 mg, 67% for two steps). $^1\text{H-NMR}$ (500 MHz, DMSO-D_6) δ 7.60 (s, 4H), 7.32 (s, 2H), 5.20 (d, $J=5.4$ Hz, 2H), 4.98 (d, $J=4.7$ Hz, 2H), 4.95 (d, $J=3.3$ Hz, 2H), 4.90 (d, $J=6.0$ Hz, 2H), 4.42 (d, 2H), 4.29 (dd, $J=12.0, 6.9$ Hz, 2H), 4.08 (m, 2H), 3.63 (td, $J=9.2, 5.2$ Hz, 2H), 3.34 (m, 2H), 3.24 (td, $J=9.2, 5.7$ Hz, 2H), 2.62 (q, $J=7.4$ Hz, 8H), 1.67 (t, $J=7.3$ Hz, 12H); $^{13}\text{C-NMR}$ (125 MHz, DMSO-D_6) δ 165.87, 144.31, 132.44, 129.94, 125.91, 93.25, 72.90, 71.68, 70.29, 69.88, 63.99, 15.43; HRMS (ESI+, m/z) calcd for $(\text{M}+\text{NH}_4)^+$: 680.3282; found: 680.3267.

6,6'-bis(trans-2,3-dimethoxycinnamoyl)- α,α -D-trehalose (UM1018).—The diaryl ester was prepared according to the general procedure for esterification via carbodiimide mediated coupling and silyl deprotection to obtain 6,6'-bis(trans-2,3-dimethoxycinnamoyl)- α,α -D-trehalose (212.8 mg, 78% for two steps). $^1\text{H-NMR}$ (500 MHz, DMSO-D_6) δ 7.84 (d, $J=16.2$ Hz, 2H), 7.36 (d, $J=7.4$ Hz, 2H), 7.11 (m, 4H), 6.59 (d, $J=16.2$ Hz, 2H), 5.15 (d, $J=5.4$ Hz, 2H), 4.93 (b, 4H), 4.83 (d, $J=6.0$ Hz, 2H), 4.35 (d, $J=10.4$ Hz, 2H), 4.18 (dd, $J=11.7, 5.5$ Hz, 2H), 3.99 (m, 2H), 3.80 (s, 6H), 3.75 (s, 6H), 3.60 (td, $J=9.3, 5.1$ Hz, 2H), 3.32 (m, 2H), 3.22 (td, $J=9.4, 5.1$ Hz, 2H); $^{13}\text{C-NMR}$ (125 MHz, DMSO-D_6) δ 166.25, 152.74, 147.74, 138.63, 127.54, 124.42, 119.04, 118.96, 114.89, 93.43, 72.79, 71.49, 70.15, 69.77, 63.57, 60.87, 55.81; HRMS (ESI+, m/z) calcd for $(\text{M}+\text{NH}_4)^+$: 740.2766; found: 740.2758.

6,6'-bis(trans-2,3-dimethoxycinnamoyl)- α,α -D-trehalose (UM1019).—The diaryl ester was prepared according to the general procedure for esterification via carbodiimide mediated coupling and silyl deprotection to obtain 6,6'-bis(trans-2,3-dimethoxycinnamoyl)- α,α -D-trehalose (222 mg, 71% for two steps). $^1\text{H-NMR}$ (500 MHz, DMSO-D_6) δ 7.57 (d, $J=16.1$ Hz, 2H), 7.35 (s, 2H), 7.22 (d, $J=8.6$ Hz, 2H), 6.97 (d, $J=8.6$ Hz, 2H), 6.54 (d, $J=16.1$ Hz, 2H), 5.12 (d, $J=5.1$ Hz, 2H), 4.92 (d, $J=4.3$ Hz, 2H), 4.89 (d, $J=3.4$ Hz, 2H), 4.86 (d, $J=6.0$ Hz, 2H), 4.29 (d, $J=10.8$ Hz, 2H), 4.21 (dd, 2H), 4.00 (dd, $J=11.8, 5.2$ Hz, 2H), 3.80 (s, 6H), 3.79 (s, 6H), 3.59 (m, 2H), 3.33 (m, 2H), 3.23 (m, 2H); $^{13}\text{C-NMR}$ (125 MHz, DMSO-D_6) δ 166.59, 151.02, 149.02, 144.83, 126.89, 123.10, 115.53, 111.52, 110.34, 93.71, 71.83, 71.50, 70.06, 69.80, 63.28, 55.66, 55.59; HRMS (ESI+, m/z) calcd for $(\text{M}+\text{NH}_4)^+$: 740.2766; found: 740.2755.

6,6'-bis(trans-3,4,5-trimethoxycinnamoyl)- α,α -D-trehalose (UM1020).—The diester was prepared according to the general procedure for esterification via carbodiimide mediated coupling and silyl deprotection to obtain 6,6'-bis(trans-3,4,5-trimethoxycinnamoyl)- α,α -D-trehalose (231 mg, 55% for two steps). $^1\text{H-NMR}$ (500 MHz, DMSO-D_6) δ 7.58 (d, J = 15.6, 2H), 7.06 (s, 4H), 6.64 (d, J = 15.7 Hz, 2H), 5.14 (br, 2H), 4.95 (br, 2H), 4.89 (d, J = 3.5, 4H), 4.30 (d, J = 10.4 Hz, 2H), 4.24 (dd, J = 11.7, 4.9, 2H), 4.01 (m, 2H), 3.81 (s, 12H), 3.68 (s, 6H), 3.60 (t, J = 8.9 Hz, 2H), 3.24 (t, J = 8.5 Hz, 2H); $^{13}\text{C-NMR}$ (125 MHz, DMSO-D_6) δ 166.85, 166.43, 153.13, 144.89, 144.80, 139.49, 129.67, 129.63, 117.31, 119.13, 106.00, 93.78, 72.84, 71.52, 70.02, 69.80, 63.35, 60.11, 56.08, 51.43; HRMS (ESI+, m/z) calcd for $(\text{M}+\text{NH}_4)^+$: 800.2977; found: 800.2990.

6,6'-bis(caffoyl)- α,α -D-trehalose (UM1021).—The diester was prepared according to the general procedure for esterification via carbodiimide mediated coupling. The aryl silyl ethers were removed with 1,8-diazabicyclo(5.4.0)undec-7-ene in aqueous ACN (95:5) at 60 °C³¹ and the remaining silyl deprotection was accomplished per the general procedure to provide 6,6'-bis(caffoyl)- α,α -D-trehalose (74.4, 32% for three steps as a green solid). $^1\text{H-NMR}$ (400 MHz, TFA-D) δ 7.73 (d, J = 15.8 Hz, 2H), 7.22 (s, 2H), 7.11 (d, J = 8.4 Hz, 2H), 6.95 (d, J = 8.4 Hz, 2H), 6.38 (d, J = 15.8 Hz, 2H), 5.53 (br, 2H), 4.73–4.60 (m, 4H), 4.48 (t, J = 9.6 Hz, 2H), 4.41 (d, J = 11.1 Hz, 2H), 4.49 (d, J = 9.1 Hz, 2H), 4.00 (t, J = 9.6 Hz, 2H); $^{13}\text{C-NMR}$ (125 MHz, DMF-D₇) δ 167.90, 150.4, 147.28, 146.31, 127.17, 122.59, 116.86, 115.74, 115.25, 95.32, 74.76, 73.44, 72.04, 71.41, 64.62; HRMS (ESI+, m/z) calcd for $(\text{M}+\text{NH}_4)^+$: 684.2140; found: 684.2162.

6,6'-bis(2,3-methoxy-6-methyl-benzoyl)- α,α -D-trehalose (UM1022).

General procedure for esterification via triflate.: 2,3,4,2',3',4'-Hexakis(trimethylsilyl)-6,6'-dihydroxy- α,α -D-trehalose²⁹ (596 mg, 0.77 mmol) was dissolved in methylene chloride (15 mL) under a nitrogen atmosphere. Pyridine (0.37 mL, 4.58 mmol) was added with magnetic stirring and the solution was cooled to 0–5 °C in an ice bath. Trifluoromethanesulfonic anhydride was added and the reaction was stirred for 1.5 hours maintaining a temperature of to 0–5 °C. The reaction was quenched with dilute hydrochloric acid, washed saturated aqueous sodium bicarbonate, dried over sodium sulfate, filtered and concentrated. Separately, 2,3-methoxy-6-methyl-benzoic acid (450 mg, 2.30 mmol) in THF (10 mL) was treated with potassium trimethylsilanolate for 30 minutes. The potassium salt was precipitated with diethyl ether and dried under high vacuum. The crude trehalose triflate intermediate was dissolved in toluene (15 mL) and combined with the acid potassium salt, 18-crown-6 (284 mg, 0.77 mmol) and the mixture was heated to 80 °C overnight. The reaction was washed with aqueous sodium bicarbonate, dried over sodium sulfate, concentrated, and the product isolated by chromatography on silica gel with a heptane to ethyl acetate gradient yielding 733 mg (84%) 2,3,4,2',3',4'-hexakis-*O*-trimethylsilyl-6,6'-bis-(2,3-methoxy-6-methyl-benzoyl)- α,α -D-trehalose. This product was deprotected as described the general preparation of UM1013 6,6'-bis(2,3-methoxy-6-methyl-benzoyl)- α,α -D-trehalose (345.7 mg, 81%). $^1\text{H-NMR}$ (500 MHz, DMSO-D_6) δ 6.45 (s, 2H), 6.41 (s, 2H), 5.09(d, J = 4.7 Hz, 2H), 4.88 (d, J = 4.5 Hz, 2H), 4.84 (br, 2H), 4.78 (d, J = 5.8 Hz, 2H), 4.40 (d, J = 11.6 Hz, 2H), 4.24 (dd, J = 4.6, 11.6 Hz, 2H), 3.96 (br, 2H), 3.77 (s, 6H), 3.73 (s, 6H), 3.57 (br, 2H), 3.23 (br, 2H), 3.19 (br, 2H), 2.18 (s, 3H); $^{13}\text{C-NMR}$ (125 MHz, DMSO-D_6) δ

167.12, 160.95, 157.69, 137.39, 116.17, 106.81, 96.17, 93.48, 72.68, 71.40, 70.06, 69.77, 63.57, 55.73, 55.31, 19.39; HRMS (ESI+, m/z) calcd for (M+Na)⁺: 721.2320; found: 721.2329.

6,6'-bis(3,5-diisopropylsalasyloyl)- α,α -D-trehalose (UM1023).—The diester was prepared according to the general procedure for esterification via triflate to obtain 6,6'-bis(3,5-diisopropylsalasyloyl)- α,α -D-trehalose (57.7 mg, 27% for two steps). ¹H-NMR (500 MHz, DMSO-D₆) δ 10.80 (s, 2H), 7.50 (s, 2H), 7.33 (s, 2H), 5.26 (d, J = 5.1 Hz, 2H), 5.02 (d, J = 4.8 Hz, 2H), 4.96 (d, J =3.4 Hz, 2H), 4.92 (d, J = 5.9 Hz, 2H), 4.50 (d, J = 10.1 Hz, 2H), 4.37 (dd, J = 11.2, 6.1 Hz, 2H), 4.09 (m, 2H), 3.63 (td, J = 9.9, 4.6 Hz, 2H), 3.24 (m, 4H), 2.82 (m, 2H), 1.16 (m, 24H); ¹³C-NMR (125 MHz, DMSO-D₆) δ 168.85, 156.46, 138.72, 135.95, 131.07, 123.76, 111.19, 93.15, 72.73, 71.63, 70.26, 69.65, 64.48, 32.59, 26.33, 23.91, 23.70, 22.45, 22.23; HRMS (ESI+, m/z) calcd for (M+NH₄)⁺: 768.3806; found: 768.3790.

Synthesis of 6,6'-bis-(3,5-di-*tert*-butylsalisate)- α,α -D-trehalose (UM1024).—The diester was prepared according to the general procedure for esterification via triflate to provide 6,6'-bis-(3,5-di-*tert*-butylsalisate)- α,α -D-trehalose (523 mg, 85%). ¹H-NMR (500 MHz, DMSO-D₆) δ 11.30 (s, 2H), 7.86 (s, 2H), 7.49 (s, 2H), 5.27 (d, J = 5.5 Hz, 2H), 5.02 (d, J = 4.6 Hz, 2H), 4.99 (d, J = 3.1 Hz, 2H), 4.90 (d, J = 5.9 Hz, 2H), 4.55 (d, J = 10.7 Hz, 2H), 4.31 (dd, J =11.4, 5.9 Hz, 2H), 4.08 (td, J = 9.1, 4.8 Hz, 2H), 3.64 (m, 2H), 3.29 (m, 2H), 3.25 (m, 2H), 1.37 (s, 18H), 1.21 (s, 18H); ¹³C-NMR (125 MHz, DMSO-D₆) δ 170.88, 158.46, 140.72, 137.02, 130.51, 123.85, 111.65, 93.47, 73.05, 72.03, 70.76, 70.07, 65.07, 36.25, 35.17, 34.25, 31.45, 29.65; HRMS (ESI+, m/z) calcd for (M+NH₄)⁺: 824.4432; found: 824.4427.

6,6'-bis(2,6-hydroxy-3,5-ditertbutylbenzoyl)- α,α -D-trehalose (UM1046).—The diester was prepared according to the general procedure for esterification via triflate to obtain 6,6'-bis(2,6-hydroxy-3,5-ditertbutylbenzoyl)- α,α -D-trehalose (219.7 mg, 28% for two steps). ¹H-NMR (500 MHz, DMSO-D₆) δ 10.31 (s, 4H), 7.34 (s, 2H), 5.04 (d, J = 3.4 Hz, 4H), 4.64 (d, J =11.3 Hz, 2H), 4.55 (dd, J = 11.5, 5.4 Hz, 2H), 4.20 (m, 2H), 3.62 (t, J = 9.3 Hz, 2H), 3.20 (dd, J = 9.4, 3.6 Hz, 8H), 3.18 (t, J = 9.3 Hz, 2H), 1.33 (s, 36H); ¹³C-NMR (100 MHz, DMSO-D₆) δ 170.20, 156.80, 131.10, 125.62, 100.57, 94.24, 79.78, 72.38, 71.44, 70.42, 69.04, 65.40, 34.25, 29.46; HRMS (ESI+, m/z) calcd for (M+NH₄)⁺: 856.4331; found: 856.4356.

6,6'-bis(3,5-ditertbutylbenzoyl)- α,α -D-trehalose (UM1047).—The diester was prepared according to the general procedure for esterification via triflate to obtain 6,6'-bis(3,5-ditertbutylbenzoyl)- α,α -D-trehalose (199.0 mg, 70% over two steps). ¹H-NMR (500 MHz, DMSO-D₆) δ 7.78 (s, 2H), 7.66 (s, 1H), 5.24 (d, J = 5.4, 2H), 5.00 (br, 4H), 4.84 (d, J =6.0 Hz, 2H), 4.50 (d, J =11.0 Hz, 2H), 4.22 (dd, J = 11.6, 6.6 Hz, 2H), 4.05 (td, J = 9.1, 4.8 Hz, 2H), 3.65 (m, 2H), 3.23 (m, 2H), 1.27 (s, 36H); ¹³C-NMR (100 MHz, DMSO-D₆) δ 166.03, 150.83, 129.24, 127.13, 123.06, 92.85, 79.78, 72.72, 71.64, 70.43, 69.82, 64.07, 34.52, 31.01; HRMS (ESI+, m/z) calcd for (M+NH₄)⁺: 792.45342; found: 792.4532.

6,6'-bis(2-hydroxy-5-tertbutylbenzoyl)- α,α -D-trehalose (UM1048).—The diester was prepared according to the general procedure for esterification via triflate to obtain 6,6'-bis(2-hydroxy-5-tertbutylbenzoyl)- α,α -D-trehalose (93.0 mg, 34% over two steps). ^1H -NMR (500 MHz, DMSO- D_6) δ 10.32 (s, 2H), 7.62 (s, 2H), 7.90 (d, J = 8.6 Hz, 2H), 6.92 (d, J = 8.5 Hz, 2H), 5.26 (d, J = 5.3 Hz, 2H), 5.00 (br, 4H), 4.89 (d, J = 5.6 Hz, 2H), 4.54 (d, J = 11.3 Hz, 2H), 4.29 (dt, J = 11.6, 6.2 Hz, 2H), 4.09 (m, 2H), 3.65 (td, J = 9.1, 4.6 Hz, 2H), 3.33 (s, 6H), 3.24 (m, 2H), 1.21 (s, 18H); ^{13}C -NMR (100 MHz, DMSO- D_6) δ 168.88, 158.30, 141.47, 133.34, 125.53, 117.14, 111.74, 93.08, 79.77, 72.61, 71.58, 70.35, 69.61, 64.51, 33.67, 30.93; HRMS (ESI+, m/z) calcd for $(\text{M}+\text{NH}_4)^+$: calcd: 712.3180; found: 712.3182.

6,6'-bis(2,4-dihydroxy-6-isopropylbenzoyl)- α,α -D-trehalose (UM1068).—The diester was prepared according to the general procedure for esterification via triflate to obtain 6,6'-bis(2,4-dihydroxy-6-isopropylbenzoyl)- α,α -D-trehalose (50% over two steps). ^1H NMR (400 MHz, DMSO- d_6) δ 10.64 (s, 1H), 7.55 (s, 1H), 6.49 (s, 1H), 5.19 (d, J = 5.4 Hz, 1H), 5.00 (d, J = 3.8 Hz, 2H), 4.84 (d, J = 6.1 Hz, 1H), 4.44 (d, J = 10.9 Hz, 1H), 4.12 – 4.21 (m, 1H), 4.00 – 4.08 (m, 1H), 3.60 – 3.69 (m, 1H), 3.19 – 3.32 (m, 2H), 1.09 (s, 6H); ^{13}C -NMR (100 MHz, DMSO- D_6) δ 169.2, 162.5, 161.4, 128.4, 126.6, 104.2, 99.7, 79.5, 79.2, 78.9, 73.1, 71.8, 70.5, 69.8, 64.3, 22.4; HRMS (ESI+, m/z) calcd for $(\text{M}+\text{NH}_4)^+$: 716.2761; found: 716.2760.

6,6'-bis(4-hydroxy-3,5-ditertbutylbenzoyl)- α,α -D-trehalose (UM1087).—The diester was prepared according to the general procedure for esterification via triflate to obtain 6,6'-bis(4-hydroxy-5-tertbutylbenzoyl)- α,α -D-trehalose (88% for the two steps). ^1H NMR (400 MHz, DMSO- d_6) δ 7.78 (s, 2H), 7.75 (br. s., 1H), 5.20 (d, J = 5.5 Hz, 1H), 5.00 (t, J = 4.6 Hz, 2H), 4.85 (d, J = 6.1 Hz, 1H), 4.43 (d, J = 11.8 Hz, 1H), 4.11–4.18 (m, 1H), 3.9–4.07 (m, 1H), 3.60–3.69 (m, 1H), 3.27–3.32 (m, 1H), 3.16 – 3.27 (m, 1H), 1.33–1.41 (m, 18H); ^{13}C NMR (125MHz,DMSO- d_6) δ 166.0, 158.5, 138.2, 126.1, 120.5, 92.8, 72.7, 71.5, 70.4, 69.8, 63.7, 34.3, 29.9; HRMS (ESI+, m/z) calcd for $(\text{M}+\text{NH}_4)^+$: 824.4427; found 824.4424.

Transgenic HEK cell SEAP assays.—Human and mouse Mincle expressing HEK cells were obtained from Invivogen (San Deigo, CA). Cells were cultured according to the manufacturer's instructions in DMEM with 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, 100 mg/ml Normocin, 2 mM L-glutamine, 30 $\mu\text{g}/\text{ml}$ blasticidin, 1 $\mu\text{g}/\text{ml}$ puromycin and 1x HEK-Blue™ CLR Selection. For assay, indicated compounds were serially diluted in diluent (50% IPA, 50% isooctane), 20 μl of a 10x final concentration were applied to the bottom of a 96-well tissue culture plate and the solvent was evaporated for > 1 hr in a biosafety hood. HEK cells were applied to the plates at a density of 3×10^5 cells/well and incubated for 18–24 h at 37°C. Cell supernatants were harvested and analyzed via the manufacturer's instructions using Hek-Blue™ Detection. SEAP activity was assessed by reading the optical density (OD) at 620–655 nm with a microplate reader; data are expressed as the fold change in OD over vehicle treated cells.

Isolation of PBMC.—Peripheral blood samples were collected from healthy adult donors. The samples were collected after approval by the University of Montana Institutional Review Board (43–16) and signed written informed consent was obtained from each donor. Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood using Ficoll-Paque, as previously reported.^{40–41}

Cytokine analysis.—Cells were treated with the indicated compound concentrations by addition to plates containing serial dilution of the stock compound in diluent (50% isopropanol/50% isooctane), as described above in HEK cell section. For DMSO experiments compounds dissolved at in 10mM in DMSO and added to cells. Supernatants were harvested from treated cells 18–24 h post cell application. Supernatants were analyzed using either a DuoSet TNF α ELISA (R&D Systems, Minneapolis, MN) or a Luminex multiplex panel for analytes TNF α , IL-1 β , IL-6, and IL-23 (R&D Systems) per the manufacturer's instructions. Multiplex analysis was performed using a Luminex 200 instrument (Luminex Corporation). ELISAs were read on a plate reader at 450 nm and cytokine concentration was calculated by fitting the standard curve OD values to a 4-parameter logistical model using curve fitting software (XLfit, IDBS, Alameda, CA).

In vivo experiments.—Animal studies were carried out in an AAALAC accredited facility in accordance with University of Montana guidelines for the care and use of laboratory animals. Groups of 8 female Balb/c mice were vaccinated intramuscularly with 1 μ g M72 and 10 nmol indicated CLR adjuvant candidates formulated as DDA:DSPC liposomes in 50 μ l total volume per injection. After 14 days, a secondary vaccination was administered. At day 28 (14 days post-secondary vaccination) blood was collected for the measurement of M72 specific humoral immunity.

ELISA for anti-M72 antibody quantification.—Sera was collected and diluted according to the expected antibody response (between 1:10 and 1:5000). Plates were coated with 100 μ l of the full-length M72 protein at 1 μ g/mL. Following washing and blocking, plates were incubated with diluted serum for 1 hour followed by anti-mouse IgG, IgG1 or IgG2a-HRP secondary antibody (Bethyl Laboratories) and TMB substrate (KPL). Plates were read at 450 nm. Antibody titers were determined by calculating titer of each sample at OD 0.3.

Splenocyte restimulation and cell-mediated immunity analysis.—Spleens were harvested from vaccinated mice 5 days after secondary injections and splenocytes were harvested by disruption of the spleens through a 100 μ m filter. Red blood cells were lysed by incubation with red blood cell lysis buffer (Sigma) for 5 min followed by washing in 1 $\times$ PBS. Cells were plated in a 96 well plate at 5 $\times$ 10⁶ cells/well in 200 μ L complete RPMI1640 media. Cells were incubated with 1 μ g/mL M72 antigen for 7 hours at 37°C. After 7 hours, GolgiPlug (Brefeldin A, BD Biosciences) was added to each well and incubated at 37°C for a further 8 hours. Following incubation, cells were stained with the cell surface antibodies against CD3e PerCP-Cy5.5 (Tonbo Biosciences, 145–2C11), CD4a APC-Cy7 (Tonbo Biosciences, RM4–5) and CD8a PE-Cy7 (Tonbo Biosciences, 53–6.7) and viability stain (Ghost 510, Tonbo Biosciences). Cells were treated with cytofix/cytoperm (BD) and stained

with anti-IFN γ PE-CF594 (BD, XMG1.2), anti-IL2 FITC (Biolegend, JES6–5H4), anti-IL-5 PE (BD, TRFK5), anti-IL17A BV421 (Biolegend, TC11–18H10.1), and anti-TNF α APC (Invitrogen, MP6-XT22). Data was collected using an LSRII flow cytometer (BD) and analyzed using FlowJo 10.0 software (TreeStar).

Computational modeling procedures.—UM1024 was docked using GOLD docking software (version 5.2.2, www.ccdc.cam.ac.uk) into hMincle (protein data bank accession 3WH2) and bovine Mincle (protein data bank accession 4KZV), using the bound trehalose from the 4KZV crystal structure to define the binding site in both human and bovine Mincle. Docked poses were scored with ChemScore, with high-ranking poses filtered according to their conformational similarity to the bound trehalose in 4KZV. We included bound Ca²⁺ in all docking runs, and designated the following receptor side chains as flexible: glu136, glu169, asn171, arg183, asn193, leu199, tyr201 for bovine Mincle (4KZV), and glu135, asp165, glu168, leu172, arg182, asp183, phe198, met200 for hMincle (3WH2). We defined the binding site as within 10 Å from the bound trehalose from the 4KZV crystal structure, and 10 Å from a structural overlay of trehalose in the 3WH2 crystal structure. We used PyMol (pymol.org) to visualize the UM1024 poses and for the structural overlay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Th17	T-helper cell 17
IL-6	Interleukin 6
IL-1β	Interleukin 1 Beta
IL-23	Interleukin-23
RPMI	Roswell Park Memorial Institute
IgG	Immunoglobulin G

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Figure 1.
Representative Mincle ligands TDM, sTDCM, TDB, Vizantin, Brartemicin and C18MeBrar.

Figure 2.
Bovine Mincle (Protein data bank 4KZV) with trehalose (light blue) CRD.

Figure 3.

TNF α cytokine production in response to synthetic aryl trehalose compounds and TDB as reference in murine assays. A) The indicated compound was dissolved in IPA, serially diluted in IPA and then dried to the bottom of a tissue culture plate. B) The indicated compound was dissolved in DMSO, serially diluted in cell culture media. RAW cells were added to both conditions and incubated at 37 °C 18–24 hr followed by assessment of TNF α production by ELISA. C) The indicated compound was dissolved in isopropanol, serially diluted in vehicle and then dried to the bottom of a tissue culture plate. D) The indicated compound was dissolved in DMSO and serially diluted in cell culture media. Freshly prepared human PBMCs were added to both conditions and incubated at 37 °C. TNF α production was measured by ELISA, n=3 experimental replicates for A, B displayed as the average. Data presented in C, D are from an individual human donor and is representative of 3 donors evaluated.

Figure 4.

Cytokine production from primary human mononuclear cells in response to stimulation with synthetic trehalose diester compounds. The indicated compound was dissolved in 50% isopropanol/isooctane, serially diluted in vehicle and then dried to the bottom of a tissue culture plate. Cryopreserved PBMCs were thawed and applied to the compound-coated plates and incubated at 37°C; supernatant was harvested 24 h later and analyzed for TNF α , IL-1 β , IL-23, and IL-6 via a multiplex cytokine array (Luminex). Data presented are from an individual representative donor and are shown as percent maximal cytokine induced at each dose level.

Figure 5.

Cytokine production of UM1046–48, UM1068 and UM1087, Brartemycin, TDB and UM1024. A) The indicated compound was dissolved in ethanol, and dried to the bottom of a tissue culture plate. RAW cells were added and incubated at 37 °C for 18–24 hours. TNF α secretion was measured by ELISA. B) The indicated compound was dissolved in DMSO and serially diluted in media. RAW cells were added to culture wells and incubated at 37 °C for 18–24 hours. TNF α secretion was measured by ELISA. C) The indicated compound was dissolved in ethanol, and dried to the bottom of a tissue culture plate. hPBMCs were added and incubated at 37 °C for 18–24 hours. IL-6 secretion was measured by ELISA. D) The indicated compound was dissolved in DMSO and serially diluted in media and added to fresh hPBMCs. Cells were incubated at 37 °C for 18–24 hours after which IL-6 secretion was measured by ELISA. n=3 for all experiments and displayed as the average.

Figure 6.

Activation of mouse and human Mincle in response TDM, TDB and UM1024. The indicated compounds were plate coated and HEK cells transfected with human or mouse Mincle and an NF- κ B-driven SEAP reporter were incubated with the compounds for 24 h followed by assessment of the supernatants for SEAP levels. Data are represented as fold change in OD650 over vehicle treated cells. Graphs are mean values from three independent experiments $\pm$ SEM.

Figure 7. High-ranking docking pose of UM1024 (purple) in bovine Miniclin (Protein data bank 4KZV) overlays with trehalose (light blue) and maintains similar interactions with bound calcium (yellow).

Figure 8.

Serum antibody levels from mice in response to vaccination with recombinant M72 antigen and various MinCLE stimulating adjuvants. BALB/c mice, 10 per group, were vaccinated twice, IM, with 1 μ g antigen plus 10 nmol of the indicated adjuvant or volume-matched blank liposomes. Serum was analyzed 14 days post-secondary vaccination for presence of anti-M72 total IgG, IgG1, and IgG2a antibodies via ELISA.

Figure 9.

Induction of IL-17 producing CD4⁺ T cells from UM1024 adjuvanted M72 vaccine.

BALB/c mice, 10 per group, were immunized two times i.m. with 0.1 µg antigen plus 2, 10 or 50 nmol of the indicated adjuvant. Spleens were harvested from 3 mice per group at 5 days post-secondary vaccination. Splenocytes were restimulated with 1 µg whole antigen and transport inhibitors followed by surface staining for CD3, CD4 and CD8 and intracellular cytokine staining for IL-17A. Data represent percentage of live, CD3⁺/CD4⁺ cells that are also positive for IL-17A upon antigen restimulation.

Scheme 1.

Synthesis of aryl trehalose library

Reagents and conditions: (a) Aryl acid, *N,N'*-Dicyclohexylcarbodiimide, DPTS, methylene chloride; (b) Dowex 50W8X, methylene chloride, methanol.

Scheme 2.

Coupling of 2-hydroxy benzoic ads to trehalose

Reagents and conditions: (a) Aryl acid, potassium trimethylsilanolate; (b) i, trimethylsilyltriflate, methylene chloride, pyridine; ii, **2**, toluene, 18-crown-6, 80 °C; (c) Dowex 50W8X, methylene chloride, methanol.

Scheme 3.

Preparation of trehalose caffeic acid derivative UM1021

Reagents and conditions: (a) Aryl acid, DCC, DPTS, CH₂Cl₂; (b) DBU (c) Dowex 50W8X, CH₂Cl₂, MeOH

Scheme 4.

Expanded SAR of lead Miniclincholic acid agonist UM1024

Reagents and conditions: (a) Aryl acid, potassium trimethylsilanolate; (b) i, trimethylsilyltriflate, methylene chloride, pyridine; ii, **2**, toluene, 18-crown-6, 80 °C; (c) Dowex 50W8X, methylene chloride, methanol.