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Lipid length and iso-branching of trehalose diesters influences Mincle agonist activity

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ABSTRACT

We report on the efficient synthesis of linear trehalose diesters (TDEs) and iso-branched TDEs (maradolipids or iso-TDEs) and their ability to activate bone marrow-derived macrophages (BMDMs) as determined by cytokine (IL-1β, IL-12, IL-6, IL-10) and chemokine (MIP-2) production. Both classes of TDEs were found to activate BMDMs in a Mincle-dependent manner, with longer-chain (≥C18) lipids leading to a robust inflammatory response. On the whole, the iso-branched TDEs led to greater cytokine production and a faster immune response when compared to their linear counterparts. Moreover, C12-TDE and iso-C12-TDE elicited the production of MIP-2 by BMDMs, thereby providing the first example of TDEs with a chain length of ≤ C12 leading to a Mincle-dependent immune response and one that is less inflammatory in nature.

1. Introduction

Since the identification of the Macrophage inducible C-type lectin (Mincle, Clec4e, or Clecf9)[1,2] and the knowledge that the mycobacterial glycolipid, trehalose dimycolate (TDM, **1**, Fig. 1), binds and activates this receptor,[3] there has been much interest in the potential of Mincle agonists as immunostimulators and in understanding how changes to the ligand structure influences the ensuing immune response.[4] Notably, trehalose dibehenate (TDB, **2**, $n = 20$), which is a simplified trehalose diester (TDE), activates Mincle in a manner similar to TDM, with induction of the FcRγ-Syk-Card9-Bcl10-Malt1 signalling axis and a T helper (Th)-1-polarised immune response.[5–7] When formulated in dimethyldioctadecyl ammonium (DDA) liposomes,[8] TDB has found wide application as a vaccine adjuvant in a number of pre-clinical and clinical vaccination studies.[9,10] Other synthetic Mincle agonists, such as 6′-acylated mannose and 6′-acylated glucose sugars,[11] homogeneous TDMs, trehalose monomycolate and glucose mycolate,[12] also exhibit promising adjuvant activity, while β-GlcCer[13] and cholesterol derivatives[14,15] were recently identified as endogenous Mincle ligands, thereby highlighting the breadth of compounds found to activate this receptor.

To better understand the scope and specificity of the Mincle-binding site and how ligand binding correlates to a functional immune response, a number of potential Mincle ligands have been synthesised and their immunomodulatory properties assessed.[11,12,16–23] Early studies by our group revealed that TDEs with a carbon chain of ≥ C18 (but not ≤ C12) elicited a pro-inflammatory response by bone marrow derived macrophages (BMDMs),[16] and that trehalose monoesters activate macrophages in a Mincle-dependent manner.[17] The immunomodulatory profile of the Mincle ligands β-gentiobiosyl diacylglyceride,[18] glucose monocorynomycolate,[19] and 6′-acylated glucose and mannose monoesters[11] were also found to be influenced by lipid length. To a lesser extent, changing the mycolic acid structure altered the cytokine response of macrophages and dendritic cells (DCs) to TDMs[12,20] and trehalose monomycolates,[12] with modifications to stereochemistry also affecting Mincle agonist activity for both mycolic acid-containing glucolipids[12] and glycerol monocorynomycolates.[21]

Given the potential of TDEs to bind and activate Mincle, we became interested in exploring the ability of maradolipids to act as Mincle agonists. Maradolipids consist of a mixture of symmetrical

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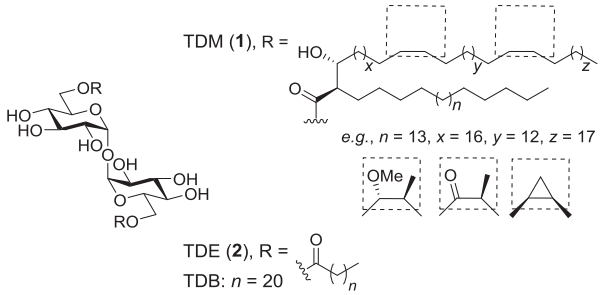


Fig. 1. TDM (**1**) and TDE (**2**).

and asymmetrical glycolipids containing mainly iso-branched and straight chain fatty acids (Fig. 2), and are produced by the dauer larva of the nematode *Caenorhabditis elegans*.[24,25] While maradolipids are structurally very similar to TDEs, structure-activity studies from previous Mincle-agonist work indicates that the incorporation of the iso-branch might be sufficient to give compounds with distinct immunomodulatory properties. Accordingly, we sought to prepare symmetrical iso-branched maradolipids **3a-f** (*i*Cn+1) and the linear TDEs (**2a**, $n = 9$; **2b**, $n = 11$; **2c**, $n = 15$) to assess their immunomodulatory profiles by measuring cytokine production by wild-type and Mincle$^{-/-}$ BMDMs. Herein, we determine that lipid length, as well as the presence or absence of the iso-branch, has a remarkable effect on the response of macrophages to TDEs.

2. Results and discussion

To synthesise the iso-branched and linear TDEs we envisioned using the strategy of Toubiana and co-workers to prepare 2,3,4,2′,3′,4′-hexa-*O*-trimethylsilyl-α,α′-trehalose in one step from α,α′-trehalose,[26] with subsequent elaboration to the target glycolipid.[16,27] To this end, bis(trimethylsilyl)acetamide (BSA) and catalytic tetrabutylammonium fluoride (TBAF) were used to persilylate α,α′-D-trehalose (**4**), with the most labile TMS groups at the 6- and 6′-positions being subsequently removed via the agency of K_2CO_3 to afford hexa-TMS protected trehalose **5** in a moderate yield (67% over two-steps, Scheme 1). Esterification with the commercially available iso-branched fatty acids **6a** and **6c-f**, or the C_{14+1} fatty acid **6b**, which can be synthesised via a Wittig reaction using the triphenylphosphonium salt derived from 11-bromoundecanoic acid and Ph_3P, and isobutyraldehyde, followed by hydrogenation,[28] then occurred under the mediation of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and 4-dimethylaminopyridene (DMAP) in toluene at 56 °C for 8–10 h to give the corresponding esters **8a-f** in good to excellent yield (71–90%) following purification by silica gel flash column chromatography. For each glycolipid, Heteronuclear Multiple Bond Correlations (HMBCs) between H-6a and H-6b of the trehalose moiety with the carbonyl carbons of the lipids confirmed the successful conjugation of the lipid moieties. The protected linear TDEs (**9a, b** and **d**) were prepared in a similar manner via the conjugation of hexa-TMS protected trehalose **5** with the commercially available carboxylic acids **7a**, ($n = 9$), **7b** ($n = 11$), and **7d** ($n = 15$).

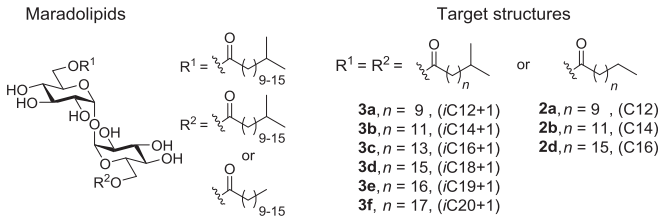
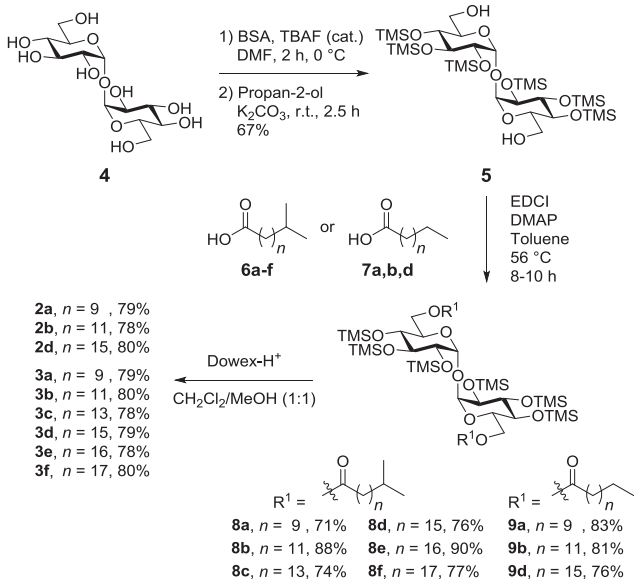


Fig. 2. Maradolipids and target structures.



Scheme 1. Synthesis of iso-branched maradolipids (iso-TDEs) and linear TDEs.

Deprotection of protected iso-TDEs **8a-f** was then achieved by using Dowex-H^+ in DCM and MeOH (1:1), which provided excellent yields of the desired iso-TDEs **3a-f** after purification by silica gel flash column chromatography. Again, 1D- and 2D-NMR techniques were used to analyse the products, and the presence of only one signal for the anomeric centres (at *ca.* $\delta = 5.9$ ppm) confirmed the formation of symmetric products. Formation of the linear TDEs (**2a, 2b** and **2d**) was achieved in an analogous manner, with the spectral data for **2b** and **2d** matching those previously reported.[16,29]

With the iso-branched maradolipids in hand, we then assessed the production of cytokines and chemokines by granulocyte-monocyte colony-stimulating factor (GM-CSF) BMDMs upon stimulation with the ligands. Here, the linear TDEs **2a** (C12), **2b** (C14), and **2d** (C18) could be directly compared to **3a** (*i*C12 + 1), **3b** (*i*C14 + 1) and **3d** (*i*C18 + 1) to ascertain whether the incorporation of a single methyl group affected the type and magnitude of immune response, while the C4 linear TDE, which we had previously synthesised and found not to activate BMDMs,[16,17] served as a negative control. TDB and LPS served as positive controls.

It is known that TDM and TDB lead to a pro-inflammatory Th-1 immune response upon the binding and activation of Mincle.[6,7] Accordingly, we first determined the production of the pro-inflammatory cytokine interleukin (IL)-1β by BMDMs upon stimulation with the glycolipids (Fig. 3A). To this end, the TDEs were assessed for their ability to activate BMDMs at two different concentrations (20 μM and 40 μM), with cytokine production being measured at 24 h and 48 h. Here, maradolipids **3a**,**b** (*i*C12 + 1 and *i*C14 + 1) and the corresponding linear TDEs (C12 and C14) did not result in significant IL-1β production at either time point, however cytokine production was observed by all TDEs with a lipid length ≥ C16. The iso-branched maradolipid **3d** (*i*C18 + 1) led to greater IL-1β production when compared to the analogous linear C18, while cytokine production upon BMDM stimulation with the longest iso-TDE (*i*C20 + 1) was similar to, if not better than, TDB. In all instances, IL-1β production was concentration dependent and

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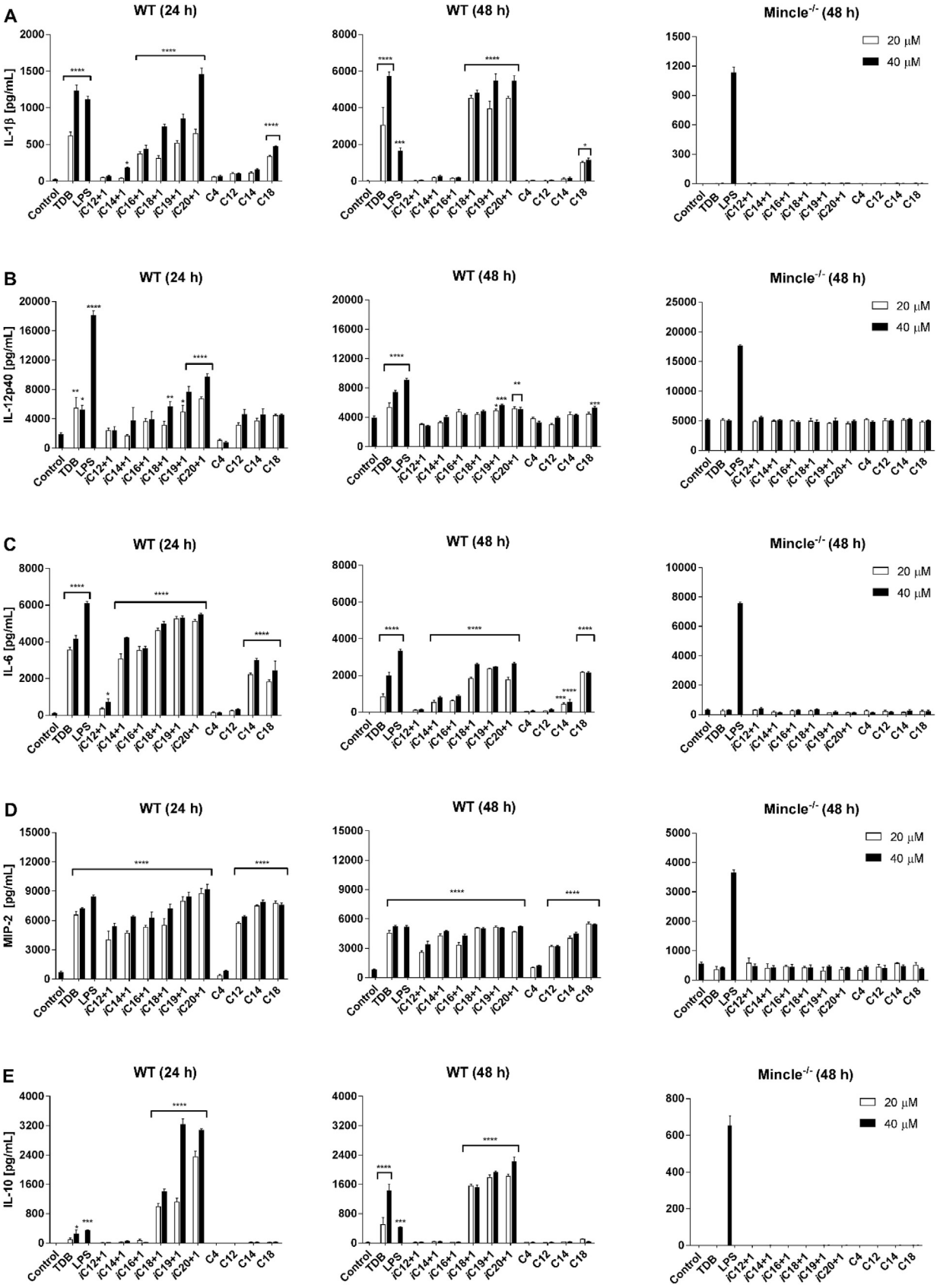

Fig. 3. WT and Mincle$^{-/-}$ BMMs were stimulated with linear or iso-branched TDEs (20 μM, 40 μM), or LPS (100 ng/mL) as a positive control. The production of IL-1β (**A**), IL-12p40 (**B**), IL-6 (**C**), MIP-2 (**D**) and IL-10 (**E**) were measured at 24 and 48 h using ELISA. Mean ± SEM of triplicate samples from a representative experiment of three performed are shown. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.005; ****P ≤ 0.001. Statistical significance was calculated in comparison to unstimulated cells.

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was also dependent on Mincle.

Next, the production of IL-12 by BMDMs in response to the TDEs was determined by measuring IL-12p40, which is the 40 kDa heavy-chain that makes up the IL-12 heterodimer.[30] The cytokine IL-12 has an important role in the development of the Th-1 immune response, and in particular, in inducing IFN-γ production by activated natural killer (NK) and T cells.[30] After 24 h, there was significant IL-12 production by BMDMs in response to the iso-TDEs with a chain length of ≥ C18, however, of the linear TDEs, only TDB led to a statistically significant increase in IL-12 (Fig. 3B). The amount of IL-12 produced by the BMDMs at 24 h in response to 40 μg/mL of maradolipids **3e** (*i*C19 + 1) and **3f** (*i*C20 + 1) was also significantly greater than that produced by TDB, however after 48 h, IL-12 production by BMDMs in response to the maradolipids had reached a plateau. Notwithstanding, IL-12 production at 72 h was greater for BMDMs stimulated with **3f** (*i*C20 + 1) than for cells stimulated with TDB at both concentrations of glycolipid (*data not shown*). Once again, IL-12 production was Mincle-dependent. Taken together, this suggests that the iso-TDEs elicit a greater and more rapid immune response than their non-branched counterparts.

The ability of GM-CSF BMDMs to produce the cytokine IL-6 and the macrophage inflammatory protein-2 (MIP-2 or CXCL2) upon stimulation with the glycolipids was then explored. Although mostly regarded as a pro-inflammatory cytokine, IL-6 also has regenerative or anti-inflammatory activities,[31] while MIP-2 is a chemotactic for polymorphonuclear leukocytes, such as neutrophils, and hematopoietic stem cells, including monocytes and macrophages.[32–34] At 24 h and 48 h, the iso- and linear-TDEs with chain lengths ≥ C14 led to the production of IL-6 in a Mincle-dependent manner (Fig. 3C). At 24 h, the production of IL-6 by BMDMs in response to the iso-TDEs **3a** (*i*C14 + 1), **4b** (*i*C16 + 1) and **4d** (*i*C18 + 1) was greater than that elicited by the corresponding TDE analogues **2a** (C14), **3b** (C16) and **3d** (C18), while comparable amounts of IL-6 were produced at 48 h for both the branched and non-branched TDEs. Absolute levels of IL-6 also appeared to decline for both the iso-TDEs and the linear-TDEs at 48 h. The production of MIP-2 by BMDMs upon stimulation with the glycolipids was also greater at 24 rather than 48 h, with both the iso- and linear-TDEs leading to comparable levels of this chemokine in a Mincle-dependent manner (Fig. 3D). In contrast to the production of cytokines IL-1β, IL-12 and IL-6, MIP-2 is produced by BMDMs in response to TDEs with a shorter chain length. Notably, the ability of both C12 TDE (**2a**) and *i*C12 + 1 (**3a**) to induce MIP-2 production by BMDMs with no (*i.e.* C12) or minor (*i.e.* *i*C12 + 1) production of any of the aforementioned cytokines is unprecedented.

Finally, the ability of the linear- and iso-TDEs to skew the immune response to more anti-inflammatory phenotype was explored, as indicated by the production of the potent anti-inflammatory cytokine IL-10.[35] Here, further differences between the responses elicited by BMDMs following stimulation with the TDEs and the iso-TDEs was observed (Fig. 3E). The iso-TDEs with chain length ≥ C18 led to the production of IL-10 in a Mincle-dependent manner at both the 24 h and 48 h time points, while the C18 linear TDE was unable to induce IL-10 production. Moreover, IL-10 production by BMDMs in response to *i*C18 + 1, *i*C19 + 1 and *i*C20 + 1 was greater than that produced by the BMDMs upon stimulation with TDB (**2**), particularly at lower concentrations of the glycolipids. It is well established that IL-10 plays an essential role in down-regulating the immune response to cytokines such as IL-12, so as to limit chronic and acute inflammation that could be detrimental to the host.[35,36] Given that the iso-TDEs appear to result in a more rapid immune response than their linear counterparts, it is not surprising that

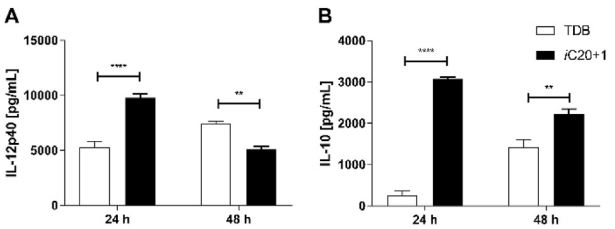

Fig. 4. A comparison of the production of IL-12p40 (**A**) and IL-10 (**B**) by WT BMMs, treated with 40 μM of TDB and *i*C20 + 1 at 24 and 48 h. Mean ± SEM of triplicate samples from a representative experiment of three performed.

the regulation of the inflammatory response via the production of IL-10 occurs at an earlier time point as well. This is illustrated by the more direct comparison of IL-12 and IL-10 production at 24 and 48 h for two representative TDEs, TDB and *i*C20 + 1 (Fig. 4A and B), whereby both IL-12 and IL-10 production by *i*C20 + 1 is greater at 24 rather than 48 h, which is also reflected in the amount of IL-10 produced at these time points. Conversely, TDB appears to have slower kinetics, with slower onsets of both IL-12 and IL-10. There have only been few studies on IL-10 production by macrophages in response to TDB, with IL-10 being produced by the human THP-1 cell line upon treatment with TDB,[37] and with beads coated with TDB promoting IL-10 production by BMDMs, thereby down-regulating IL-12 secretion in response to the Toll-like receptor (TLR)-2 ligand Pam_3CSK_4.[38] Thus, our findings support these earlier observations and further demonstrate the reciprocal relationship between the production of IL-12 and IL-10.

Taken together, our findings further support the observation that minor changes to Mincle-ligands can affect the activation status of macrophages. In particular, we demonstrate that lipid length and the iso-branching of TDEs can result in immuno stimulatory compounds with quite distinct biological profiles. As was first described in our earlier studies exploring the ability of linear TDEs to activate BMDMs,[16] the pro-inflammatory immune response is most notable for both the linear- and iso-branched TDEs of medium to long lipid length. Herein, we also demonstrate that this immune response is Mincle-dependent for both classes of TDE. While there are subtle differences between the type and amount of cytokines produced for each respective glycolipid, on the whole, the most robust pro-inflammatory immune responses are observed for the linear TDEs with a lipid length of ≥C18, while iso-TDEs of a slightly shorter chain length (*i.e.* ≥ C14) are able to induce a similar response. Moreover, on the whole, a more rapid immune response is observed in response to the iso-TDEs, as compared to their linear counterparts, with cytokine production at 24 h typically being higher for the iso-derivatives. When directly comparing cytokine and chemokine production elicited by BMDMs in response to *i*C18 + 1 and the linear C18 TDE, *i*C18 + 1 generally leads to a stronger immune response at both 24 and 48 h. The reason for this is unclear, and while one could speculate that the lipophilic iso-branch is better accommodated in the major hydrophobic grove adjacent to the carbohydrate-binding domain of the Mincle-binding site,[39–41] this major hydrophobic groove is best suited for binding the first few methylenes of the lipid, not so much their termini. Thus, it is possible that the difference in immune response between the linear and iso-branched TDEs is physiochemical in nature, and is due to the increased fluidity that iso-branching typically confers on the membrane.[42] Notwithstanding, our data indicates that while lipid length remains an important consideration in the development of Th-1-promoting Mincle-agonist, the addition of an iso-branch further supports agonist activity *in vitro*. More advanced *in vivo* studies would be required to determine

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whether the ability of the longer chain iso-TDEs to more rapidly engage the immune response is beneficial in terms of vaccine development, however, it would seem intuitive that this is the case given that the innate immune response enhances the activity of antigen-presenting cells and the ensuing T cell response.[43]

Another key observation from our studies is the ability of medium length TDEs, such as C12 and *i*C12 + 1, to lead to the induction of the chemokine MIP-2, and to a lesser extent IL-6, by BMDMs in the absence of other inflammatory cytokines. In the case of C12-TDE, only MIP-2 was produced by the BMDMs. MIP-2 elicits chemotaxis, and thus the ability of C12- and *i*C12 + 1 to recruit immune cells such as neutrophils and macrophages in the absence of inflammatory cytokines may have unique applications, including wound healing. Inflammatory signals are required to recruit immune cells for tissue repair and the removal of debris, however, sustained inflammation can lead to maladaptive processes like loss of functional parenchyma, fibrosis and carcinogenesis in a variety of organs.[44] In the same way that Toll-like-receptor (TLR) ligands can impair or contribute to the healing process depending on the exact type of TLR and its associated ligand,[45] it is possible that C12-TDE or *i*C12 + 1 could enhance wound healing in situations where immune cell recruitment but a limited inflammatory response are required. Moreover, the production of cytokines and/or chemokines by BMDMs in response to C12 and *i*C12 + 1 illustrates, for the first time, that medium-length TDEs can activate macrophages in a Mincle-dependent manner. Thus, to fully explore the potential of TDEs as adjuvants or other immunostimulating agents, it is prudent to assay for a variety of cytokines and chemokines.

In conclusion we have synthesised a variety of linear- and iso-branched-TDEs in good yields and determined that both classes of TDEs activate macrophages in a Mincle-dependent manner. Notably, the iso-TDEs lead to greater cytokine production by BMDMs when compared to their analogous linear counterparts, and moreover, the iso-TDEs had more rapid kinetics. A long lipid (≥C18) is required for a robust inflammatory immune response by both the linear and iso-TDEs, however it was observed that the C12 and *i*C12 + 1 TDEs elicited the production of MIP-2 by BMDMs. This is the first time that TDEs with a chain length of ≤C12 have been shown to lead to a Mincle-dependent functional immune response and one that is less inflammatory in nature. While the translation of this work to more advanced *in vivo* assays remains, our findings nonetheless suggest that long-chain iso-TDEs may have superior adjuvant activity, while the C12 linear and iso-branched TDEs may have application in situations such as wound healing, where immune cell recruitment along with a minimal inflammatory response is required.

3. Experimental

3.1. General chemical

All reactions were performed under an atmosphere of argon. Prior to use, methanol (Fisher Scientific) was distilled. Toluene (ROMIL) was dried and stored over Na wire, and the following solvents were distilled: acetone, ethyl acetate (EtOAc) and petroleum ether (PE). α,α′-D-Trehalose dihydrate (Sigma), behenic acid (BDH Biochem), $MgSO_4$ (Pure Science), NaCl (Chem Solute), Et_2O (LabServ), BSA (Aldrich), $Pd(OH)_2/C$ (Acros Organics), DMAP (Lab Supply), EDCI (Chem Impex), AcOH (Pancreac), $NaHCO_3$ (Pure Science), HCl (Chem Solute), isopropanol (Fischer Scientific), $KMnO_4$ (AnalR), 11-bromoundecanoic acid (Aldrich), 11-methyldodecanosanoic acid (Larodan AB), 15-methylhexadecanoic acid (Larodan AB), 17-methyloctadecanoic acid (Larodan AB), 18-methylnonadecanoic acid (Larodan AB), 19-methyleicosanoic acid (Larodan AB), PPh_3 (Acros Organics), BuLi (2 M in cyclohexane, Alridch), isobutyraldehyde (Aldrich), pyridine (ROMIL), C_5D_5N (Apollo), CD_3OD (Cambridge Isotopes Laboratories Inc.), $CDCl_3$ (Aldrich) were used as received. Reactions were monitored by TLC analysis on Macherey-Nagel silica gel coated plastic sheets (0.20 mm with fluorescent indicator UV_{254}) via detection by UV absorbtion (254 nm) where relevant and dipping in 10% H_2SO_4 in EtOH followed by charring or dipping in a solution of $KMnO_4$ (0.05 M), K_2CO_3 (0.4 M), and NaOH (0.06%) in water. Column chromatography was performed using Pure Science silica gel (40–63 μm). All solvents were removed by evaporation under reduced pressure. High resolution mass spectra were recorded on an Agilent 6530 Q-TOF mass spectrometer utilising a JetStream™ electro-spray ionisation (ESI) source in positive or negative mode. Optical rotations were recorded on a Autopol II (Rudolph Research Analytical) at 589 nm (sodium Dline). Infrared (IR) spectra were recorded as thin films using either a Bruker Platinum-ATR spectrometer and are reported in wave numbers (cm^{-1}). Nuclear magnetic resonance spectra were obtained at 20 °C in $CDCl_3$, C_5D_5N, D_2O, or CD_3OD using a Varian INOVA operating at 500 MHz. Chemical shifts are given in ppm (δ) relative to the solvent residual peak. NMR peak assignments were made using COSY, HSQC, and HMBC 2D experiments.

3.2. 13-Methyltetradecanosanoic acid (**6b**)

11-Bromoundecanoic acid (500 mg, 2 mmol) and triphenyl phosphine (530 mg, 2 mmol) were combined and heated to 100 °C for 10 min under an Ar atmosphere. The resulting yellow oil was dissolved in 10 mL of dry DMSO and heated for 10 min. The solution was cooled to room temperature and butyl lithium (2.0 M in cyclohexane, 2.5 mL, 5 mmol) was added drop wise over the course of 1 h, with each addition leading to an orange colouration appearing in the reaction mixture. The reaction was stirred for half an hour and then isobutyraldehyde (0.50 mL, 5.0 mmol) was added slowly. The reaction remained yellow after the addition of isobutyraldehyde was completed and the mixture was stirred for 2 h. The suspension was diluted with EtOAc, and washed with 1 M HCl and brine solution. The combined aqueous layers were extracted with EtOAc (2 × 20 mL), and the combined organic phases were dried with anhydrous magnesium sulfate, filtered, and concentrated *in vacuo* to give a yellow oil. The crude product was purified by silica gel flash chromatography (PE to PE/Acetic acid, 99:1, *v/v*) to yield *E*/*Z*-13-methyltetradec-11-enoic acid as a white solid. *E*/*Z*-13-methyltetaredec-11-enoic acid was dissolved in DCM (5 mL), $Pd(OH)_2/C$ (57.6 mg, 22 wt% Pd) was added, and the black suspension put under an atmosphere of H_2 and stirred for 21 h. The suspension was filtered through Celite and concentrated to give a white solid. The crude product was recrystallised from acetone to give 13-methyltetradecanoic acid as a crystalline white solid (223 mg, 0.92 mmol, 46%). R_f = 0.24 (PE/EtOAc, 9:1, v/v); Mp = 51.0–51.9 °C (lit.[28] 51.5–51.7 °C); IR (film) = 2917, 2850, 1698, 1472 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 2.3 (t, $J_{2,3}$ = 7.7 Hz, 2H, H-2), 1.63 (p, $J_{3,2} = J_{3,4}$ = 7.3 Hz, 2H, H-3), 1.51 (m, 1H, H-13), 1.37–1.21 (m, 16H, H-4–H-11), 1.18–1.12 (m, 2H, H-12), 0.86 (d, $J_{13,14}$ = 6.7 Hz, 6H, H-14); ^{13}C NMR (125 MHz, $CDCl_3$) δ 179.0 (C-1), 39.0 (C-12), 33.89 (C-2), 29.9, 29.69, 29.65, 29.64, 29.59, 29.4, 29.2, 29.1 (C-4–C10), 28 (C-13), 27.4 (C-11), 24.7 (C-3), 22.7 (C-14); HRMS (ESI) *m/z* calculated for $[C_{15}H_{29}O]^-$: 241.2173 found 241.2168. The data corresponded to those published.[28]

3.3. General esterification procedure

Maradolipids of different chain lengths were synthesised

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according to a procedure by Khan et al.[16] 2,2′,3,3′,4,4′-hexa-*O*-trimethylsilyl-α,α′-D-trehalose (1 mmol, 1 equiv.) and carboxylic acid (4.4 mmol, 4 equiv.) were co-evaporated together with dry toluene (1 mL), then suspended in dry toluene (5 mL). To the reaction mixture, EDCI (6.6 mmol, 6.6 equiv.) and DMAP (1 mmol, 1 equiv.) were added and the resulting suspension was heated to 56 °C until TLC analysis (PE/EtOAc, 9:1, v/v) showed complete conversion of the starting material to a higher running product. The reaction was cooled to r.t. and diluted with of Et_2O (5 mL). The organic layer was then washed with water (5 mL), sat. aq. $NaHCO_3$ (5 mL), and brine (5 mL). The combined aqueous phases were re-extracted with of Et_2O (5 mL) and the combined organic phases were dried with anhydrous $MgSO_4$, filtered, and concentrated *in vacuo*. The product was purified using gradient silica-gel column chromatography (PE to PE/EtOAc, 9:1, *v/v*).

3.4. 6,6′-Di-O-11-methyldodecanoyl-2,2′,3,3′,4,4′-hexa-O-trimethylsilyl-α,α′-D-trehalose (**8a**)

By subjecting trehalose diol **5** (80 mg, 0.10 mmol), 11-methyldodecanoic acid **6a** (97 mg, 0.44 mmol), EDCI (130 mg, 0.66 mmol) and DMAP (12.6 mg, 0.103 mmol) to the general procedure for esterification (8 h), the title compound **8a** was obtained as a yellow oil (52.9 mg, 0.072 mmol, 71%). $R_f = 0.8$ (PE/EtOAc, 9:1, v/v); $[\alpha]_D^{23.1} = +50$ ($c = 0.1$ $CHCl_3$); IR (film) = 2954, 2924, 2854, 1738, 1459, 1250, 1163, 1110, 1075, 1044, 1009, 963, 897, 871, 841, 747, 683 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 4.92 (d, $J_{1,2} = 3.1$ Hz, 2H, H-1), 4.27 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6a} = 2.0$ Hz, 2H, H-6a), 4.06 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6b} = 4.5$ Hz, 2H, H-6b), 4.02–3.97 (m, 2H, H-5), 3.90 (t, $J_{2,3} = J_{3,4} = 9.0$ Hz, 2H, H-3), 3.48 (t, $J_{3,4} = J_{4,5} = 9.0$ Hz, 2H, H-4), 3.44 (dd, $J_{2,3} = 9.0$ Hz, $J_{1,2} = 3.0$ Hz, 2H, H-2), 2.40–2.28 (m, 4H, H-8), 1.66–1.58 (m, 4H, H-9), 1.54–1.47 (m, 2H, H-17), 1.35–1.21 (m, 24H, H-10–H-15), 1.18–1.11 (m, 4H, H-16), 0.86 (d, $J_{17,18} = 6.7$, 12H, H-18), 0.15 (s, 18H, TMS), 0.135 (s, 18H, TMS), 0.130 (s, 18H, TMS); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.8 (C-7), 94.3 (C-1), 73.4 (C-3), 72.6 (C-2), 71.8 (C-4), 70.7 (C-5), 63.2 (C-6), 39.0 (C-16), 34.1 (C-8), 29.8, 29.6, 29.4, 29.3, 29.1 (C-10–C-14), 27.9 (C-17), 27.4 (C-15), 24.7 (C-9), 22.6 (C-18), 1.0, 0.8, 0.1 (TMS); HRMS (ESI) *m/z* calculated for $[C_{56}H_{118}NaO_{13}Si_6]^+$: 1189.7080, found 1189.7083.

3.5. 6,6′-Di-O-13-methyltetradecanoyl-2,2′,3,3′,4,4′-hexa-O-trimethylsilyl-α,α′-D-trehalose (**8b**)

By subjecting trehalose diol **5** (80 mg, 0.103 mmol), 13-methyltetradecanoic acid **6b** (112.5 mg, 0.46 mmol), EDCI (130 mg, 0.66 mmol) and DMAP (12.6 mg, 0.10 mmol) to the general procedure for esterification (8 h), the title compound **8b** was obtained as a colourless oil (111.7 mg, 0.091 mmol, 88%). $R_f = 0.6$ (PE/EtOAc, 9:1, v/v); $[\alpha]_D^{21.8} = +60$ ($c = 0.1$ $CHCl_3$); IR (film) = 2923, 2853, 1741, 1250, 1164, 1076, 871, 843, 747 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 4.93 (d, $J_{1,2} = 3.1$ Hz, 2H, H-1), 4.29 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6a} = 2.0$ Hz, 2H, H-6a), 4.07 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6b} = 4.5$ Hz, 2H, H-6b), 4.03–3.99 (m, 2H, H-5), 3.92 (t, $J_{2,3} = J_{3,4} = 9.0$ Hz, 2H, H-3), 3.49 (t, $J_{3,4} = J_{4,5} = 9.0$ Hz, 2H, H-4), 3.45 (dd, $J_{2,3} = 9.0$ Hz, $J_{1,2} = 3.0$ Hz, 2H, H-2), 2.41–2.29 (m, 4H, H-8), 1.66–1.58 (m, 4H, H-9), 1.57–1.48 (m, 2H, H-19), 1.37–1.23 (m, 32H, H-10–H-17), 1.19–1.13 (m, 4H, H-18), 0.87 (d, $J_{19,20} = 6.7$, 12H, H-20), 0.16 (s, 18H, TMS), 0.15 (s, 18H, TMS), 0.14 (s, 18H, TMS); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.8 (C-7), 94.3 (C-1), 73.5 (C-3), 72.6 (C-2), 71.9 (C-4), 70.7 (C-5), 63.2 (C-6), 39.0 (C-18), 34.6 (C-8), 29.9, 29.7, 29.6, 29.4, 29.3, 29.1 (C-10–C-16), 27.9 (C-19), 27.4 (C-17), 24.8 (C-9), 22.6 (C-20), 1.0, 0.8, 0.1 (TMS); HRMS (ESI) *m/z* calculated for $[C_{60}H_{126}NaO_{13}Si_6]^+$: 1245.7706, found 1245.7700.

3.6. 6,6′-Di-O-15-methylhexadecanoyl-2,2′,3,3′,4,4′-hexa-O-trimethylsilyl-α,α′-D-trehalose (**8c**)

By subjecting diol **5** (65 mg, 0.084 mmol), 15-methylhexadecanoic acid **6c** (98 mg, 0.4 mmol), EDCI (106 mg, 0.55 mmol) and DMAP (10 mg, 0.084 mmol) to the general procedure for esterification (8 h), the title compound **8c** was obtained as a colourless oil (79.6 mg, 0.062 mmol, 74%). $R_f = 0.8$ (PE/EtOAc, 9:1, v/v); $[\alpha]_D^{23} = +140$ ($c = 0.1$, $CHCl_3$); IR (film) = 2953, 2924, 2841, 1741, 1249, 1109, 1099, 1075, 1008, 897, 870, 837, 746 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 4.92 (d, $J_{1,2} = 3.1$ Hz, 2H, H-1), 4.27 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6a} = 2.0$ Hz, 2H, H-6a), 4.05 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6b} = 4.4$ Hz, 2H, H-6b), 4.02–3.97 (m, 2H, H-5), 3.90 (t, $J_{2,3} = J_{3,4} = 9.0$ Hz, 2H, H-3), 3.48 (t, $J_{3,4} = J_{4,5} = 9.0$ Hz, 2H, H-4), 3.44 (dd, $J_{2,3} = 9.0$ Hz, $J_{1,2} = 3.0$ Hz, 2H, H-2), 2.39–2.28 (m, 4H, H-8), 1.69–1.58 (m, 4H, H-9), 1.56–1.47 (m, 2H, H-21), 1.38–1.20 (m, 40H, H-10–H-19), 1.19–1.11 (m, 4H, H-20), 0.86 (d, $J_{21,22} = 6.7$, 12H, H-22), 0.15 (s, 18H, TMS), 0.135 (s, 18H, TMS), 0.130 (s, 18H, TMS); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.8 (C-7), 94.5 (C-1), 73.5 (C-3), 72.6 (C-2), 71.9 (C-4), 70.7 (C-5), 63.2 (C-6), 39.05 (C-20), 34.1 (C-8), 29.9, 29.68, 29.6, 29.4, 29.3, 29.1 (C-10–C-18), 27.9 (C-21), 27.4 (C-19), 24.7 (C-9), 22.6 (C-22), 1.0, 0.8, 0.1 (TMS); HRMS (ESI) *m/z* calculated for $[C_{64}H_{134}NaO_{13}Si_6]^+$: 1301.8332, found 1301.8363.

3.7. 6,6′-Di-O-17-methyloctadecanoyl-2,2′,3,3′,4,4′-hexa-O-trimethylsilyl-α,α′-D-trehalose (**8d**)

By subjecting diol **5** (60 mg, 0.077 mmol), 17-methyloctadecosanoic acid **6d** (101 mg, 0.34 mmol), EDCI (98 mg, 0.5 mmol) and DMAP (9.4 mg, 0.077 mmol) to the general procedure for esterification (10 h), the title compound **8d** was obtained as a colourless oil (86 mg, 0.064 mmol, 76%). $R_f = 0.8$ (PE/EtOAc, 9:1, v/v); $[\alpha]_D^{23.9} = +96$ ($c = 0.1$, $CHCl_3$); IR (film) = 2923, 1742, 1249, 1163, 1075, 1008, 870, 837, 747 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 4.92 (d, $J_{1,2} = 3.1$ Hz, 2H, H-1), 4.27 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6a} = 2.0$ Hz, 2H, H-6a), 4.06 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6b} = 4.4$ Hz, 2H, H-6b), 4.02–3.98 (m, 2H, H-5), 3.90 (t, $J_{2,3} = J_{3,4} = 9.0$ Hz, 2H, H-3), 3.48 (t, $J_{3,4} = J_{4,5} = 9.0$ Hz, 2H, H-4), 3.44 (dd, $J_{2,3} = 9.0$ Hz, $J_{1,2} = 3.0$ Hz, 2H, H-2), 2.40–2.28 (m, 4H, H-8), 1.66–1.58 (m, 4H, H-9), 1.56–1.47 (m, 2H, H-23), 1.38–1.19 (m, 48H, H-10–H-21), 1.18–1.11 (m, 4H, H-22), 0.86 (d, $J_{23,24} = 6.7$, 12H, H-24), 0.15 (s, 18H, TMS), 0.135 (s, 18H, TMS), 0.130 (s, 18H, TMS); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.8 (C-7), 94.3 (C-1), 73.5 (C-3), 72.6 (C-2), 71.9 (C-4), 70.7 (C-5), 63.3 (C-6), 39.0 (C-22), 34.1 (C-8), 29.9, 29.73, 29.7, 29.6, 29.4, 29.3, 29.1 (C-10–C-20), 27.9 (C-23), 27.4 (C-21), 24.7 (C-9), 22.6 (C-24), 1.0, 0.8, 0.1 (TMS); HRMS (ESI) *m/z* calculated for $[C_{68}H_{142}NaO_{13}Si_6]^+$: 1357.8953, found 1357.8918.

3.8. 6,6′-Di-O-18-methylnonadecanoyl-2,2′,3,3′,4,4′-hexa-O-trimethylsilyl-α,α′-D-trehalose (**8e**)

By subjecting diol **5** (60 mg, 0.077 mmol), 18-methylnonadecanoic acid **6e** (106 mg, 0.34 mmol), EDCI (98 mg, 0.5 mmol) and DMAP (9.4 mg, 0.077 mmol) to the general procedure for esterification (10 h), the title compound **8e** was obtained as a colourless oil (95.5 mg, 0.069 mmol, 90%). $R_f = 0.6$ (PE/EtOAc, 9:1, v/v); $[\alpha]_D^{24.7} = +150$ ($c = 0.1$, $CHCl_3$); IR (film) = 2923, 2853, 1742, 1250, 1164, 1076, 1010, 842, 841, 748 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 4.92 (d, $J_{1,2} = 3.1$ Hz, 2H, H-1), 4.28 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6a} = 2.0$ Hz, 2H, H-6a), 4.06 (dd, $J_{6a,6b} = 12.0$ Hz, $J_{5,6b} = 4.4$ Hz, 2H, H-6b), 4.02–3.97 (m, 2H, H-5), 3.90 (t, $J_{2,3} = J_{3,4} = 9.0$ Hz, 2H, H-3), 3.48 (t, $J_{3,4} = J_{4,5} = 9.0$ Hz, 2H, H-4), 3.44 (dd, $J_{2,3} = 9.0$ Hz, $J_{1,2} = 3.0$ Hz, 2H, H-2), 2.40–2.28 (m, 4H, H-8), 1.66–1.58 (m, 4H, H-9), 1.56–1.47 (m, 2H, H-24), 1.36–1.20 (m, 52H, H-10–H-22),

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1.18–1.10 (m, 4H, H-23), 0.86 (d, $J_{24,25}$ = 6.7, 12H, H-25), 0.15 (s, 18H, TMS), 0.135 (s, 18H, TMS), 0.130 (s, 18H, TMS); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.8 (C-7), 94.3 (C-1), 73.5 (C-3), 72.6 (C-2), 71.9 (C-4), 70.7 (C-5), 63.3 (C-6), 39.0 (C-23), 34.1 (C-8), 29.9, 29.73, 29.70, 29.69, 29.66, 29.6, 29.4, 29.3, 29.1 (C-10–C-21), 27.9 (C-24), 27.4 (C-22), 24.7 (C-9), 22.6 (C-25), 1.0, 0.87, 0.18 (TMS); HRMS (ESI) *m/z* calculated for $[C_{70}H_{146}NaO_{13}Si_6]^+$: 1185.9271, found 1185.9294.

3.9. 6,6′-Di-O-19-methyleicosanoyl-2,2′,3,3′,4,4′-hexa-O-trimethylsilyl-α,α′-D-trehalose (**8f**)

By subjecting diol **5** (60 mg, 0.077 mmol), 19-methyleicosanoic acid **6f** (100 mg, 0.36 mmol), EDCI (98 mg, 0.5 mmol) and DMAP (9.4 mg, 0.077 mmol) to the general procedure for esterification (10 h), the title compound **8f** was obtained as a colourless oil (82.5 mg, 0.059 mmol, 77%). R_f = 0.6 (PE/EtOAc, 9:1, v/v); $[\alpha]_D^{25.5}$ = +121 (*c* = 0.1, $CHCl_3$); IR (film) = 2923, 2854, 1736, 1250, 1162, 1075, 870, 840, 737 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.92 (d, $J_{1,2}$ = 3.1 Hz, 2H, H-1), 4.28 (dd, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6a}$ = 2.0 Hz, 2H, H-6a), 4.06 (dd, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6b}$ = 4.4 Hz, 2H, H-6b), 4.02–3.97 (m, 2H, H-5), 3.90 (t, $J_{2,3}$ = $J_{3,4}$ = 9.0 Hz, 2H, H-3), 3.48 (t, $J_{3,4}$ = $J_{4,5}$ = 9.0 Hz, 2H, H-4), 3.44 (dd, $J_{2,3}$ = 9.0 Hz, $J_{1,2}$ = 3.0 Hz, 2H, H-2), 2.40–2.27 (m, 4H, H-8), 1.67–1.57 (m, 4H, H-9), 1.55–1.47 (m, 2H, H-25), 1.36–1.20 (m, 56H, H-10–H-25), 1.18–1.11 (m, 4H, H-24), 0.86 (d, $J_{25,26}$ = 6.7, 12H, H-26), 0.15 (s, 18H, TMS), 0.135 (s, 18H, TMS), 0.130 (s, 18H, TMS); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.8 (C-7), 94.3 (C-1), 73.5 (C-3), 72.6 (C-2), 71.9 (C-4), 70.7 (C-5), 63.3 (C-6), 39.0 (C-24), 34.1 (C-8), 29.9, 29.73, 29.70, 29.66, 29.63, 29.4, 29.3, 29.1 (C-10–C-22), 27.9 (C-25), 27.4 (C-23), 24.7 (C-9), 22.6 (C-26), 1.0, 0.8, 0.1 (TMS); HRMS (ESI) *m/z* calculated for $[C_{72}H_{150}NaO_{13}Si_6]^+$: 1413.9584, found 1413.9587.

3.10. General desilylation procedure

To a solution of TMS protected maradolipids in CH_2Cl_2:MeOH (5 mL, 1/1, v/v) Dowex-H$^+$ (10% by weight) was added and the reaction was stirred at room temperature. After 30 min, the reaction mixture was filtered and concentrated *in vacuo*. The residue was subjected to gradient silica-gel column chromatography (EtOAc to EtOAc/MeOH, 9:1, v/v).

3.11. 6,6′-Di-O-11-methyldodecanoyl-α,α′-D-trehalose (**3a**)

By subjecting **8a** (50 mg, 0.068 mmol) to the general procedure for desilylation, the title compound **3a** was obtained as a white solid (40 mg, 0.054 mmol, 79%). R_f = 0.8 (EtOAc/MeOH, 9:1, v/v); $[\alpha]_D^{21.8}$ = +5 (*c* = 1.0, C_5H_5N); IR (film) = 3325, 2922, 2852, 1727, 1463, 1383, 1365, 1250, 1151, 1109, 988, 756, 721 cm^{-1}; ^{1}H NMR (500 MHz, C_5H_5N) δ 5.90 (d, $J_{1,2}$ = 3.7 Hz, 2H, H-1), 5.14–5.09 (m, 2H, H-5), 5.06–4.98 (m, 2H, H-6a), 4.85 (dd, $J_{6a,6b}$ = 11.7 Hz, $J_{5,6b}$ = 5.5 Hz, 2H, H-6b), 4.77 (t, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, 2H, H-3), 4.33 (dd, $J_{2,3}$ = 9.5 Hz, $J_{1,2}$ = 3.7 Hz, 2H, H-2), 4.19 (t, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, 2H, H-4), 2.39–2.28 (m, 4H, H-8), 1.63 (p, $J_{8,9}$ = $J_{9,10}$ = 7.3 Hz, 4H, H-9), 1.54–1.46 (m, 2H, H-17), 1.36–1.13 (m, 28H, H-10–H-16), 0.88 (d, $J_{17,18}$ = 6.5, 12H, H-18); ^{13}C NMR (125 MHz, C_5H_5N) δ 174.1 (C-7), 96.2 (C-1), 75.3 (C-3), 73.8 (C-2), 72.4 (C-4), 72.0 (C-5), 64.8 (C-6), 39.6 (C-16), 34.8 (C-8), 30.6, 30.3, 30.1, 30.0, 29.8 (C-10–C-14), 28.6 (C-17), 28.1 (C-15), 25.7 (C-9), 23.2 (C-18); HRMS (ESI) *m/z* calculated for $[C_{38}H_{74}NO_{13}]^+$: 752.5155, found 752.5153.

3.12. 6,6′-Di-O-13-methyltetradecanoyl-α,α′-D-trehalose (**3b**)

By subjecting **8b** (100 mg, 0.082 mmol) to the general procedure for desilylation, the title compound **3b** was obtained as a white solid (52 mg, 0.066 mmol, 80%). R_f = 0.63 (EtOAc/MeOH, 9:1, v/v); $[\alpha]_D^{24.8}$ = +7 (*c* = 0.1, C_5H_5N); IR (film) = 3334, 2917, 2849, 1727, 1467, 1383, 13,366, 1214, 1170, 1154, 1101, 1022, 984, 938, 912, 806, 754 cm^{-1}; ^{1}H NMR (500 MHz, C_5H_5N) δ 5.91 (d, $J_{1,2}$ = 3.7 Hz, 2H, H-1), 5.14–5.09 (m, 2H, H-5), 5.04–5.00 (m, 2H, H-6a), 4.85 (dd, $J_{6a,6b}$ = 11.7 Hz, $J_{5,6b}$ = 5.4 Hz, 2H, H-6b), 4.77 (t, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, 2H, H-3), 4.33 (dd, $J_{2,3}$ = 9.5 Hz, $J_{1,2}$ = 3.7 Hz, 2H, H-2), 4.19 (t, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, 2H, H-4), 2.39–2.28 (m, 4H, H-8), 1.63 (p, $J_{8,9}$ = J9,10 = 7.3 Hz, 4H, H-9), 1.54–1.46 (m, 2H, H-19), 1.36–1.13 (m, 36H, H-10–H-18), 0.88 (d, $J_{19,20}$ = 6.5, 12H, H-20); ^{13}C NMR (125 MHz, C_5H_5N) δ174.3 (C-7), 96.4 (C-1), 75.5 (C-3), 74.0 (C-2), 72.6 (C-4), 72.1 (C-5), 65.0 (C-6), 39.9 (C-18), 35.0 (C-8), 30.8, 30.6, 30.61, 30.5, 30.4, 30.2, 30.0 (C-10–C-16), 28.8 (C-19), 28.3 (C-17), 25.9 (C-9), 23.4 (C-20); HRMS (ESI) *m/z* calculated for $[C_{42}H_{82}NO_{13}]^+$: 808.5781, found 808.5776. The data corresponded to those published.[46]

3.13. 6,6′-Di-O-15-methylhexadecanosanoyl-α,α′-D-trehalose (**3c**)

By subjecting **8c** (70 mg, 0.055 mmol) to the general procedure for desilylation, the title compound **3c** was obtained as a white solid (36.5 mg, 0.043 mmol, 78%). R_f = 0.68 (EtOAc/MeOH, 4:1, v/v); $[\alpha]_D^{26.6}$ = +6 (*c* = 1.0, C_5H_5N); IR (film) = 3321, 2921, 2852, 1728, 1464, 1365, 1351, 1277, 1243, 1152, 1080, 1018, 983, 937, 806, 756, 721 cm^{-1}; ^{1}H NMR (500 MHz, C_5H_5N) δ 5.90 (d, $J_{1,2}$ = 3.7 Hz, 2H, H-1), 5.13–5.07 (m, 2H, H-5), 5.01 (d, $J_{6a,6b}$ = 11.7 Hz, 2H, H-6a), 4.84 (dd, $J_{6a,6b}$ = 11.7 Hz, $J_{5,6b}$ = 5.4 Hz, 2H, H-6b), 4.76 (t, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, 2H, H-3), 4.32 (dd, $J_{2,3}$ = 9.5 Hz, $J_{1,2}$ = 3.7 Hz, 2H, H-2), 4.18 (t, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, 2H, H-4), 2.39–2.28 (m, 4H, H-8), 1.64 (p, $J_{8,9}$ = $J_{9,10}$ = 7.5 Hz, 4H, H-9), 1.55–1.46 (m, 2H, H-21), 1.36–1.14 (m, 44H, H-10–H-20), 0.89 (d, $J_{21,22}$ = 6.6, 12H, H-22); ^{13}C NMR (125 MHz, C_5H_5N) δ 174.0 (C-7), 96.2 (C-1), 75.3 (C-3), 73.8 (C-2), 72.4 (C-4), 72.0 (C-5), 64.8 (C-6), 39.7 (C-20), 34.8 (C-8), 30.6, 30.48, 30.46, 30.42, 30.37, 30.2, 30.0, 29.8 (C-10–C-17), 28.6 (C-21), 28.1 (C-19), 25.7 (C-9), 23.2 (C-22); HRMS (ESI) *m/z* calculated for $[C_{46}H_{90}NO_{13}]^+$: 864.6407, found 864.6407.

3.14. 6,6′-Di-O-17-methyloctadecanoyl-α,α′-D-trehalose (**3d**)

By subjecting **8d** (78 mg, 0.058 mmol) to the general procedure for desilylation, the title compound **3d** was obtained as a white solid (41.5 mg, 0.046 mmol, 79%). R_f = 0.42 (EtOAc/MeOH, 9:1, v/v); $[\alpha]_D^{26.7}$ = +27 (*c* = 1.0, C_5H_5N); IR (film) = 3330, 2921, 2852, 1728, 1465, 1365, 1214, 1152, 1102, 1080, 1020, 983, 937, 911, 806, 755 cm^{-1}; ^{1}H NMR (500 MHz, C_5H_5N) δ 5.91 (d, $J_{1,2}$ = 3.7 Hz, 2H, H-1), 5.14–5.09 (m, 2H, H-5), 5.05–5.0 (m, 2H, H-6a), 4.85 (dd, $J_{6a,6b}$ = 11.7 Hz, $J_{5,6b}$ = 5.4 Hz, 2H, H-6b), 4.77 (t, $J_{2,3}$ = $J_{3,4}$ = 9.5 Hz, 2H, H-3), 4.33 (dd, $J_{2,3}$ = 9.5 Hz, $J_{1,2}$ = 3.7 Hz, 2H, H-2), 4.19 (t, $J_{3,4}$ = $J_{4,5}$ = 9.5 Hz, 2H, H-4), 2.40–2.29 (m, 4H, H-8), 1.64 (p, $J_{8,9}$ = $J_{9,10}$ = 7.5 Hz, 4H, H-9), 1.55–1.46 (m, 2H, H-23), 1.37–1.13 (m, 52H, H-10–H-22), 0.88 (d, $J_{23,24}$ = 6.6, 12H, H-24); ^{13}C NMR (125 MHz, C_5H_5N) δ 173.8 (C-7), 96.0 (C-1), 75.0 (C-3), 73.5 (C-2), 72.2 (C-4), 71.7 (C-5), 64.5 (C-6), 39.4 (C-22), 35.6 (C-8), 30.45, 30.24, 30.23, 30.2, 30.1, 29.9, 29.8, 29.5 (C-10–C-20), 28.4 (C-23), 27.9 (C-21), 25.4 (C-9), 22.9 (C-24); HRMS (ESI) *m/z* calculated for $[C_{50}H_{98}NaO_{13}]^+$: 920.7033, found 920.7033.

3.15. 6,6′-Di-O-18-methylnonadecanoyl-α,α′-D-trehalose (**3e**)

By subjecting **8e** (80 mg, 0.059 mmol) to the general procedure for desilylation, the title compound **3e** was obtained as a white solid (43 mg, 0.046 mmol, 78%). R_f = 0.3 (EtOAc/MeOH, 9:1, v/v); $[\alpha]_D^{16.5}$ = +81 (*c* = 1.0, C_5H_5N); IR (film) = 3331, 2918, 2850, 1741, 1467, 1365, 1251, 1153, 1103, 1078, 1015, 984, 938, 806, 720 cm^{-1}; ^{1}H

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NMR (500 MHz, C_5H_5N) δ 5.91 (d, $J_{1,2}$ = 3.7 Hz, 2H, H-1), 5.14–5.09 (m, 2H, H-5), 5.05–5.0 (m, 2H, H-6a), 4.85 (dd, $J_{6a,6b}$ = 11.7 Hz, $J_{5,6b}$ = 5.4 Hz, 2H, H-6b), 4.77 (t, $J_{2,3} = J_{3,4}$ = 9.5 Hz, 2H, H-3), 4.33 (dd, $J_{2,3}$ = 9.5 Hz, $J_{1,2}$ = 3.7 Hz, 2H, H-2), 4.19 (t, $J_{3,4} = J_{4,5}$ = 9.5 Hz, 2H, H-4), 2.40–2.29 (m, 4H, H-8), 1.64 (p, $J_{8,9} = J_{9,10}$ = 7.5 Hz, 4H, H-9), 1.55–1.46 (m, 2H, H-24), 1.37–1.13 (m, 56H, H-10–H-23), 0.88 (d, $J_{24,25}$ = 6.6, 12H, H-25); ^{13}C NMR (125 MHz, C_5H_5N) δ 174.2 (C-7), 96.4 (C-1), 75.5 (C-3), 74.0 (C-2), 72.6 (C-4), 72.2 (C-5), 65.0 (C-6), 39.9 (C-23), 35.0 (C-8), 30.8, 30.68, 30.67, 30.63, 30.5, 30.4, 30.2, 30.0 (C-10–C-21), 28.8 (C-24), 28.3 (C-22), 25.9 (C-9), 23.4 (C-25); HRMS (ESI) *m/z* calculated for $[C_{52}H_{102}NaO_{13}]^+$: 948.7346, found 948.7355.

3.16. 6,6′-Di-O-19-methylleicosanoyl-α,α′-D-trehalose (**3f**)

By subjecting **8f** (65 mg, 0.047 mmol) to the general procedure for desilylation, the title compound **3f** was obtained as a white solid (36 mg, 0.0375 mmol, 80%). R_f = 0.3 (EtOAc/MeOH, 9:1, v/v); $[\alpha]_D^{17.3}$ = +49 (*c* = 1.0, C_5H_5N); IR (film) = 3329, 2917, 2849, 1727, 1467, 1383, 1215, 1154, 1102, 1023, 984, 937, 806, 753, 665, 577 cm^{-1}; ^{1}H NMR (500 MHz, C_5H_5N) δ 5.92 (d, $J_{1,2}$ = 3.7 Hz, 2H, H-1), 5.14–5.09 (m, 2H, H-5), 5.05–5.0 (m, 2H, H-6a), 4.85 (dd, $J_{6a,6b}$ = 11.7 Hz, $J_{5,6b}$ = 5.4 Hz, 2H, H-6b), 4.77 (t, $J_{2,3} = J_{3,4}$ = 9.5 Hz, 2H, H-3), 4.33 (dd, $J_{2,3}$ = 9.5 Hz, $J_{1,2}$ = 3.7 Hz, 2H, H-2), 4.19 (t, $J_{3,4} = J_{4,5}$ = 9.5 Hz, 2H, H-4), 2.40–2.29 (m, 4H, H-8), 1.64 (p, $J_{8,9} = J_{9,10}$ = 7.5 Hz, 4H, H-9), 1.55–1.46 (m, 2H, H-25), 1.37–1.13 (m, 60H, H-10–H-24), 0.88 (d, $J_{25,26}$ = 6.6, 12H, H-26); ^{13}C NMR (125 MHz, C_5H_5N) δ 174.3 (C-7), 96.4 (C-1), 75.5 (C-3), 74.0 (C-2), 72.6 (C-4), 72.2 (C-5), 65.0 (C-6), 39.9 (C-24), 35.0 (C-8), 30.9, 30.7, 30.6, 30.6, 30.4, 30.2, 30.0 (C-10–C-22), 28.8 (C-25), 28.3 (C-23), 25.9 (C-9), 23.4 (C-26); HRMS (ESI) *m/z* calculated for $[C_{54}H_{106}NaO_{13}]^+$: 976.7659, found 92.7236.

3.17. Immunology

3.17.1. Animals

C57BL/6 wild-type mice and Mincle$^{-/-}$ mice were bred and housed in a conventional animal facility at the Malaghan Institute of Medical Research, Wellington, New Zealand. All animals used for the experiments were aged between 8 and 12 weeks. All experimental procedures were approved by the Victoria University Animal Ethics Committee in accordance with their guidelines for the care of animals.

3.17.2. Generation and stimulation of bone marrow-derived macrophages

Bone marrow cells were collected from the tibia and femur of C57BL/6, Mincle$^{-/-}$ mice and were cultured (250,000 cells/mL) in complete RPMI media [(RPMI Medium 1640-GlutaMAX™-I (Gibco) supplemented with 10% heat inactivated fetal bovine serum (Gibco), 1% penicillin-streptomycin (Gibco))]. Macrophage differentiation was induced by 50 ng/mL GM-CSF (PeproTech) added to the cRPMI. Cells were incubated at 37 °C (5% CO_2) for 8 days (cells fed on days 3 and 6). On day 8 all media was removed and fresh complete RPMI was added to the cells. Stock solutions of iso-branched TDEs and linear TDEs (1 mM in PBS containing 2% DMSO), TDB (5 mg/mL stock with 2% DMSO in DPBS) were vortexed and warmed to 50 °C for 30 min and sonicated for 20 min to ensure complete solubilisation of the compounds prior to administration to BMDM cultures. Cells were then stimulated with maradolipids (**3a-f**), linear TDEs (**2a-c**, C4,[16] TDB[16]) at 20 μM and 40 μM, or LPS (100 ng/mL) as positive control. Supernatant was collected after 24 h, and 48 h. TDB was synthesised according to the previously published procedures. All synthetic compounds were confirmed to be free of endotoxin at a sensitivity of ≤0.125 EU/mL by Limulus amebocyte lysate (LAL) assay using an endotoxin kit (Pyrotell, Limulus Amebocyte Lysate).

3.17.3. Cytokine analysis

Levels of IL-10 (R&D systems), MIP-2 (R&D systems), IL-1β (BD Biosciences), IL-12p40 (BD Biosciences), and IL-6 (BD Biosciences) were determined by sandwich ELISA according to the manufacturer's instructions.

3.17.4. Statistics

Statistical significance of differences was assessed with 2-way ANOVA with Dunnett's multiple comparisons test using Prism v7 software (GraphPad).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.tet.2017.11.076.

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