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Phosphatidylinositol mannosides: Synthesis and adjuvant properties of phosphatidylinositol di- and tetramannosides

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Abstract—Phosphatidylinositol mannosides (PIMs) isolated from mycobacteria have been identified as an important class of glycolipids with significant immune modulating properties. We present here the syntheses of phosphatidylinositol dimannoside (PIM2, **1**) and phosphatidylinositol tetramannoside (PIM4, **2**) and evaluate their adjuvant properties in a transgenic mouse model. The key step in the synthetic methodology for the synthesis of **2** relies on the selective glycosylation of diol **3** with mannosyl donor **11**. Both synthetic PIMs were effective at enhancing IFN-γ when given as adjuvants with a model antigen, with PIM2 being the more active. These data suggest that in this assay the PIM core structure is responsible for the observed biological activity.

1. Introduction

Vaccines that consist of chemically defined components, such as sub-unit vaccines, have significant advantages over live attenuated vaccines in terms of their reproducibility, safety and hence ease of registration.[1] However, relatively poor immunogenicity is a major drawback in their use. In order to elicit a strong and robust cell-mediated immune response, the co-administration of an adjuvant is required[2] and there is at present a paucity of safe, defined and effective adjuvants. Currently available immune modulators used in experimental animals, such as Complete Freund's Adjuvant (CFA) and Cholera Toxin (CT), are based on complex bacterial cell components and are crude products which are not suitable for use in humans owing to their considerable toxicities. Therefore, there is a need for the development of novel immune modulators that are safe, potent, selective and consist of chemically defined entities.

The mycobacterial vaccine Bacille Calmette–Guerin (BCG) has been used to protect humans and animals against tuberculosis. In addition, it is now known that it can also prevent the development of atopic disorders in humans and experimental animals.[3,4] The precise mechanism as to how these mycobacterial products confer protection against atopy is not well understood but is thought to involve the production of T_H1 cytokines that inhibit T_H2 cytokine release that are crucial in mediating atopy.[5] This effect has been, at least in part, attributed to the presence of certain cell surface glycolipids in the BCG vaccine such as: lipoarabinomannan (LAM), lipomannans (LM) and phosphatidylinositol mannooligosaccharides (PIMs).[6] We and others have demonstrated that the smaller PIM molecules, by themselves, retain immunomodulatory activities.[7–10] In particular, they stimulate cytokine production via key cells of the immune system and generate an inflammatory response.[7,9,11] Thus, we reasoned that PIM compounds, besides their potential as antagonists of the development of atopy, may have potential applications as adjuvants to enhance the activity of sub-unit vaccines.

One obstacle hampering the elucidation of this glycolipid based immunomodulatory effect is the lack of discrete molecules of known structure that can be used for testing. The oligosaccharide backbone of the PIM

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molecules has been rigorously investigated,[12,13] however, the precise acylation state still remains an area of uncertainty. Differently acylated PIM molecules are difficult to separate from each other when isolated from natural sources[13,14] and it is likely that subtle changes in the acylation pattern affect their bioactivity.

In recent work, a bacterial antigen that induces interferon-γ (IFN-γ) release in a CD1d restricted manner was identified as a tetra-mannosylated-dipalmitoyl phosphatidyl inositol mannoside (PIM4).[15] Also, the first crystal structure of a CD1d–PIM2 complex, where the acyl groups were palmitoyl residues, has recently been determined[16] and revealed that the mannopyranosyl residue bonded to O-6 of the inositol moiety projects away from the surface of the protein implying that CD1d could accommodate higher molecular weight ligands such as PIM4 and PIM6. Their tri- and penta-manno-oligosaccharide chains are linked to the O-6 position and would also project away from the CD1d hydrophobic binding groove. This work also revealed that the F′ binding pocket, of the CD1d protein, accommodates the sn-2 palmitoyl group of the PIM2 molecule and that this group was an optimal fit.

In our ongoing programme to elucidate the biological mechanism by which PIM compounds act, we have synthesized and investigated the biological properties of discrete PIM molecules.[8] Here, we present an improved synthesis of dipalmitoyl PIM2 (**1**), and, to our knowledge, the first chemical synthesis of dipalmitoyl PIM4 (**2**). We have also investigated the adjuvant properties of these molecules in an in vivo ovalbumin (OVA) specific transgenic mouse model.

2. Results and discussion

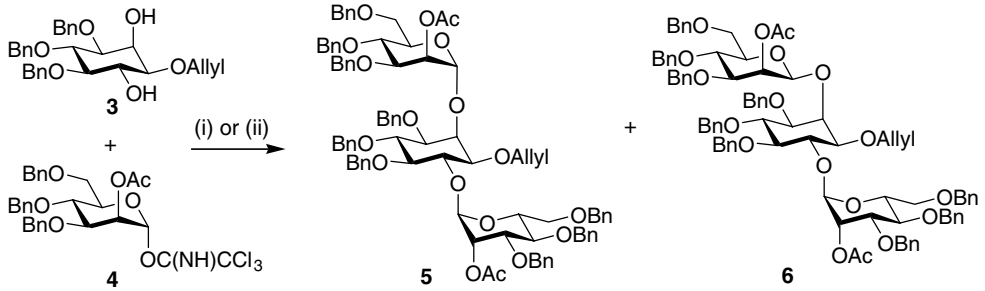
2.1. Synthesis of PIM2

A number of approaches for the synthesis of PIM1 and PIM2 compounds have been reported[8,17–24] and, very recently, the first synthesis of PIM6 has been published.[25] The synthetic methodologies used to date to obtain a suitable unsymmetrical *myo*-inositol derivative as starting material rely on its synthesis from glucose by application of the Ferrier rearrangement or the derivatization and desymmetrization of *myo*-inositol. Our approach utilizes the former methodology to give the previously used allyl diol **3**[24] which can be doubly glycosylated with mannosyl donor **4** in dichloromethane to afford the α,α-pseudo-trisaccharide **5** in 57% yield.[8] We now find the reaction applied on a larger scale affords the α,β-pseudo-trisaccharide **6** (22%) as by-product together with the desired product **5** (59%, Scheme 1). The anomeric configurations of the mannopyranose residues of **6** were established from the glycosidic C-1 ^{13}C–^{1}H coupling constants of 176 and 161 Hz which are consistent with α- and β-linkages, respectively.[26,27] A correlation between H-2 of the inositol and the anomeric carbon atom of the β-linked mannopyranosyl residue was apparent in the heteronuclear multiple bond coherence (HMBC) spectrum, establishing that the β-mannopyranosyl residue was attached to O-2 of the inositol moiety and therefore O-6 carried the α-linked sugar. The anomeric selectivity was increased by changing the synthesis solvent to diethyl ether which has been reported[28] to favour the formation of α-glycosidic bonds. Consistent with this finding, when the glycosidation reaction was repeated in this solvent the isolated yield of **5** was increased to 70% and that of **6** decreased to 10%.

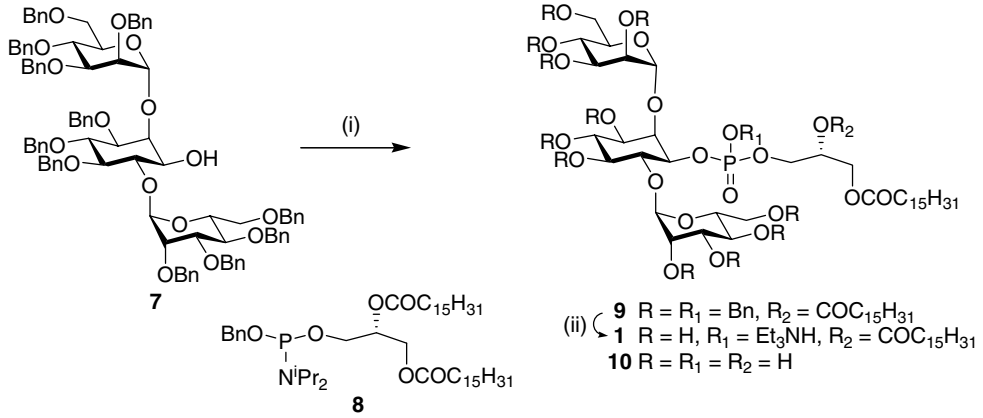
Routine protecting group manipulation as described previously[8] afforded alcohol **7** that was subsequently coupled with phosphoramidite **8** to give the protected PIM2 **9** in good yield (Scheme 2). Hydrogenolytic debenzylation of this material using reaction solvent mixtures that contained some water led to a small amount of the deacylation product **10**. This product was slightly more polar than the desired PIM2 product so could be separated by column chromatography. This side reaction was overcome by using anhydrous solvent mixtures and trapping the free acid as the triethylammonium salt.

2.2. Synthesis of PIM4

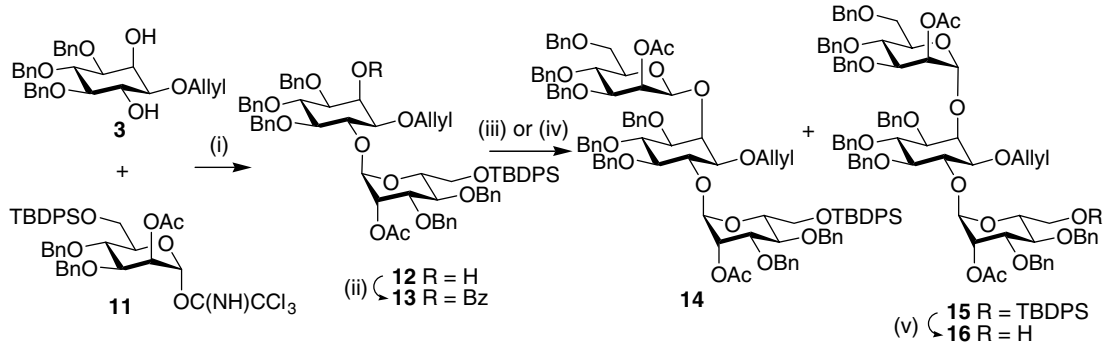
The protected inositol **3** was also used as the starting material for our synthesis of PIM4 **2**. The strategy requires either a chemo-selective O-6 glycosylation of acceptor **3** with an orthogonally protected mannosyl donor or temporary protection of one of the hydroxyl groups of **3** to establish the required unsymmetrical glycosylation pattern. As we had previously demonstrated[8] that **3** could be selectively glycosylated we chose the former approach as the latter would necessitate both protection and subsequent deprotection steps (Scheme 3). Glycosylation of the C-6 hydroxyl group of inositol



Scheme 1. Reagents: (i) TMSOTf, MS-4 Å, CH_2Cl_2 (59% for **5** and 22% for **6**); (ii) TMSOTf, MS-4 Å, Et_2O (70% for **5** and 10% for **6**).



Scheme 2. Reagents: (i) 1*H*-tetrazole, **8**, CH_2Cl_2 then *m*-CPBA (67%); (ii) Pd/C, MeOH, THF, H_2 then Et_3N (98%).



Scheme 3. Reagents: (i) TMSOTf, MS-4 Å, CH_2Cl_2 (73%); (ii) Py, BzCl, DMAP (44%); (iii) **4**, TMSOTf, MS-4 Å, CH_2Cl_2 (54% for **14**); (iv) **4**, TMSOTf, MS-4 Å, Et_2O (80% for **15**); (v) TBAF, THF (47%).

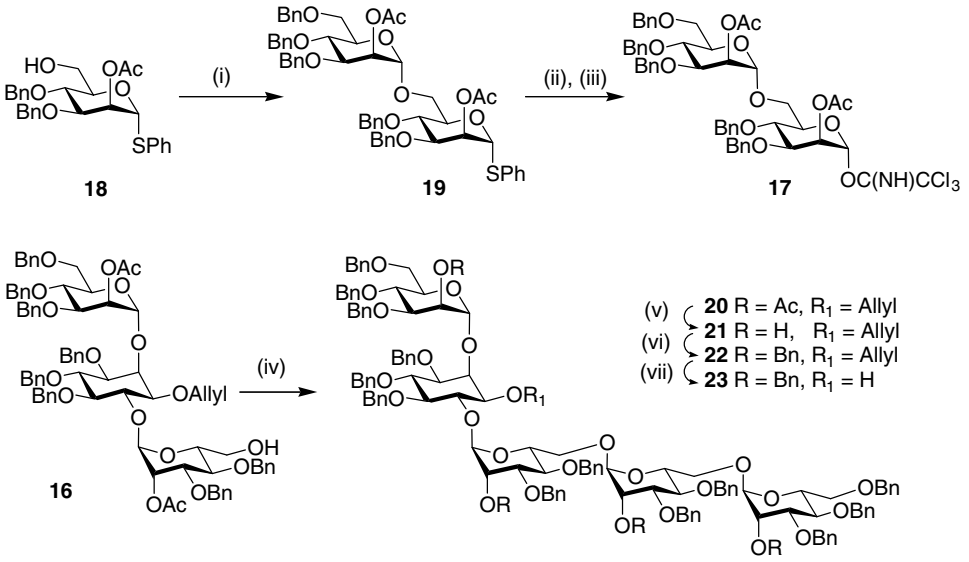
acceptor **3** with orthogonally protected donor **11** afforded the mono-mannoside **12** in good yield (Scheme 3). The glycosylation site was established by benzoylation of **12** to give the 2-*O*-benzoyl-inositol **13**. The expected downfield shift (4.18 ppm for **12** to 5.98 ppm for **13**) for the characteristic broad triplet of the inositol H-2 proton was evident in the ^1H NMR spectra. The α-anomeric configuration of glycoside **12** was established from the one-bond ^{13}C–^1H coupling constant of 177 Hz for the C-1′ resonance in the coupled ^{13}C NMR spectrum. Surprisingly, glycosylation of pseudo-disaccharide **12** with **4** in dichloromethane afforded the α,β-bis-mannoside **14** as the major product. Although we observed some formation of the β-glycoside in the preparation of PIM2 (*vide infra*), we did not expect that β-glycosylation would dominate in this reaction. Gratifyingly, the use of diethyl ether as the reaction solvent reversed the stereoselectivity of the mannosylation such that the α,α-bis-mannoside **15** was obtained in high yield. The anomeric carbons of **15** resonated at 99.0 and 98.6 ppm in the ^{13}C NMR spectrum with the one-bond ^{13}C–^1H coupling constants of 173 Hz each confirming the assignment of anomeric stereochemistry. The next step in the sequence involved unblocking the 6′-hydroxyl group for glycosylation. Removal of the TBDPS ether group to give alcohol **16** proved to be problematic. A number of reaction conditions were investigated but the yield could not be improved above 50%. Forcing reaction conditions lead to the formation of deacylation products. We are currently investigating the use of the acid-labile triisopropylsilyl group as this group was used successfully in the synthesis of PIM6.[25]

The synthesis of disaccharide donor **17** from **4** and thioglycoside **18**,[29] through the intermediacy of **19** proceeded smoothly. Glycosylation of the pseudo-trisaccharide **16** with trichloroacetimidate **17** using standard conditions gave pseudo-pentasaccharide **20** in 80% yield. The newly formed glycosidic bond was confirmed as having α-stereochemistry as all of the four one-bond C–H coupling constants of the anomeric carbon signals were in the range of 172–175 Hz. Routine manipulation of the protecting groups of **20** by deacetylation to **21**, subsequent benzylation to **22** and deallylation gave the protected PIM4 headgroup **23** in 63% overall yield for the sequence Scheme 4.

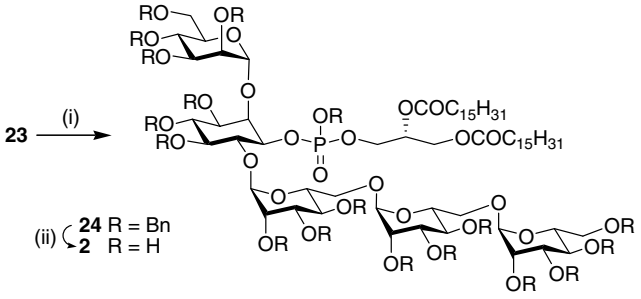
The phosphodiester linkage was introduced by coupling excess phosphoramidite **8** (4 equiv) with **23** (Scheme 5) to give protected PIM4 **24** in 52% yield as a 1:1.5 mixture of stereoisomers about the phosphorus centre. The final step involved hydrogenolytic debenzylation under anhydrous conditions to furnish dipalmitoyl PIM4 **2** in 89% yield.

2.3. Adjuvant properties of PIM2 and PIM4

Adjuvants normally generate relatively subtle quantitative changes in the immune response to an antigen. For this reason, we used an ovalbumin (OVA)-specific



Scheme 4. Reagents: (i) **4**, TMSOTf, MS-4 Å, CH_2Cl_2 (67%); (ii) NIS, TfOH, H_2O, Me_2CO (62%); (iii) DBU, Cl_3CCN, CH_2Cl_2 (87%); (iv) **17**, TMSOTf, MS-4 Å, Et_2O (80%); (v) NaOMe, MeOH (85%); (vi) NaH, BnBr, DMF (80%); (vii) $(Ph_2MeP)_2(COD)Ir^+ PF_6^-$, THF then AcCl, CH_2Cl_2, MeOH (92%).



Scheme 5. Reagents: (i) 1*H*-tetrazole, **8**, CH_2Cl_2 then *m*-CPBA (52%); (ii) $Pd(OH)_2/C$, MeOH, THF, H_2 (89%).

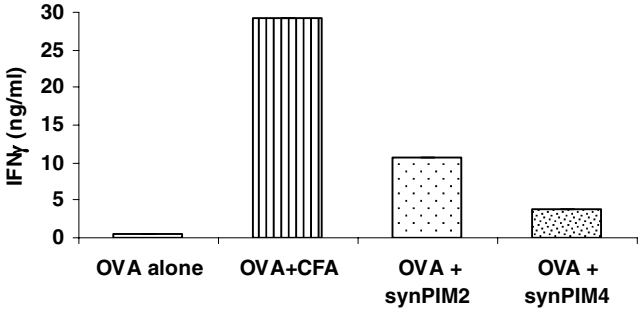


Figure 1. Effects of subcutaneous administration of PIM2 and PIM4 with the model antigen, OVA, for the development of an immune response in C57BL/6 mice which had been transferred with OT-II spleen cells. Mice were given 100 μg of OVA emulsified in CFA, or with 36 μg of PIM2 or PIM4. Axillary lymph node cells were isolated 4 days following injection. Cells were cultured for 4 days with 1 μM of OVA peptide, after which IFN-γ levels were measured in supernatants. Results are means of triplicates, repeated twice with similar results. Standard errors of the means were in the range of 5%.

MHC class II restricted αβ T cell receptor (TCR) transgenic mouse model (OT-II) for assessing the adjuvant properties of PIM2 and PIM4. Expression of the T cell receptors Vα2 and Vβ5.1 is increased to 20–40% of total T cells in these mice, compared to the wild-type C57BL/6, where expression is less than 1%.[30] This increased expression leads to a robust immune response versus OVA (used here as a model antigen), and increases the opportunities for detecting changes when testing adjuvants, that otherwise would not be apparent.

Mice were administered 100 μg of OVA by the subcutaneous route with the synthetic PIMs (36 μg) as an adjuvant, or OVA emulsified in CFA (0.1 mL) given as a positive control adjuvant. The ability of lymph node cells from the axillary lymph nodes to release IFN-γ in vitro in response to OVA was then evaluated, as a measure of the magnitude of the induction of a T_H1 response. Figure 1 shows that administration of OVA with synthetic PIM2 generated significant IFN-γ release (about 40% of that compared with CFA). In comparison, PIM4 was also able to generate significant IFN-γ release but only at about 20% of that compared with CFA. Both PIM2 and PIM4 induced significant enhancements of IFN-γ release, compared to mice given OVA alone ($P < 0.01$), although PIM2 was more efficient than PIM4 ($P < 0.01$). It was observed that injection of OVA and CFA induced the formation of substantial abscesses and fibrosis in subcutaneous tissues, whereas OVA and PIM2 and PIM4 induced only a slight reddening of tissues, with no abscesses nor fibrosis. Data were analyzed for significance using a Fischer's unprotected test with pairwise comparisons.

3. Conclusions

We have prepared synthetic samples of PIM2 **1** and PIM4 **2** and evaluated their adjuvant activities in an OVA-specific mouse model. Both synthetic molecules given as adjuvants with a model antigen resulted in significantly higher quantities of IFN-γ released by cells of

immunized mice, with PIM2 being more potent than PIM4. Significantly, in contrast to CFA, neither compound caused any local edema nor visible lesions following administration. We are currently undertaking further studies to fully evaluate the potential of this class of molecule to act as adjuvants, both when given parenterally and by the mucosal route.

4. Experimental procedures for synthesis of PIM analogues

4.1. General

Specific optical rotations, given in 10^{-1} deg cm^2 g^{-1}, were measured at ambient temperature using a Perkin-Elmer 241 polarimeter with a cell of path length 1.0 dm. ^{1}H NMR spectra were obtained at either 300 or 500 MHz and referenced to either the residual solvent peak or tetramethylsilane (0.0 ppm). ^{13}C NMR spectra were recorded at 75 or 125 MHz and referenced to the residual solvent peak (77.08). ^{31}P NMR spectra were recorded at 121.5 or 202.5 MHz. Chemical shifts are reported using the δ scale, and coupling constants (J) are reported in Hz. High resolution electro-spray ionization (ESI) mass spectra were recorded on a Waters Micromass Q-Tof Premier mass spectrometer. Elemental analyses were carried out by the Campbell Microanalytical Laboratory, University of Otago, Dunedin, New Zealand. Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} plates and was visualized under UV light and/or with a cerium sulfate–ammonium molybdate solution and subsequent heating. Flash column chromatography was carried out using Scharlau silica gel 60 (0.04–0.06 mm, 230–400 mesh). All chromatography solvents were of reagent grade. Dry solvents were purchased from Aldrich.

4.1.1. 1-*O*-Allyl-2-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-mannopyranosyl)-6-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4,5-tri-*O*-benzyl-D-*myo*-inositol (6) and 1-*O*-Allyl-2,6-di-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4,5-tri-*O*-benzyl-D-*myo*-inositol (5)[8]. TMSOTf (200 μL, 1.11 mmol) was added drop-wise to a stirred mixture of 1-*O*-allyl-3,4,5-tri-*O*-benzyl-D-*myo*-inositol (**3**) (1.02 g, 2.08 mmol), 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl trichloroacetimidate (**4**) (2.83 g, 4.44 mmol) and 4Å molecular sieves (400 mg) in CH_2Cl_2 (60 mL) cooled to −40 °C. The reaction mixture was allowed to warm to 0 °C over 90 min when Et_3N (5.0 mL) was added. The mixture was filtered through Celite® and the filter cake washed with further CH_2Cl_2 (2× 200 mL). The solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9 to 3:7) afforded the α,β-diglycoside **6** (650 mg, 0.451 mmol, 22%) as an oil. $[\alpha]_D^{20}$ − 18.4 (*c* 2.72, CH_2Cl_2). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37–7.01 (m, 45H), 6.01–5.88 (m, 1H), 5.70 (d, J = 3.1 Hz, 1H), 5.45 (s, 2H), 5.22–5.06 (m, 2H), 4.93–4.38 (m, 19H), 4.29 (d, J = 12.0 Hz, 1H), 4.23–4.17 (m, 1H), 4.00–3.90 (m, 6H), 3.79–3.66 (m, 3H), 3.53 (dd, J = 9.2, 3.3 Hz, 1H), 3.43–3.20 (m, 6H), 2.17 (s, 3H), 2.10 (s, 3H). ^{13}C NMR (75 MHz) δ 171.0, 170.4, 139.1, 138.7, 138.6, 138.3, 138.0, 137.8, 135.1, 128.6, 128.4, 128.3, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.3, 127.2, 117.5, 98.6, 98.2, 81.5, 81.3, 81.1, 80.4, 78.6, 78.2, 75.8, 75.6, 75.5, 75.2 (2× CH_2), 74.9, 74.7, 74.3, 73.5, 73.4, 73.2, 71.7, 71.5, 71.4, 70.1, 69.8, 68.9, 68.4, 67.9, 21.2. Gated decoupled ^{13}C NMR (75 MHz, $CDCl_3$) selected data, δ 98.6, $^1J_{C1'-H1'}$ 161 Hz, δ 98.2, $^1J_{C1''-H1''}$ 176 Hz. HRMS-ESI $[M+Na]^+$ calcd for $C_{88}H_{94}O_{18}Na$: 1461.6338. Found 1461.6371. Further elution afforded **5** (1.77 g, 1.23 mmol, 59%).

4.1.2. 1-*O*-Allyl-2,6-di-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4,5-tri-*O*-benzyl-D-*myo*-inositol (5).[8] TMSOTf (24 μL, 0.13 mmol) was added drop-wise to a stirred mixture of **3** (217 mg, 0.442 mmol), 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl trichloroacetimidate (**4**) (844 mg, 1.33 mmol) and 4 Å molecular sieves (100 mg) in Et_2O (20 mL) cooled to −30 °C. The reaction mixture was allowed to warm to 0 °C over 90 min when Et_3N (2.0 mL) was added. The mixture was filtered through Celite® and the filter cake washed with further Et_2O (2 × 100 mL). The solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9 to 3:7) afforded **6** (63 mg, 0.044 mmol, 10%) followed by **5** (445 mg, 0.309 mmol, 70%) as an oil. ^{1}H and ^{13}C NMR spectra were consistent with the previously reported data. Anal. ($C_{88}H_{94}O_{18}$) C, H.

4.1.3. 3,4,5-Tri-*O*-benzyl-2,6-di-*O*-2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl-1-*O*-(1,2-di-*O*-hexadecanoyl-*sn*-glycero-3-benzylphosphoryl)-D-*myo*-inositol (9). 1*H*-Tetrazole (41 mg, 0.59 mmol) was added to a stirred solution of 3,4,5-tri-*O*-benzyl-2,6-di-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl)-D-*myo*-inositol (**7**) and phosphoramidite **8** (476 mg, 0.590 mmol) in dry CH_2Cl_2 (8 mL) cooled to 0 °C under an argon atmosphere. After stirring at rt for 2 h the reaction mixture was cooled to −40 °C and a solution of *m*-CPBA (50%, 204 mg, 0.591 mmol) in CH_2Cl_2 (10 mL) was transferred by cannular into the reaction mixture. After warming to rt over 2 h, the reaction was quenched with the addition of aq Na_2SO_3 (10%, 50 mL) and the combined mixture extracted with Et_2O (100 mL). The ethereal extract was washed with aq $NaHCO_3$ (satd, 3× 50 mL) and dried ($MgSO_4$). After filtration, the solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9 to 1:4) afforded the title compound **9** (175 mg, 0.079 mmol, 67%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.39–7.01 (m, 60H), 5.49 (br s, 1H), 5.37–5.29 (m, 1H), 5.12–4.40 (m, 24H), 4.30–3.75 (m, 17H), 3.52–3.20 (m, 6H), 2.23–2.09 (m, 4H), 1.58–1.41 (m, 4H), 1.31–1.17 (m, 48H), 0.89–0.82 (m, 6H). ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 1.16, 0.94. HRMS-ESI $[M+Na]^+$ calcd for $C_{137}H_{171}O_{23}NaP$: 2238.1847. Found 2238.1833. Anal. ($C_{137}H_{171}O_{23}P$) C, H, P.

4.1.4. 2,6-(Di-*O*-α-D-mannopyranosyl)-1-*O*-(1-*O*-hexadecanoyl-*sn*-glycero-3-phosphoryl)-D-*myo*-inositol (10). $Pd(OH)_2$/C (20%, 31 mg) was added to a stirred solution of **7** (30 mg, 0.014 mmol) in $CHCl_3$/MeOH/H_2O (1:1:0.1, 2.1 mL). The mixture was stirred under a

hydrogen atmosphere for 10 h at rt when the hydrogen was replaced with argon and the mixture was filtered through Celite® and concentrated in vacuo. The residue was purified on silica gel eluting with $CHCl_3$ then $CHCl_3$/MeOH/H_2O (70:40:1 to 70:40:4) to afford PIM2 (8.0 mg, 0.0070 mmol, 50%) followed by the title compound (3.0 mg, 0.0033 mmol, 24%). ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD/D_2O, 70:40:6) δ 5.18 (br s, 1H), 5.11 (br s, 1H), 4.34 (br s, 1H), 4.18–3.93 (m, 7H), 3.87–3.58 (m, 10H), 3.59–3.42 (m, 1H), 3.31–3.25 (m, 1H), 2.36 (t, J = 7.5 Hz, 2H), 1.67–1.55 (m, 2H), 1.67–1.55 (m, 2H), 1.35–1.21 (m, 24H), 0.88 (t, J = 6.6 Hz, 3H). ^{31}P NMR (121.5 MHz, $CDCl_3$/CD_3OD/D_2O, 70:40:6) δ 1.21. HRMS-ESI $[M-H]^-$ calcd for $C_{37}H_{68}O_{22}P$: 895.3940. Found 895.3950.

4.1.5. Triethyl ammonium 2,6-(di-*O*-α-D-mannopyranosyl)-1-*O*-(1,2-di-*O*-hexadecanoyl-*sn*-glycero-3-phosphoryl)-D-*myo*-inositol (1). $Pd(OH)_2$/C (20%, 40 mg) was added to stirred solution of **7** (112 mg, 0.0505 mmol) in THF/MeOH (2:3, 5 mL). The mixture was stirred under a hydrogen atmosphere for 2.5 h at rt when the hydrogen was replaced with argon and triethylamine (1.0 mL) was added. The mixture was filtered through Celite® and concentrated in vacuo. The residue was lyophilized to afford **1** as its tetrahydrate triethylammonium salt (62 mg, 0.050 mmol, 98%) as a white powder. $[\alpha]_D^{20}$ + 38 (*c* 0.19, $CHCl_3$/CH_3OH/H_2O, 70:40:6). ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD/D_2O, 70:40:6) δ 5.31–5.22 (m, 1H), 5.13 (br s, 1H), 5.10 (br s, 1H), 4.45–4.40 (m, 1H), 4.30 (br s, 1H), 4.08–3.92 (m, 7H), 3.87–3.58 (m, 10H), 3.50–3.42 (m, 1H), 3.31–3.25 (m, 1H), 3.10 (q, J= 7.5 Hz, 6H), 2.40–2.27 (m, 4H), 1.67–1.55 (m, 4H), 1.33–1.21 (m, 57H), 0.89 (t, J = 6.9 Hz, 6H). ^{31}P NMR (121.5 MHz, $CDCl_3$/CD_3OD/D_2O, 70:40:6) δ −3.7. HRMS-ESI $[M-Et_3NH]^-$ calcd for $C_{53}H_{98}O_{23}P$: 1133.6237. Found 1133.6232. Anal. ($C_{59}H_{114}O_{23}NP$. $4H_2O$) C, H, N.

4.1.6. 1-*O*-Allyl-6-*O*-(2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-*tert*-butyldiphenylsilyl-α-D-mannopyranosyl)-3,4,5-tri-*O*-benzyl-D-*myo*-inositol (12). TMSOTf (40 μL, 0.22 mmol) was added drop-wise to a stirred mixture of **3** (1.08 g, 2.20 mmol), 2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-*tert*-butyldiphenylsilyl-α-D-mannopyranosyl trichloroacetimidate (**11**) (2.07 g, 2.64 mmol) and 4 Å molecular sieves (120 mg) in CH_2Cl_2 (30 mL) at −30 °C. After 10 min Et_3N (2.5 mL) was added. The mixture was filtered through Celite® and the filter cake washed with further CH_2Cl_2 (2× 100 mL). The solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9 to 1:4) afforded the title compound **12** (1.78 g, 1.60 mmol, 73%) as an oil. $[\alpha]_D^{20}$ + 17 (*c* 0.68, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.69 (d, J = 6.5 Hz, 2H), 7.58 (d, J = 6.5 Hz, 2H), 7.42–7.07 (m, 28H), 7.02–6.96 (m, 1H), 6.89–6.81 (m, 2H), 6.04–5.91 (m, 1H), 5.42–5.40 (m, 2H), 5.32–5.5.20 (m, 2H), 4.92–4.66 (m, 7H), 4.61–4.56 (m, 3H), 4.18 (t, J = 2.6 Hz, 1H), 4.15–3.83 (m, 7H), 3.57–3.49 (m, 2H), 3.38 (dd, J = 9.6, 2.4 Hz, 1H), 3.30–3.22 (m, 2H), 2.38 (br s, 1H), 2.10 (s, 3H), 1.03 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.4, 139.2, 138.6, 138.3, 138.1, 138.0, 136.1, 135.6, 134.5, 134.3, 133.5, 129.5, 129.4, 128.6, 128.4, 128.3, 128.1, 128.1, 128.0. 127.7, 127.6, 127.5, 127.3, 127.2, 118.1, 98.2, 81.4, 81.2, 80.6, 79.8, 78.1, 75.9, 75.5, 75.3, 75.1, 74.2, 72.8, 72.4, 71.8, 71.4, 69.2, 66.9, 62.4, 26.8, 21.0, 19.4. Gated decoupled ^{13}C NMR (75 MHz, $CDCl_3$) selected data, δ 98.2, $^1J_{C1'-H1'}$ 176 Hz. HRMS-ESI $[M+Na]^+$ calcd for $C_{68}H_{76}O_{12}NaSi$: 1135.5004. Found 1135.5034.

4.1.7. 1-*O*-Allyl-6-*O*-(2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-*tert*-butyldiphenylsilyl-α-D-mannopyranosyl)-2-*O*-benzoyl-3,4,5-tri-*O*-benzyl-D-*myo*-inositol (13). Benzoyl chloride (22 μL, 0.19 mmol) was added drop-wise to a stirred solution of alcohol **12** (69 mg, 0.062 mmol) and DMAP (1.0 mg, 0.008 mmol) in pyridine (5 mL) cooled to 0 °C. After being stirred for 10 h at rt, the solvent was removed in vacuo and the residue partitioned with H_2O (50 mL) and Et_2O (100 mL). The ethereal extract was washed with aq HCl (0.5 M, 50 mL), aq $NaHCO_3$ (satd, 50 mL) and brine (50 mL). After drying ($MgSO_4$) and filtration the solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:4 to 3:7) afforded the title compound **13** as an oil (33 mg, 0.027 mmol, 44%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.07–7.99 (m, 2H), 7.66–7.52 (m, 5H), 7.40–7.10 (m, 30H), 7.06–6.98 (m, 1H), 6.90–6.84 (m, 2H), 6.04–5.90 (m, 2H), 5.44–5.42 (m, 2H), 5.31–5.24 (m, 2H), 4.96–4.54 (m, 10H), 4.25–3.88 (m, 7H), 3.65–3.61 (2H, m), 3.56 (dd, J = 9.9, 2.7 Hz, 1H), 3.47 (dd, J = 9.6, 2.7 Hz, 1H), 3.38 (t, J = 9.6 Hz, 1H), 2.07 (s, 3H), 1.02 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.4, 166.0, 139.2, 138.6, 138.3, 137.8, 137.7, 136.0, 135.6, 134.4, 134.3, 133.4, 133.1, 129.9, 129.5, 128.6, 128.4, 128.3, 128.2, 127.9, 127.8 127.7, 127.6, 127.4, 127.3, 118.3, 98.2, 81.8, 81.2, 78.7, 78.4, 78.1, 76.2, 75.8, 75.3, 74.2, 72.5, 72.2, 71.8, 71.1, 69.0, 66.9, 62.4, 26.8, 21.1, 19.4. HRMS-ESI $[M+Na]^+$ calcd for $C_{75}H_{80}O_{13}NaSi$: 1239.5266. Found 1239.5304.

4.1.8. 1-*O*-Allyl-2-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-mannopyranosyl)-6-*O*-(2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-*tert*-butyldiphenylsilyl-α-D-mannopyranosyl)-3,4,5-tri-*O*-benzyl-D-*myo*-inositol (14). TMSOTf (7.0 μL, 0.04 mmol) was added drop-wise to a stirred mixture of **12** (208 mg, 0.187 mmol), trichloroacetimidate **4** (237 mg, 0.373 mmol) and 4 Å molecular sieves (400 mg) in CH_2Cl_2 (15 mL) at −30 °C. After 10 min, Et_3N (0.5 mL) was added and the mixture was filtered through Celite®. The solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with acetone/toluene (1:49 to 1:19) afforded title compound **14** (160 mg, 0.101 mmol, 54%) as an oil. $[\alpha]_D^{20}$ + 5.0 (*c* 0.80, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.71–7.68 (m, 2H), 7.60–7.57 (m, 2H), 7.40–7.10 (m, 43H), 7.01 (t, J = 7.4 Hz, 1H), 6.91 (t, J = 7.7 Hz, 2H), 6.07–5.98 (m, 1H), 5.74 (d, J = 3.4 Hz, 1H), 5.48–5.46 (m, 1H), 5.44–5.42 (m, 1H), 5.22 (dd, J = 17.2, 1.7 Hz, 1H), 5.16–5.12 (m, 1H), 4.93–4.75 (m, 8H), 4.70 (d, J = 11.2 Hz, 1H), 4.61–3.51 (m, 7H), 4.40 (d, J = 11.2 Hz, 1H), 4.36 (t, J = 2.3 Hz, 1H), 4.26–4.20 (m, 2H), 4.05–3.90 (m, 4H), 3.83–3.66 (m, 4H), 3.55–3.41 (m, 4H), 3.36 (dd,

J = 9.8, 2.1 Hz, 1H), 3.28 (t, J = 9.4 Hz, 1H), 3.23 (dd, J = 9.7, 2.6 Hz, 1H), 2.13 (s, 3H), 2.05 (s, 3H), 1.06 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.9, 170.3, 139.2, 138.7, 138.4, 138.2, 138.2, 138.1, 137.8, 136.0, 135.6, 135.3, 134.3, 133.4, 129.5, 129.4, 128.6, 128.5, 128.4, 128.4, 128.4, 128.3, 128.2, 128.1, 128.0. 128.0, 127.9, 127.9, 127.8, 127.7, 127.6, 127.6, 127.5, 127.5, 127.2, 127.2, 127.0, 117.7, 99.2, 98.4, 81.4, 81.2, 81.0, 80.5, 78.5, 78.3, 76.2, 75.8, 75.5, 75.3, 75.0, 74.5, 73.9, 73.6, 73.4, 72.7, 72.3, 71.7, 71.4, 70.3, 69.9, 69.0, 67.9, 62.3, 26.9, 21.1, 21.1, 19.5. Gated decoupled ^{13}C NMR (125 MHz, $CDCl_3$) selected data, δ 99.2, $^1J_{C1'-H1'}$ 160 Hz, δ 99.2, $^1J_{C1''-H1''}$ 176 Hz. HRMS-ESI $[M+Na]^+$ calcd for $C_{97}H_{106}O_{18}NaSi$: 1609.7046. Found 1609.7087. Further elution afforded the di-α mannoside **15** (119 mg, 0.0749 mmol, 40%), characterized below.

4.1.9. 1-*O*-Allyl-2-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-6-*O*-(2-*O*-acetyl-3,4-di-*O*-benzyl-6-*O*-*tert*-butyldiphenylsilyl-α-D-mannopyranosyl)-3,4,5-tri-*O*-benzyl-D-*myo*-inositol (15). TMSOTf (30 μL, 0.17 mmol) was added drop-wise to a stirred mixture of **12** (920 mg, 0.828 mmol), trichloroacetimidate **4** (1.05 g, 1.65 mmol) and 4 Å molecular sieves (400 mg) in Et_2O (20 mL) at −30 °C. The reaction mixture was allowed to warm to 0 °C over 30 min when Et_3N (2.5 mL) was added. The mixture was filtered through Celite® and the filter cake washed with further CH_2Cl_2 (2× 100 mL). The solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with acetone/toluene (1:49 to 3:97) afforded the title compound **15** (1.05 g, 0.661 mmol, 80%) as an oil. $[\alpha]_D^{20}$ + 42.0 (c 1.06, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70–7.67 (m, 2H), 7.58–7.55 (m, 2H), 7.41–7.12 (m, 43H), 7.03 (t, J = 7.4 Hz, 1H), 6.92 (t, J = 7.7 Hz, 2H), 6.01–5.92 (m, 1H), 5.50–5.41 (m, 3H), 5.29–5.19 (m, 3H), , 4.95–4.70 (m, 8H), 4.64–4.57 (m, 5H), 4.53 (d, J = 11.2 Hz, 1H), 4.41 (d, J = 10.7 Hz, 1H), 4.33 (t, J = 2.3 Hz, 1H), 4.30 (d, J = 12.0 Hz, 1H), 4.25–3.93 (m, 8H), 3.88 (d, J = 9.8 Hz, 1H), 3.79 (t, J = 9.6 Hz, 1H), 3.56–3.47 (m, 3H), 3.31–3.22 (m, 4H), 2.19 (s, 3H), 2.15 (s, 3H), 1.04 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.4, 170.0, 139.2, 138.8, 138.4, 138.3, 138.2, 138.0, 137.9, 137.9, 136.0, 135.6, 134.2, 134.1, 133.2, 129.5, 129.4, 128.6, 128.5, 128.4, 128.4, 128.3, 128.2, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.7, 127.6, 127.6, 127.5, 127.4, 127.3, 117.8, 99.0, 98.6, 81.5, 81.2, 80.9, 78.7, 78.4, 76.7, 75.7, 75.7, 75.1, 75.0, 74.1, 74.0, 73.4, 72.6, 72.4, 71.7, 71.7, 71.6, 71.4, 68.8, 68.6, 68.5, 62.3, 26.9, 21.3, 21.2, 19.4. Gated decoupled ^{13}C NMR (125 MHz, $CDCl_3$) selected data, δ 99.0, $^1J_{C1'-H1'}$ 173 Hz, δ 98.6, $^1J_{C1''-H1''}$ 173 Hz. HRMS-ESI $[M+Na]^+$ calcd for $C_{97}H_{106}O_{18}NaSi$: 1609.7046. Found 1609.7014.

4.1.10. 1-*O*-Allyl-2-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-6-*O*-(2-*O*-acetyl-3,4-di-*O*-benzyl-α-D-mannopyranosyl)-3,4,5-tri-*O*-benzyl-D-*myo*-inositol (16). Tetrabutylammonium fluoride (1 M in THF, 2.00 mL, 2.00 mmol) was added to stirred solution of the silyl ether **15** (450 mg, 0.283 mmol) and acetic acid (17 μL, 0.29 mmol) in THF (10 mL) heated to 40 °C. After 5 d, the mixture was diluted with EtOAc (100 mL) and washed with HCl (0.5 M, 50 mL), aq $NaHCO_3$ (satd, 50 mL) and brine (50 mL). After drying ($MgSO_4$) and filtration, the solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (3:7 to 1:1 to 3:1) afforded the starting material **15** (88 mg, 0.055 mmol, 19%) followed by the title compound **16** (180 mg, 0.133 mmol, 47%) as an oil. $[\alpha]_D^{20}$ + 99 (c 0.88, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.41–7.09 (m, 40H), 5.94–5.82 (m, 1H), 5.49–5.41 (m, 3H), 5.25–5.13 (m, 3H), 4.92–4.52 (m, 14H), 4.42 (d, J = 10.8 Hz, 1H),4.34–4.29 (m, 2H), 4.14–3.79 (m, 10H), 3.50–3.23 (m, 7H), 2.17 (s, 3H), 2.16 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.1, 138.8, 138.7, 138.4, 138.2, 138.1, 137.9, 137.9, 133.9, 128.4, 128.4, 128.3, 128.3, 128.2, 128.3, 127.9, 127.9, 127.9, 127.8, 127.7, 127.6, 127.5, 127.5, 127.4, 127.3, 117.6, 98.8, 98.2, 81.4, 81.1, 78.8, 77.9, 77.5, 76.1, 75.7, 75.6, 75.1, 75.0, 74.1, 73.4, 72.5, 71.8, 71.6, 71.5, 71.5, 71.1, 68.7, 68.6, 68.6, 61.5, 21.1, 21.0. HRMS-ESI $[M+Na]^+$ calcd for $C_{81}H_{88}O_{18}Na$: 1371.5868. Found 1371.5873.

4.1.11. Phenyl 2-*O*-acetyl-6-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4-di-*O*-benzyl-1-thio-α-D-mannopyranoside (19). TMSOTf (55 μL, 0.30 mmol) was added drop-wise to a stirred mixture of **18** (1.15 g, 2.33 mmol), trichloroacetimidate **4** (1.92 g, 3.02 mmol) and 4 Å molecular sieves (150 mg) in CH_2Cl_2 (35 mL) cooled to −30 °C. The reaction mixture was allowed to warm to 0 °C over 90 min when Et_3N (2.0 mL) was added. The mixture was filtered through Celite® and the filter cake washed with further CH_2Cl_2 (2× 100 mL). The solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9 to 1:4 to 1:3) afforded the title compound **19** (1.52 g, 1.56 mmol, 67%) as an oil. $[\alpha]_D^{20}$ + 60 (c 1.1, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.43–7.09 (m, 30H), 5.61–5.58 (m, 1H), 5.45–5.42 (m, 2H), 4.95–4.83 (m, 3H), 4.74–4.41 (m, 8H), 4.31–4.25 (m, 1H), 3.97–3.58 (m, 9H), 2.13 (s, 3H), 2.12 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.4, 170.3, 138.6, 138.3, 137.9, 137.6, 133.9, 131.5, 129.3, 128.6, 128.5, 128.4, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6, 98.1, 86.3, 78.6, 78.0, 75.2, 74.4, 74.3, 73.4, 72.3, 71.9, 71.7, 71.6, 70.3, 68.8, 68.5, 66.2, 21.2, 21.0. Gated decoupled ^{13}C NMR (125 MHz, $CDCl_3$) selected data, δ 98.1, $^1J_{C1'-H1'}$ 172 Hz, δ 86.3, $^1J_{C1-H1}$ 169 Hz. HRMS-ESI $[M+Na]^+$ calcd for $C_{57}H_{60}O_{12}NaS$: 991.3703. Found 991.3664.

4.1.12. 2-*O*-Acetyl-6-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-3,4-di-*O*-benzyl-α-D-mannopyranosyl trichloroacetimidate (17). *N*-Iodosuccinimide (414 mg, 1.84 mmol) followed by triflic acid (5.0 μL, 0.06 mmol) was added to a stirred solution of **19** (595 mg, 0.614 mmol) in acetone/H_2O (9:1, 20 mL). After stirring at rt for 12 h, the reaction mixture was diluted with CH_2Cl_2 (100 mL). The organic phase was washed with aq $Na_2S_2O_3$ (10%, 50 mL), aq $NaHCO_3$ (satd, 50 mL), brine (50 mL) and dried ($MgSO_4$). After filtration, the solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (3:7 to 1:1) afforded the

intermediate hemiacetal (332 mg, 0.379 mmol, 62%) that was dissolved in CH_2Cl_2 (15 mL) and cooled to 0 °C. Trichloroacetonitrile (380 μL, 3.79 mmol) and DBU (6 μL, 0.04 mmol) were then added. After 2 h, the solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:5 to 3:7) afforded the title compound **17** (336 mg, 0.329 mmol, 87%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.68 (s, 1H), 7.34–7.12 (m, 25H), 6.21 (br s, 1H), 5.50–5.42 (m, 2H), 4.96–4.43 (m, 11H), 3.92–3.62 (m, 10H), 2.18 (s, 3H), 2.12 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 170.1, 159.6, 138.6, 138.4, 138.0, 137.9, 137.5, 128.5, 128.4, 128.3, 128.2, 128.2, 128.1, 128.0, 127.9, 127.8, 127.5, 97.8, 95.0, 90.8, 77.9, 75.3, 75.1, 74.2, 73.9, 73.5, 73.4, 72.1, 71.6, 71.5, 68.8, 68.4, 67.4, 65.5, 21.1, 20.9. Gated decoupled ^{13}C NMR (125 MHz, $CDCl_3$) selected data, δ 97.8, $^1J_{C1'-H1'}$ 172 Hz, δ 95.0, $^1J_{C1-H1}$ 179 Hz. HRMS-ESI $[M+Na]^+$ calcd for $C_{53}H_{56}O_{13}Cl_3NNa$: 1042.2715. Found 1042.2715.

4.1.13. 2-*O*-(2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-6-*O*-[(2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-(1 → 6)-(2-*O*-acetyl-3,4-di-*O*-benzyl-α-D-mannopyranosyl)-(1 → 6)-(2-*O*-acetyl-3,4-di-*O*-benzyl-α-D-mannopyranosyl)]-1-*O*-allyl-3,4,5-tri-*O*-benzyl-D-*myo*-inositol (20). TMSOTf (5 μL, 0.03 mmol) was added drop-wise to a stirred mixture of **16** (194 mg, 0.144 mmol), trichloroacetimidate **17** (257 mg, 0.252 mmol) and 4 Å molecular sieves (150 mg) in Et_2O (15 mL) cooled to −30 °C. After 10 min, Et_3N (1.5 mL) was added, the mixture filtered through Celite® and the filter cake washed with further CH_2Cl_2 (2× 100 mL). The solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9 to 1:4 to 3:7) afforded the title compound **20** (253 mg, 0.115 mmol, 80%) as an oil. $[\alpha]_D^{20}$ + 47 (*c* 1.4, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43–7.05 (m, 65 H), 5.97–5.88 (m, 1H), 5.54–5.40 (m, 5H), 5.26–5.16 (m, 3H), 5.05 (d, *J* = 11.0 Hz, 1H), 4.95–4.58 (m, 19H), 4.54 (d, *J* = 10.7 Hz, 1H), 4.47 (d, *J* = 11.3 Hz, 1H), 4.44–4.40 (m, 4H), 4.35–4.29 (m, 4H), 4.19 (d, *J* = 8.5 Hz, 1H), 4.12 (dd, *J* = 12.5, 5.6 Hz, 1H), 4.05 (dd, *J* = 12.5, 5.7 Hz, 1H), 4.02–3.84 (m, 11H), 3.79 (t, *J* = 9.6 Hz, 1H), 3.60–3.43 (m, 5H), 3.34–3.23 (m, 7H), 3.16 (d, *J* = 11.0 Hz, 1H), 2.17 (s, 3H), 2.14 (s, 6H), 2.13 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.3, 170.1, 170.0, 169.9, 138.9, 138.8, 138.7, 138.6, 138.3, 138.2, 138.1, 138.0, 138.0, 137.7, 137.7, 134.0, 128.7, 128.6, 128.5, 128.4, 128.4, 128.3, 128.3, 128.2, 128.2, 128.2, 128.1, 128.1, 128.1, 128.0, 127.9, 127.9, 127.8, 127.8, 127.6, 127.6, 127.5, 127.5, 127.4, 127.3, 127.2, 127.1, 126.8, 117.8, 99.0, 98.8, 98.2, 98.0, 81.3, 81.2, 80.7, 78.7, 77.7, 77.7, 76.9, 75.8, 75.8, 75.1, 75.0, 74.8, 74.5, 74.1, 74.1, 73.7, 73.4, 73.4, 72.6, 71.7, 71.6, 71.6, 71.5, 71.4, 71.3, 71.2, 70.9, 70.7, 68.6, 68.3, 68.1, 67.8, 65.2, 65.1, 21.1, 21.0. Gated decoupled ^{13}C NMR (125 MHz, $CDCl_3$) selected data, δ 99.0, $^1J_{C1'-H1'}$ 174 Hz, δ 98.8, $^1J_{C1''-H1''}$ 175 Hz, $^1J_{C1'''-H1'''}$ 173 Hz, $^1J_{C1''''-H1''''}$ 172 Hz. HRMS-ESI $[M+Na]^+$ calcd for $C_{132}H_{142}O_{30}Na$: 2229.9484. Found 2229.9512.

4.1.14. 1-*O*-Allyl-2-*O*-(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-6-*O*-[(3,4,6-tri-*O*-benzyl-α-D-mannopyranosyl)-(1 → 6)-(3,4-di-*O*-benzyl-α-D-mannopyranosyl)-(1 → 6)-(3,4-di-*O*-benzyl-α-D-mannopyranosyl)]-3,4,5-tri-*O*-benzyl-D-*myo*-inositol (21). Sodium methoxide in MeOH (30%, approx 0.05 mL) was added drop-wise to a stirred solution of tetraacetate **20** (251 mg, 0.114 mmol) in CH_2Cl_2/MeOH (1:9, 15 mL). After being stirred for 24 h, the reaction mixture was diluted with aq NH_4Cl (satd, 50 mL). The aqueous phase was extracted with $CHCl_3$ (3× 40 mL) and the combined organic extracts were washed with H_2O (100 mL). After drying ($MgSO_4$) and filtration, the solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:1 to 3:1) afforded title compound **21** (197 mg, 0.097 mmol, 85%) as an oil. $[\alpha]_D^{20}$ + 70 (*c* 0.80, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43–7.14 (m, 65H), 5.94–5.86 (m, 1H), 5.41 (s, 1H), 5.32–5.20 (m, 3H), 5.01 (d, *J* = 11.0 Hz, 1H), 4.95–4.55 (m, 21H), 4.49–4.39 (m, 6H), 4.36 (d, *J* = 12.0 Hz, 1H), 4.19–4.06 (m, 6H), 4.00–3.73 (m, 12H), 3.64–3.54 (m, 3H), 3.51–3.46 (m, 2H), 3.41–3.28 (m, 6H), 3.23–3.17 (m, 2H), 2.22 (br s, 4H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.7, 138.7, 138.6, 138.4, 138.3, 138.3, 138.1, 138.0, 138.0, 138.0, 137.9, 137.8, 133.8, 128.7, 128.6, 128.5, 128.5, 128.4, 128.4, 128.3, 128.3, 128.3, 128.3, 128.2, 128.2, 128.2, 128.0, 128.0, 128.0, 128.0, 127.9, 127.8, 127.8, 127.7, 127.6, 127.5, 127.5, 127.5, 127.4, 127.4, 127.3, 127.1, 117.9, 100.5, 100.1, 100.1, 99.8, 81.5, 81.5, 81.3, 80.5, 79.8, 79.6, 79.3, 78.9, 75.9, 75.8, 75.1, 75.0, 74.7, 74.2, 73.8, 73.6, 73.4, 73.4, 72.4, 72.0, 71.9, 71.7, 71.6, 71.3, 71.2, 71.1, 70.6, 70.6, 70.5, 68.7, 68.4, 68.0, 67.8, 65.8. HRMS-ESI $[M+Na]^+$ calcd for $C_{124}H_{134}O_{26}Na$: 2061.9061. Found 2061.9070.

4.1.15. 1-*O*-Allyl-2-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl)-6-*O*-[(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl)-(1 → 6)-(2,3,4-tri- *O*-benzyl-α-D-mannopyranosyl)-(1 → 6)-(2,3,4-tri- *O*-benzyl-α-D-mannopyranosyl)]-3,4,5-tri-*O*-benzyl-D-*myo*-inositol (22). Sodium hydride (60% dispersion in mineral oil, 30 mg, 0.75 mmol) was added to a stirred solution of tetraol **21** (188 mg, 0.092 mmol) in DMF (15 mL) cooled to 0 °C. After 20 min benzyl, bromide (66 μL, 0.55 mmol) was added and the reaction mixture stirred at rt for 14 h when aq NH_4Cl (satd, 50 mL) was added. The mixture was extracted with ether (2× 50 mL) and the combined ethereal extracts were washed with H_2O (2× 50 mL). After drying ($MgSO_4$) and filtration, the solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9 to 1:4 to 1:3) afforded the title compound **22** as an oil (178 mg, 0.074 mmol, 80%). $[\alpha]_D^{20}$ + 40 (*c* 0.71, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40–7.09 (m, 85H), 5.78–5.70 (m, 1H), 5.44 (s, 1H), 5.24–5.19 (m, 2H), 5.11–5.03 (m, 3H), 4.96–4.77 (m, 8H), 4.70–4.34 (m, 27H), 4.18–3.82 (m, 18H), 3.63–3.50 (m, 5H), 3.40–3.25 (m, 6H), 3.19–3.14 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 139.0, 138.9, 138.9, 138.9, 138.6, 138.6, 138.6, 138.5, 138.4, 138.4, 138.4, 138.3, 138.3, 138.1, 134.0, 128.6, 128.4, 128.4, 128.3, 128.3, 128.3, 128.2, 128.2, 128.2, 128.2, 128.2, 128.1, 128.1, 128.1, 128.1, 128.0, 128.0,

127.9, 127.8, 127.8, 127.7, 127.7, 127.6, 127.5, 127.5, 127.4, 127.4, 127.4, 127.3, 127.3, 127.2, 127.2, 127.1, 127.0, 117.7, 99.1, 98.8, 98.4, 98.3, 81.6, 81.4, 81.2, 80.7, 79.6, 79.2, 79.1, 78.8, 75.8, 75.7, 75.3, 75.0, 75.0, 74.9, 74.7, 74.7, 74.3, 73.8, 73.4, 73.3, 72.7, 72.6, 72.6, 72.3, 72.2, 72.2, 72.0, 71.9, 71.9, 71.6, 71.3, 71.2, 71.0, 70.4, 69.1, 69.0, 65.6, 65.5. HRMS-ESI $[M+Na]^+$ calcd for $C_{152}H_{158}O_{26}Na$: 2422.0939. Found 2422.0933.

4.1.16. 2-*O*-(2,3,4,6-Tetra-*O*-benzyl-α-D-mannopyranosyl)-6-*O*-[(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl)-(1 → 6)-(2,3,4-tri- *O*-benzyl-α-D-mannopyranosyl)-(1 → 6)-(2,3,4-tri- *O*-benzyl-α-D-mannopyranosyl)]-3,4,5-tri-*O*-benzyl-D-*myo*-inositol (23). (1,5-Cyclooctadiene)-bis(methyldiphenylphosphine)iridium(I) hexafluorophosphate (10 mg, 0.01 mmol) was added to a stirred solution of allyl ether **22** (68 mg, 0.028 mmol) in THF (5 mL) under argon. This atmosphere was replaced with hydrogen for ca 1 min and then, in turn, the hydrogen was replaced with argon. The mixture was stirred at 20 °C for 70 min, the solvent was removed in vacuo and the residue dissolved with stirring in CH_2Cl_2/MeOH (2:1, 9 mL). Acetyl chloride (100 μL, 0.26 mmol) was added to this solution and stirring was continued for 12 h when solid $NaHCO_3$ (200 mg, 2.38 mmol) was added. The mixture was stirred for an additional 5 min when H_2O (50 mL) was added. This mixture was extracted with $CHCl_3$ (2× 60 mL), and after drying ($MgSO_4$) and filtration the solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:4 to 3:7) afforded the title compound **23** as an oil (61 mg, 0.026 mmol, 92%). $[\alpha]_D^{20}$ +37.5 (*c* 1.22, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$) δ 7.39–7.12 (m, 85H), 5.46 (s, 1H), 5.23 (s, 1H), 5.15 (s, 1H), 4.93–4.85 (m, 7H), 4.76–4.42 (m, 27H), 4.30–4.27 (m, 2H), 4.12–3.78 (m, 17H), 3.70–3.51 (m, 8H), 3.39–3.31 (m, 3H), 3.23 (t, $J = 9.5$ Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.8, 138.7, 138.6, 138.5, 138.5, 138.5, 138.4, 138.4, 138.3, 138.2, 138.1, 138.0, 128.5, 128.5, 128.4, 128.4, 128.3, 128.3, 128.3, 128.3, 128.3, 128.2, 128.2, 128.2, 128.1, 128.1, 128.1, 128.0, 127.9, 127.8, 127.8, 127.8, 127.7, 127.7, 127.7, 127.6, 127.6, 127.5, 127.5, 127.5, 127.5, 127.4, 127.4, 127.4, 127.3, 99.0, 98.5, 98.3, 96.6 (br), 81.3, 80.6, 80.2, 79.2, 78.9, 78.7, 75.6, 75.4, 75.0, 75.0, 74.9, 74.8, 74.7, 74.1, 73.4, 73.3, 73.0, 72.5, 72.3, 72.3, 72.1, 72.1, 72.0, 71.9, 71.8, 71.8, 71.7, 71.7, 71.3, 69.2, 69.1, 66.4, 65.6. HRMS-ESI $[M+Na]^+$ calcd for $C_{149}H_{154}O_{26}Na$: 2382.0626. Found 2382.0732.

4.1.17. 2-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl)-6-*O*-[(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl)-(1 → 6)-(2,3,4-tri- *O*-benzyl-α-D-mannopyranosyl)-(1 → 6)-(2,3,4-tri- *O*-benzyl-α-D-mannopyranosyl)]-3,4,5-tri-*O*-benzyl-1-*O*-(1,2-di-*O*-hexadecanoyl-*sn*-glycero-3-benzylphosphoryl)-D-*myo*-inositol (24). 1*H*-Tetrazole (13 mg, 0.19 mmol) was added to a stirred solution of **23** (90 mg, 0.038 mmol) and phosphoramidite **8** (154 mg, 0.191 mmol) in CH_2Cl_2 (5 mL) cooled to 0 °C under an argon atmosphere. After stirring at rt for 2 h, the reaction mixture was cooled to −40 °C and a solution of *m*-CPBA (50%, 66 mg, 0.191 mmol) in CH_2Cl_2 (5 mL) was transferred by cannular into the reaction mixture. After warming to rt over 2 h, the reaction was quenched with the addition of aq Na_2SO_3 (10%, 50 mL) and the combined mixture was extracted with CH_2Cl_2 (2× 30 mL). The combined organic extract was washed with aq $NaHCO_3$ (satd, 3× 50 mL) and dried (Na_2SO_4). After filtration, the solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9 to 1:4) followed by a further purification with fresh silica gel eluting with EtOAc/CH_2Cl_2 (1:49 to 1:24) afforded the title compound **24** (61 mg, 0.020 mmol, 52%) as an oil. $[\alpha]_D^{20}$ +27.0 (*c* 1.22, $CHCl_3$). ^1H NMR (500 MHz, $CDCl_3$), mixture of isomers, δ 7.38–7.05 (m, 80 H), 5.42–5.5.40 (1H, m), 5.31–5.27 (m, 1H), 5.18–4.26 (m, 40H), 4.23–3.78 (m, 21H), 3.61–3.42 (m, 6H), 3.34–3.24 (m, 5H), 3.13–3.08 (m, 1H), 2.24–2.13 (m, 4H), 1.58–1.48 (m, 4H), 1.34–1.18 (m, 48H), 0.89 (t, $J = 6.5$ Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$), mixture of isomers, δ 173.1, 173.0, 172.8, 172.6, 139.1, 139.0, 139.0, 138.9, 138.8, 138.6, 138.6, 138.5, 138.4, 138.4, 138.4, 138.2, 138.0, 137.9, 137.8, 135.2, 135.2, 135.1, 135.0, 129.1, 128.9, 128.8, 128.6, 128.4, 128.4, 128.4, 128.3, 128.3, 128.2, 128.2, 128.2, 128.2, 128.1, 128.1, 128.1, 128.0, 128.0, 127.9, 127.8, 127.8, 127.7, 127.6, 127.6, 127.5, 127.5, 127.4, 127.4, 127.4, 127.3, 127.3, 127.2, 127.2, 127.1, 126.9, 99.6, 99.1, 99.0, 98.4, 98.2, 98.2, 81.0, 80.7, 80.6, 80.3, 80.2, 79.6, 79.2, 79.0, 78.7, 78.5, 76.0, 75.8, 75.7, 75.6, 75.0, 74.9, 74.7, 74.7, 74.6, 74.1, 73.9, 73.8, 73.7, 73.3, 72.9, 72.6, 72.6, 72.4, 72.3, 72.2, 71.9, 71.7, 71.6, 71.3, 71.3, 71.2, 70.2, 70.2, 69.5, 69.4, 69.1, 68.8, 66.1, 66.1, 65.5, 61.5, 34.1, 34.1, 34.0, 33.9, 32.0, 29.8, 29.7, 29.6, 29.4, 29.4, 29.2, 29.2, 24.8, 22.8, 14.2. ^{31}P NMR (121.5 MHz, $CDCl_3$) δ 1.11, 0.80. HRMS-ESI $[M+Na]^+$ calcd for $C_{191}H_{227}O_{33}NaP$: 3012.5720. Found 3012.5745.

4.1.18. 1-*O*-(1,2-di-*O*-Hexadecanoyl-*sn*-glycero-3-phosphoryl)-2-*O*-(α-D-mannopyranosyl)-6-*O*-[(α-D-mannopyranosyl)-(1 → 6)- (α-D-mannopyranosyl)-(1 → 6)-(α-D-mannopyranosyl)]-D-*myo*-inositol (2). $Pd(OH)_2$/C (20%, 25 mg) was added to a stirred solution of **24** (59 mg, 0.019 mmol) in THF/MeOH (2:3, 5 mL). The mixture was stirred under hydrogen atmosphere for 4 h at rt when the hydrogen was replaced with argon. The mixture was filtered through Celite® and concentrated in vacuo. The residue was lyophilized to afford **2** (25 mg, 0.017 mmol, 89%) as a white powder. $[\alpha]_D^{20}$ +37 (*c* 0.30, D_2O). ^1H NMR (500 MHz, $CDCl_3$/CD_3OD/D_2O, 1.0:1.5:0.5), δ 5.32–5.26 (m, 1H), 5.15 (br s, 1H), 5.11 (br s, 1H), 4.88 (br s, 1H), 4.86 (br s, 1H), 4.40–3.48 (m, 36H), 2.40–2.31 (m, 4H), 1.67–1.57 (m, 4H), 1.35–1.20 (m, 48H), 0.89 (t, $J = 6.9$ Hz, 6H). ^{31}P NMR (202.5 MHz, $CDCl_3$/CD_3OD/D_2O, 1.0:1.5:0.5) δ 0.5. HRMS-ESI $[M-H]^-$ calcd for $C_{65}H_{118}O_{33}P$: 1457.7293. Found 1457.7284.

4.2. Experimental Procedures for the Adjuvant activity of PIM2 and PIM4

Female C57BL/6 mice were purchased from the Hercus Taieri Resource Unit, Dunedin, New Zealand. Female

OVA 323-339 specific MHC class II restricted T cell receptor transgenic mice on the C57BL/6 background[30] were donated by Dr. J. Kirman of the Malaghan Institute, Wellington, New Zealand, and bred in our facility. Mice were housed in containment facilities and used between 6 and 12 weeks of age. All procedures on the mice had the approval of the Wallaceville Research Centre Animal Ethics Committee (Upper Hutt, New Zealand). Spleen cells from transgenic animals were harvested, pooled and 2.5×10^7cells per mouse were transferred to naïve C57BL/6 mice by intravenous injection via the tail vein. Successful cellular transfer was verified by flow cytometry with antibodies against OVA-specific Vα2 and Vβ5.1 T cell receptors. Mice were vaccinated 3 days after the cellular transfer. Mice were injected subcutaneously between the shoulder blades with 100 μg of OVA (Sigma, St. Louis, MO, USA) with or without PIMs (36 μg) in PBS or emulsified in 0.1 mL of Complete Freund's adjuvant (CFA, Difco, Detroit, MI, USA). Mice were euthanized by cervical dislocation and axillary lymph node cells prepared by passage through an 80 gauge wire mesh sieve. After washing, cells were cultured in triplicate into flat-bottomed 96- well plates at a concentration of 5×10^5 cells/well (200 μL volumes) with 1 μM of OVA-specific peptide (Auspep, Parkville, Victoria, Australia) and incubated at 37 °C and 10% CO_2. The peptide used for the in vitro stimulation was endotoxin-free, as assessed by the Limulus amoebocyte assay (Cape Cod Associates, Falmouth, MA, USA). Supernatants were removed from cultures after 4 days incubation and frozen at −80 °C until assayed. Levels of IFN-γ in culture supernatants were measured by ELISA. Pairs of monoclonal antibodies against mouse IFN-γ were purchased from Pharmingen (San Jose, CA, USA). IFN-γ responses were measured with an avidin horseradish peroxidase conjugate and *o*-phenylenediamine (OPD) substrate (Sigma).

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