Towards the development of synthetic vaccines against tuberculosis

Marino, L.

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Mincle/TLR2 co-stimulation using synthetic ligands

Laura Marino [1], Susan J.F. van den Eeden [2], Krista E. van Meijgaarden [2], Nico J. Meeuwenoord [1], Dmitri V. Filippov[1], Gijs A. van der Marel [1], Jeroen D. C. Codée [1], Tom H.M. Ottenhoff [2]

[1] Department of Bioorganic Synthesis, Leiden University, Leiden, The Netherlands

[2] Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands

Abstract

C-type lectin receptors (CLRs) and Toll-like receptors (TLRs) are proteins expressed on antigen-presenting cells, involved in pathogen recognition by the host. CLRs and TLRs can act in synergy by interacting with different microbial ligands thus inducing amplified immune responses, which may result in the production of pro-inflammatory cytokines to a significantly higher extent than that resulting from interaction with a single ligand. Given the importance of the macrophage inducible C-type lectin receptor (Mincle) and the Toll-like receptor 2 (TLR2) in mycobacterial recognition, with Mincle shown to interact with cell-wall glycolipids and TLR2 interacting with lipoproteins from *Mycobacterium tuberculosis*, the effects of Mincle/TLR2 co-stimulation on murine and human dendritic cells was investigated. Dendritic cells are key players in the induction of innate and adaptive immune responses, and their activation can result in induction of both pro- and anti-inflammatory cytokines. In the present study two synthetic compounds were selected, namely trehalose distearate (TDS, a Mincle ligand) and UPam, a TLR2 ligand, and by using a matrix of concentrations for the two ligands, identified TDS/UPam combinations which resulted in increased production of pro-inflammatory, as well as anti-inflammatory cytokines by human monocyte-derived dendritic cells (moDCs). On the basis of these results, it was investigated whether Mincle/TLR2 co-stimulation would also affect (positively or negatively) adaptive immune responses by studying antigen presentation to human monoclonal $CD4^+$ Th1 T cells specific for peptide epitopes from the mycobacterial Heat shock protein 65. These results showed that, upon stimulation of moDCs in the presence of cognate antigen, the addition of combinations of TDS and UPam did not affect (neither increased nor decreased) the level of T cell proliferative responses compared to single pathogen recognition receptor stimuli.

Introduction

The immune system is able to detect microbial pathogens through pathogen recognition receptors (PRRs), proteins expressed by antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages, amongst others. It is well established that different PRRs can be simultaneously engaged by infecting pathogens, and several groups have investigated synergistic interactions between different PRRs.[1–3]

Toll-like receptors (TLRs) recognise a plethora of microbial structures and they are arguably the most studied PRRs for their effects of co-stimulation on the induction of inflammatory responses. For example, Napolitani *et al.* measured the induction of several pro-inflammatory cytokines in human and murine DCs via ELISA and RT-PCR, showing that TLR3 and TLR4 activating ligands can act in synergy with ligands for three other receptors, namely TLR7, TLR8 and TLR9.[4] On the other hand, much less is currently known about the interaction of TLRs with C-type lectin receptors (CLRs), a class of PRRs specialized in detection of microbial carbohydrates. The investigation of TLRs/CLRs interactivity is of interest, since it can provide insights into the interaction of two distinct signalling pathways.[5,6]

During mycobacterial infections, host immune cells are exposed to mycobacterial lipoproteins as well as glycolipids. These pathogen-associated molecular patterns (PAMPs) can engage the TLR2 and the macrophage inducible C-type lectin receptor (Mincle), which are expressed on the cell surface of human and murine antigen presenting cells. TLR2 engagement induces pro-inflammatory cytokine production via the MyD-88/TIRAP signalling pathway, while Mincle signals via ITAM/Syk/Card9, and both pathways converge on NF-κB activation.[7–9] Interestingly it has been shown that expression of the Mincle receptor is increased upon TLR2 stimulation. Schick *et al.* have shown that glycolipid extracts of *C. diphtheriae* and of *C. ulcerans*, as well as the commercially available TLR2 ligand lipopeptide Pam_3CSK_4 upregulated Mincle expression by murine bone marrow-derived macrophages (BMMs) in a TLR2-dependent manner.[10] Matsumura *et al.* proposed that TLR2-induced production of IL-6 enhanced Mincle expression on IFN-γ-producing immature myeloid cells.[11] More information on the cooperation between Mincle and TLR2 was provided by Lee *et al.* who stimulated murine BMMs with Pam_3CSK_4 and trehalose 6-6'-dimycolate. Using RNA sequencing they compared the changes in mRNA transcriptional profiles by stimulating BMMs either with the single ligands or the combination of the two, and concluded that Mincle signalling at the initial stage of cell activation synergistically modulated the transcription of most TLR2-regulated genes towards pro-inflammatory anti-mycobacterial responses (*e.g.,* iNOS, IL-12 and CCL2), at least in mice. They also showed that, at this stage, type I interferon responses were selectively inhibited, while at a later stage,

continuous Mincle/TLR2 stimulation inhibited the general translational machinery, possibly leading to inflammation control.[12] These results point to a potential double role of trehalose 6-6'-dimycolate in admixture with TLR2 ligands in initiation and control of host responses to infection.

Given the key role that DCs play in the induction of innate and adaptive immune responses, the effect of co-stimulation of human dendritic cells with well-defined, synthetic TLR2 and Mincle ligands was here investigated. Trehalose-6,6'-distearate (TDS) and 1-tetradecylcarbamyl-Cys((RS)-2,3-di-(palimitoyloxy)propyl)-Ser-Lys-Lys-Lys-Lys-NH_2 (UPam) were used to investigate whether this combination can result in synergistic activation of murine and human dendritic cells (the chemical structures are shown in Figure 1). The ligands used in the present study were selected because of their known ability to interact with murine and human Mincle and TLR2, respectively.[13–15] Moreover, these compounds are readily produced via synthetic pathways and can be easily modified for conjugation to antigens, making them versatile tools for the generation of subunit vaccines.

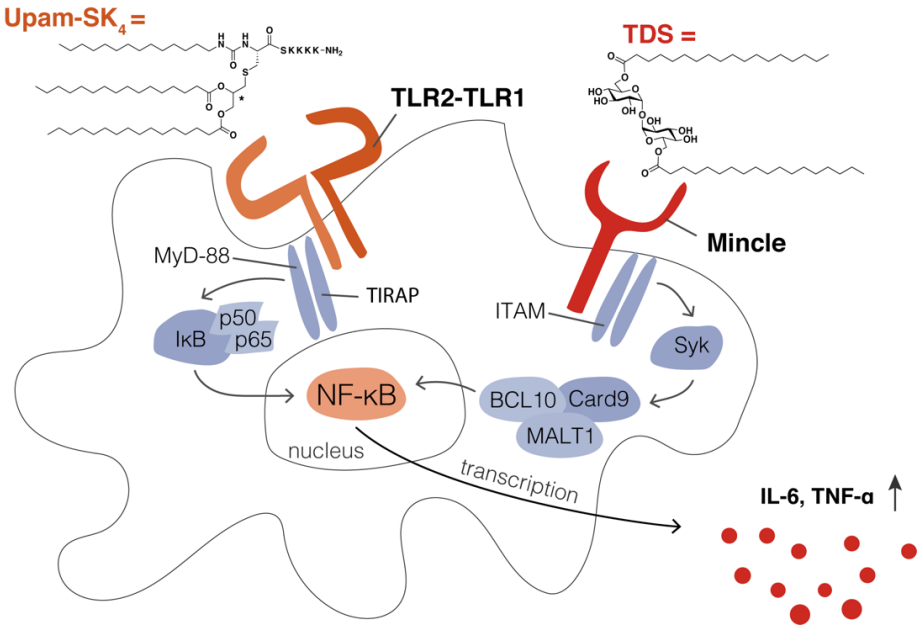

Figure 1 – Visual representation of a dendritic cell and its interaction with UPam and TDS via TLR2/TLR1 and Mincle, respectively. Key proteins involved in signaling through the two receptors are shown. Chemical structures of the synthetic adjuvants selected for this study are also presented.

Three cellular systems were selected to investigate the effects of single vs. double ligand activation: a D1 murine cell line system, which is a long-term immature DC cell line from cultured mouse spleen cells;[16] human monocyte-derived dendritic cells (moDCs); and two cloned human T cell lines specific for mycobacterial Heat shock protein 65.[17] Flow cytometry was employed to assess if double vs. single stimulation would result in increased expression of activation markers and T cell co-stimulatory molecules on APCs. Analysis of the level of pro- and anti-inflammatory cytokines released in the cell supernatants upon stimulation with nine different mixtures of UPam and TDS, was performed in order to identify potential synergistic combinations of the two ligands. Antigen-presentation studies using the two T cell clones were also performed to analyse the influence of selected single vs. double stimulations with UPam and/or TDS on T cell proliferation.

Results

In vitro results using murine D1 cells system

First, the expression of Mincle on the D1 cell line was verified using a cross species reactive anti-Mincle antibody (anti-CLEC4E Clone 16E3). As shown in supporting Figure S1, Mincle is expressed on the cell surface of immature D1 cells. Subsequently, D1 cells were assessed for their responsiveness to Mincle ligands, following stimulation with either synthetic TDS or commercial trehalose 6-6'-dibehenate (TDB). ODN1826, a TLR9 ligand was taken along as positive control. Upon treatment with these ligands, the cells shifted from an immature DC-like phenotype to mature DC-like phenotype which resulted in increased expression of CD40 and CD86 activation markers, as determined by flow cytometry (see supporting Figure S1). Stimulation of D1 cells using TDS and TDB also resulted in production of IL-12p40, as determined by ELISA (see supporting Figure S2).

In line with previous results,[13] UPam stimulation resulted in an increased number of $CD40^{+}CD86^{+}$ cells and production of IL-12p40 as compared to the unstimulated control (Figure 2A and 2B). Notably, TDS stimulation induced an even stronger cellular activation with significantly higher levels of IL-12p40 production as compared to UPam stimulation. Combinations of UPam/TDS were investigated using a 3 by 3 concentration matrix, with concentrations ranging from 50 - 10 - 2 μM for both ligands. As shown in Figure 2C, stimulation of the cells with 10 μM UPam in combination with TDS (50 - 10 - 2 μM) resulted in increased numbers of $CD40^{+}CD86^{+}$ cells as compared to stimulation with UPam or TDS alone. However, no increase in IL-12p40 production was observed (Figure 2D).

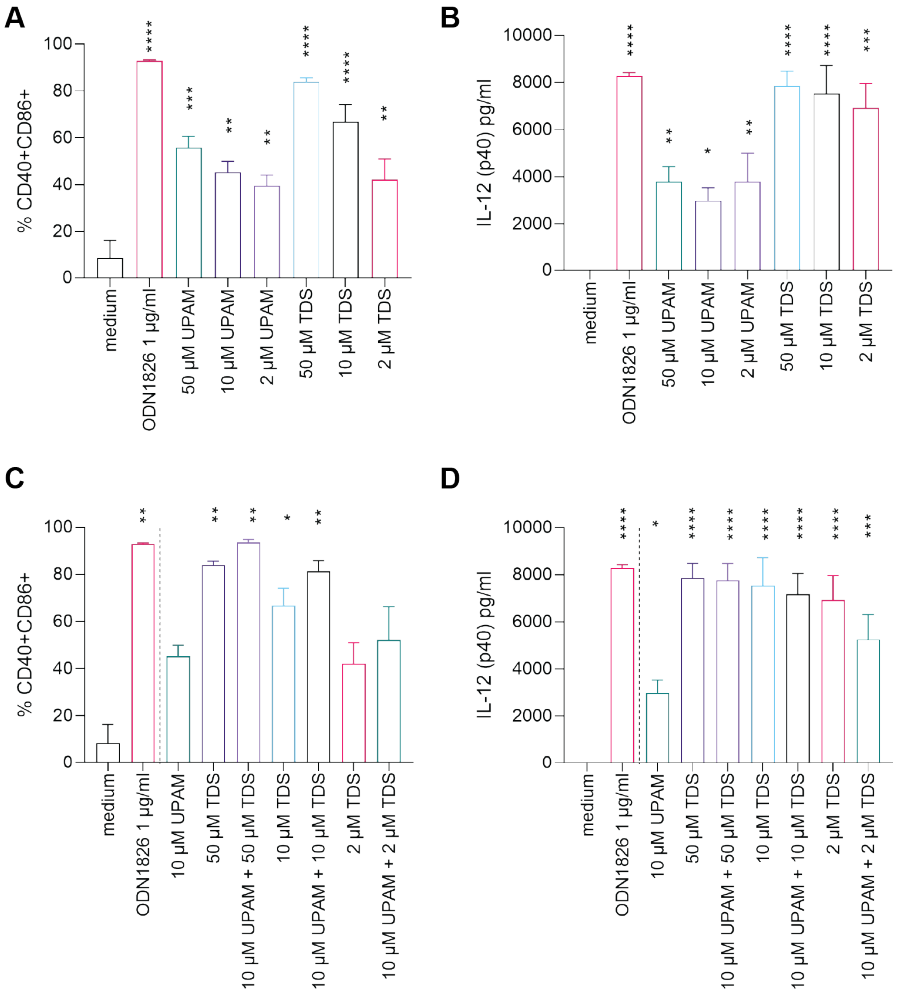

Figure 2 - Effects of UPam/TDS stimulation on D1 DC cells. Bar plots depicting: **(A)** percentage of CD40-CD86 double positive D1 cells as measured by flow cytometry; **(B)** amount of IL-12p40 cytokine detected in the supernatant as measured by ELISA after 20 hours stimulation with UPam or TDS; **(C)** percentage of CD40-CD86 double positive D1 cells as measured by flow cytometry and **(D)** amount of IL-12p40 cytokine detected in the supernatant as measured by ELISA after 20 hours stimulation with UPam and TDS or their combination. Error bars represent mean + SD of two independent experiments as calculated using GraphPad Prism. Statistical significance with reference to cells exposed to medium + DMSO was calculated through one-way ANOVA method (****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05). CpG ODN1826 (1 µg/ml) was used as positive control.

In vitro results using a human moDCs cell system

Immature monocyte-derived dendritic cells (DC) were differentiated as previously described and analysed for correct differentiation by expression of CD14, CD163, CD11b, CD1a.[18,19]

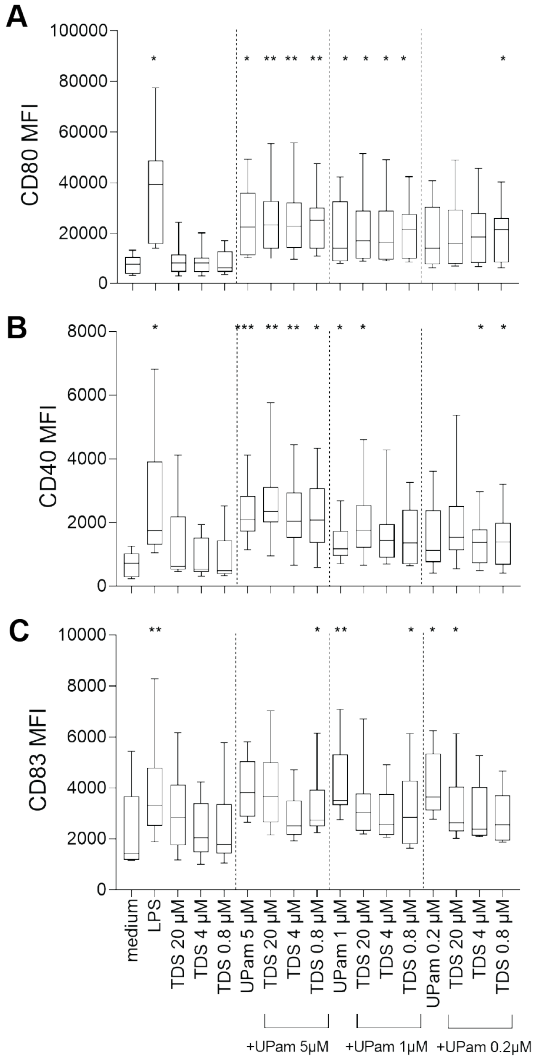

Figure 3 - Box and whiskers plots depicting the expression of selected surface markers from 9 different donors, as measured by flow cytometry. Box plots indicate the median value of the dataset (n = 9 donors) with error bars from min to max values as calculated using GraphPad Prism. Statistical significance with reference to cells exposed to medium + DMSO was calculated through one-way ANOVA method (***p < 0.001; **p < 0.01; *p < 0.05). LPS (100 ng/ml) was used as a positive control.

In order to verify that this human cell model could be used to investigate the response to the Mincle/TLR2 ligands, immature moDCs were further characterised for expression of human Mincle and TLR2. As shown in supporting Figure S3, both receptors were expressed in immature moDCs, and the expression of TLR2 but not Mincle further increased upon stimulation by UPam and LPS.

Studies conducted with immature moDCs showed that stimulation with a concentration range of UPam (5 - 1 - 0.2 μM) resulted in cell activation with an increased expression of CD40 and CD80 T cell co-stimulatory markers and CD83 activation marker. By contrast, stimulation with a concentration range of TDS (20 - 4 - 0.8 μM) resulted only in an increase in CD83 but not CD40 or CD80 expression.

To determine the immunogenic profile of simultaneous Mincle/TLR2 receptor stimulation, moDCs were stimulated using matrixed combinations of UPam (5 - 1 - 0.2 μM) and TDS (20 - 4 - 0.8 μM). When TDS was added to UPam, only minor changes were observed for CD83 and CD40 (Figure 3C, 3B). Interestingly, TDS did not promote CD40 expression, although co-stimulation using UPam and TDS, for the two lowest concentrations (1 and 0.2 μM) of UPam selected in this study, led to upregulation of this surface protein to somewhat higher levels than the corresponding UPam stimulus. In the case of CD80 (Figure 3A), upregulation of this marker could be observed when the cells were treated with UPam, in a dose dependent manner. In contrast TDS stimulation, by itself, did not lead to an increase in CD80 expression. The simultaneous stimulation using TDS and UPam resulted in increased expression of CD80 as compared to single UPam stimulation only for the suboptimal 0.2 and 0.8 μM concentrations of UPam and TDS, respectively.

Cell supernatants were analysed by ELISA for the quantification of IL-12p40, IL-10, IL-6 cytokines and by Luminex for TNF-α, IL-8, IL-1β and GM-CSF. Neither UPam nor TDS stimulation resulted in detectable IL-1β and GM-CSF production (data not shown). Interestingly, when moDCs were stimulated using UPam, medium to high levels of IL-12p40, IL-10, IL-8, IL-6 and TNF-α were released in the cell supernatant after 20 hours (see Figure 4). Neither of these cytokines could be detected in the supernatants of TDS-stimulated cells.

Stimulation of DCs using mixtures of TDS 0.8 μM with UPam (5 - 1 - 0.2 μM) resulted in increased production of IL-6 and TNF-α as compared to stimulation by single ligand, with statistically significant differences for one defined concentration of ligands. In the selected concentration window, the release of IL-6 in the cell supernatants tended to increase, with twice as much IL-6 produced when UPam 1 μM was combined with TDS 0.8 μM as compared to the amount of cytokine induced by UPam 1 μM alone (see Figure 4). For the same concentrations of ligands, also the amount of TNF-α increased significantly, doubling the amount released upon UPam stimulation of DCs. The amount of IL-12p40 and IL-10 increased slightly upon double ligand activation, although comparison to the single ligand activation did not result

in a statistically significant change (Figure 4C and 4D). However, the amount of IL-8 slightly decreases upon TLR2/Mincle stimulation as opposed to single TLR2 stimulation in the studied concentration range (see supporting Figure S4).

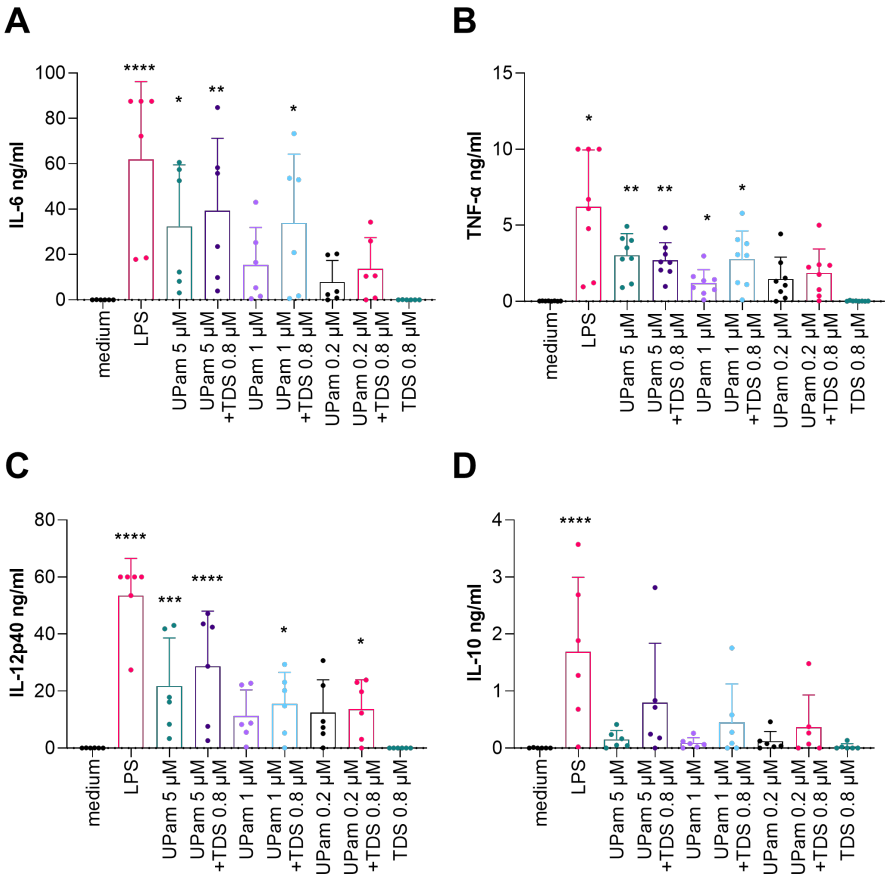

Figure 4 - Cytokine production profile of human moDCs stimulated for 20 hours using single adjuvants or mixtures thereof, as measured by ELISA or milliplex. (A, C, D) Dots shown in the scatter dot plots represent IL-6, IL-12p40 and IL-10 data from 6 different donors (each dot corresponds to the mean of duplicate measurements), as measured by ELISA; **(B)** Dots shown in the scatter dot plots represent TNF-α data from 8 different donors, as measured by milliplex. Bars indicate mean value + SD. Statistical significance with reference to cells exposed to medium + DMSO was calculated through one-way ANOVA method (****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05). LPS (100 ng/ml) is used as a positive control.

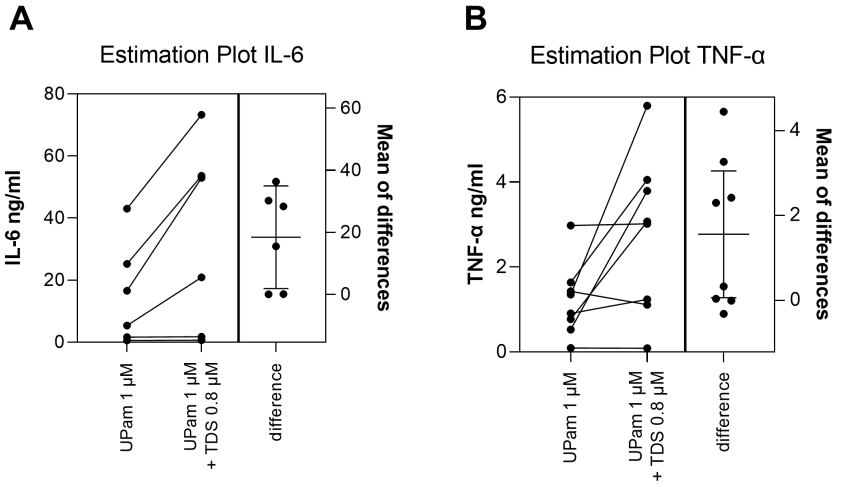

Figure 5 – Estimation plots for cytokines produced by human moDCs using UPam 1 µM or UPam 1 µM in combination with TDS 0.8 µM. Comparison of change in cytokine concentration for depicted cytokines is visually facilitated connecting with a line the cytokine concentration per each donor after stimulation with single or double adjuvants. The difference in cytokine concentration of the two selected conditions per each donor is visualized on the right side of each plot. **(A)** Dots shown in the scatter dot plots represent IL-6 data from 6 different donors (each dot corresponds to the mean of duplicate measurements), as measured by ELISA; **(B)** Dots shown in the scatter dot plots represent TNF-α data from 8 different donors, as measured by Luminex. Error bars indicate mean value of differences + 95% confidence interval, as calculated by GraphPad Prism.

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For comparison purposes, paired t-test for each relevant pair of conditions was performed and the resulting estimation plots for the statistically significant difference are shown in Figure 5. These plots clearly show that dendritic cells from 4 out of 6 donors produced a higher amount of IL-6 when stimulated with the mixture of ligand as compared to UPam only. In contrast, UPam stimulation on cells from the remaining 2 donors resulted in very low amounts of this cytokine, and addition of TDS did not increase production. Additionally, TNF-α production increased for 4 out of 8 donors for double ligand stimulation as opposed to UPam stimulation (Figure 5B).

In vitro results using human T cell clones

To study the effect of TDS/UPam co-stimulation on antigen-presentation by monocyte-derived dendritic cells of peptide antigen to T cells, a T cell proliferation assay was performed. Here several combinations of TDS (0.8 and 4 μM) and UPam

(0.2, 1 and 5 μM) were selected in the presence of varying amounts of antigen. Two T cell clones were selected, namely R2F10, a DR2-restricted T cell clone generated against the peptide p418-427 from *Mycobacterium leprae* heat shock protein 65, and Rp15 1-1, a DR3-restricted T cell clone generated against the peptide p3-13 from *Mycobacterium leprae/Mycobacterium tuberculosis* heat shock protein 65 matching the HLA DR type of the moDC donor.

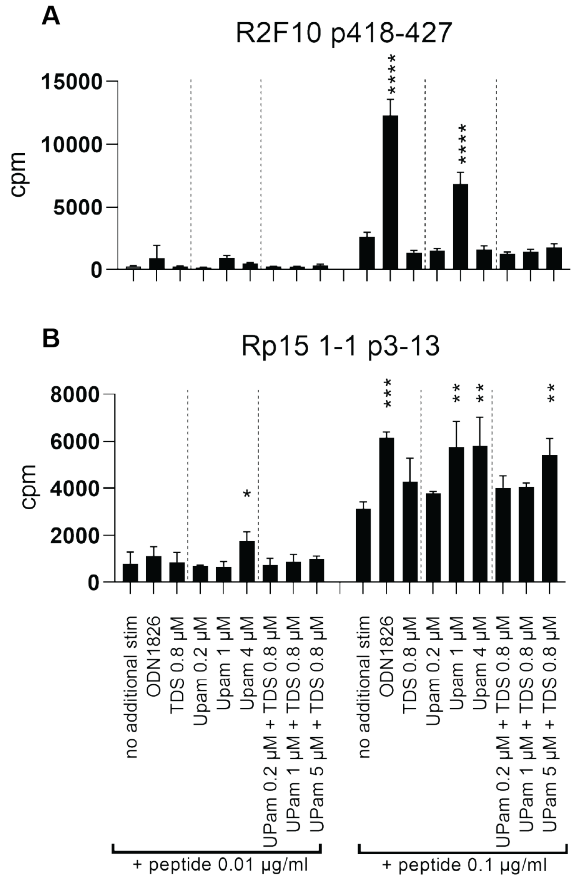


Figure 6 - Antigen presentation experiment using moDCs and T cell clones. T cell proliferation for the R2F10 p418-427 clone (subplot A) and for the Rp15 1-1 p3-13 clone (subplot B) was measured on a Microbetaplate counter after 18 hours pulsation with [^{3}H]thymidine and it is expressed as counts per minute (cpm). Values represent mean + SD of triplicate measurements from one experiment as calculated using GraphPad Prism. Statistical significance with reference to cells exposed to reference peptide was calculated through one-way ANOVA method (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$). Peptide (at described concentration) is used as positive control.

Only UPam at a very narrow single concentration (1 μM) (seen for both T cell clones) and ODN1826, a TLR9 ligand used as positive control, induced an increase in proliferation of T cells from the R2F10 clone (see Figure 6). For clone Rp15 1-1 there was a slight to moderate increase in T cell proliferation by addition of all ligands, but no synergistic effect was observed, indicating the effect on the antigen presenting capacity of the treated moDC not to be interfering nor beneficial for T cell antigen presentation.

Discussion

There is increasing evidence that vaccine adjuvants containing PAMPs not only function to amplify an immune signal,[20] but can also influence and direct the type of immune response.[3,21] Single-molecule PRR ligands allow for the study of these immune pathways and represent valuable tools to deepen our understanding of how the immune system responds to single PAMP molecules. When a pathogen interacts with cells from the host, several immunostimulatory molecules are present simultaneously that activate different receptors expressed by antigen-presenting cells.[22] It has been shown that distinct combinations of pathogenic molecules lead to very specific responses.[5] PRRs can cooperate to produce amplified responses, a phenomenon known as synergistic activation. Many combinations of ligands acting synergistically have been identified within the TLR family.[4,23–30] Recently, combinations of nucleotide-binding oligomerization domain-like receptors, retinoic acid-inducible gene-I-like receptors, CLRs and TLRs have also been studied.[31–34] Not all combinations lead to synergy.[35,36] Here, the effect of simultaneous TLR2/Mincle stimulation on murine and human DCs as key players in innate and adaptive immune responses was studied, because the effect of co-stimulation by ligands for Mincle and TLR2 on this type of antigen-presenting cells had not been investigated previously. Synthetic small molecules, namely TDS, a synthetic analogue of mycobacterial cord factor and an established Mincle ligand, and UPam, a synthetic ligand very similar in structure to Pam_3CSK_4 which is able to activate the TLR1/TLR2 heterodimer[13,15] were selected for this study. One of the advantages of using small molecules is their synthetic and commercial potential, in addition to the ease of chemical modification, which allows for the generation of multiple epitope/adjuvant conjugates, the insertion of molecular scaffolds to optimise the immune response and/or their facile incorporation in nanoparticles. Two model cellular systems were used, the murine D1 DC cell line and human monocyte-derived DCs for the study of potential Mincle/TLR2 co-stimulation. It was first verified that both cell types expressed Mincle in their immature state. The D1 DC cells expressed Mincle and they responded to TDS stimulation by upregulating the expression of CD40 and CD86, and by producing the pro-inflammatory cytokine IL-12p40. As expected,[13] also UPam stimulation resulted in upregulation of selected activation markers and production

of IL-12p40, although to a lower extent than when stimulating these cells with TDS. No synergy, however, was detected when stimulating the D1 DC cells with the two ligands. Human DCs, generated by culturing monocytes in the presence of IL-4 and GM-CSF, were stimulated using a matrix of nine combinations of the two ligands and the effects of co-stimulation were assessed using flow-cytometry to study the expression of activation markers and T cell co-stimulatory molecules, and ELISA and Luminex to detect cytokine-production. Notably, TDS stimulation induced only CD83 upregulation in moDCs and no production of IL-10, IL-6, IL-8, IL-12 nor TNF-α. When Decout *et al.* treated human monocyte-derived DCs or peripheral blood monocytes with TDB, a synthetic compound structurally very similar to TDS, they also observed that no TNF-α nor IL-6 was detected in the cell supernatants.[37] A different study by Smith *et al.* reported on Brartemicin derivatives, which are synthetic aryl-6,6'-trehaloses, being able to induce the production of cytokines, including TNF-α and IL-6, by human peripheral blood monocytes.[38] It is possible that the physicochemical properties of the different trehalose derivatives influence the interaction with Mincle and the uptake by APCs. This has also been suggested by Kiyotake *et al.* for cholesterol in the soluble or crystalline form.[39] Additionally, Hunter *et al.* have described how different supra-molecular structures of trehalose dimycolate are responsible for very different activities on human or murine macrophages.[40] Further studies, including structure-activity relationship studies using human Mincle, are required to fully explain the absence of cytokine production by human moDCs stimulated with TDS, as observed in our experiments. It is possible that the interaction to Mincle of the solubilized/suspended ligand differs from the interaction of the same ligand in a plate-bound form. However, with the overarching goal of including TDS or analogues in a single molecule vaccine modality containing two adjuvants, the studies reported in this Chapter required evaluation of the immune-stimulatory properties of the solubilized/suspended form of this compound.

On the contrary, UPam stimulation, performed as described in the materials and methods section of this Chapter, induced upregulation of all selected markers and production of both pro-inflammatory and anti-inflammatory cytokines. These results are in agreement with published data, where it has been shown that stimulation of moDCs by UPam induced upregulation of CD83, CD86 and HLA-DR in addition to production of several chemokines and cytokines, including IL-12, IL-8 and IL-10.[14] Interestingly, mixtures of the two ligands, in the assessed concentration window, induced increased production of TNF-α and IL-6 (up to 2-fold), as compared to the corresponding amounts released by only UPam stimulation. High inter-donor variability in terms of extent of the cytokine response to stimuli, with some DCs producing high amounts of cytokines and others responding with low production, was observed and this may be related to TLR1 polymorphisms.[41–43] Future studies should include DNA analysis to detect TLR1 polymorphisms with the aim of selecting donors who may better respond to UPam treatment.

Finally, to study the effect of TDS/UPam co-stimulation on antigen-presentation by monocyte-derived dendritic cells in the presence of peptide antigen, and to exclude inhibitory effects on T cell activation, a T cell proliferation assay was performed. No decrease or increase in T cell proliferation was observed upon co-stimulation with the two PRR ligands, suggesting that the combination of UPam and TDS, in the selected concentration range, does not improve antigen presentation by dendritic cells. However, antigen presentation of peptide p418-427 to the R2F10 clone was strongly enhanced in the presence of single adjuvant UPam (1 μM) and CpG control as compared to stimulation with peptide alone, and this was the case to a lesser extent for the p3-13 specific T cell clone. TLR ligands, such as CpG oligonucleotides and Pam_3CSK_4, have been shown to enhance T cell activation and proliferation when combined with anti-CD3 stimulation but this work did not address physiological HLA class II dependent antigen-presentation.[44–46]

In summary, the present study describes the immune-stimulatory effect of TLR2/Mincle co-stimulation using synthetic PRR ligands on murine and human dendritic cells, key players in the induction of T cell immune responses. Experiments performed using the murine D1 DC cell line, indicated that admixture of TDS and UPam induced a minor further increase in cell activation as compared to single ligand stimulation. Detailed quantification of cytokine production by human moDCs, however, allowed for the identification of only one synergistic/additive combination of the two synthetic PRR ligands, although minimal or no upregulation was observed for CD80, CD83 or CD40. Functional ELISA and Luminex assays identified concentrations of TDS and UPam which induced increased production of TNF-α and IL-6, as compared to the corresponding amount released following UPam stimulation alone. Notably, TDS alone failed to induce detectable amounts of any of these cytokines. Further studies are required to discover which cellular mechanisms may be responsible for these observations. Taken together, these first results suggest the potential of co-stimulation of the human TLR2 and Mincle receptor for the enhancement of immune responses typically associated with TLR2 stimulation.

Materials and methods

Synthesis of ligands

The two synthetic ligands employed in this study were synthesized according to published procedures.[13,15] Synthetic compounds were tested using LAL assay to exclude the possibility of LPS contamination.

Culturing and stimulation of murine D1 cells

The D1 cell line[16] was obtained from the department of Immunohematology and Blood Transfusion of the Leiden University Medical Centre. The cells were cultured in IMDM medium (Lonza, Belgium) containing 10% heat inactivated FBS (Sigma, St.Louis, MO, USA), 2 mM GlutaMAX™ (Gibco, PAA, Linz, Austria), 50 μM β-mercaptoethanol (Sigma, St.Louis, MO, USA) and 30% supernatant from R1 cells (mouse fibroblast NIH/3T3 cells transfected with mouse GM-CSF gene), which was collected from confluent cultures and filtered. Cells were harvested using PBS containing 2 mM EDTA (Sigma, St.Louis, MO, USA), counted and transferred to 96 well-plates (round bottom, Corning Costar TC-Treated Microplates, Corning, NY) at approximately 50.000 cells/well. Immediately after plating, the cells were stimulated at described concentrations (see results section) of UPam, trehalose 6-6'-distearate and combination thereof. Synthetic compounds were dissolved in DMSO (Sigma, St.Louis, MO, USA) at a concentration of 5 nmol/μL, further diluted and premixed in culture medium. ODN1826 (1 μg/ml; 5'-TCCATGACGTTCCTGACGTT-3'; InvivoGen, San Diego, CA) and TDB (50 – 10 – 2 μM; Avanti Polar Lipids, Alabama) were used as positive control for cell stimulation. Supernatants were harvested 20 hours after the addition of stimuli and cells were stained as described below.

Generation and stimulation of immature human moDCs

Buffy coats of healthy human Blood Bank donors were purchased from Sanquin, Amsterdam, the Netherlands. $CD14^+$monocytes were isolated from whole blood using Ficoll-Paque density gradient followed by purification on autoMACS® Pro Separator instrument using CD14 MicroBeads (MACS, Miltenyi Biotec, Bergisch Gladsbach, Germany). The monocytes were differentiated to monocyte-derived dendritic cells (moDCs) at a concentration of 106 cells/mL in RPMI 1640 (Gibco, PAA, Linz, Austria) medium containing 10% FCS (HyClone, GE Healthcare Life Sciences, Eindhoven, the Netherlands), 2 mM GlutaMAX™ (Gibco, PAA, Linz, Austria), 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies-Invitrogen), 10 ng/mL GM-CSF (Life Technologies-Invitrogen, Bleiswijk, the Netherlands) and 10 ng/mL IL-4 (Peprotech, Rocky Hill, NJ). On day 3 of culturing, fresh medium (RPMI 1640 + 10% FCS, 2 mM GlutaMAX™ + 100 U/ml penicillin + 100 µg/ml streptomycin) supplemented with 30 ng/mL GM-CSF and 30 ng/mL IL-4 was added to the cell culture plates (0.5 mL fresh medium per 1 ml old medium). Cells were differentiated for a total of 5 days at 37°C and in a 5% CO_2 atmosphere. Differentiated $CD14^+$ monocytes were harvested, counted and transferred to 96 wells plates (round bottom, Corning Costar TC-Treated Microplates, Corning, NY) at approximately 50.000 cells/well. The next day, cells were stimulated with described concentrations (see results section) of UPam, trehalose 6-6'-distearate and combination thereof. Synthetic compounds were dissolved in DMSO (Sigma, St.Louis, MO, USA) at a concentration of 5 nmol/μL, further diluted and premixed in RPMI 1640 medium containing

10% FCS, 2 mM GlutaMAX™, 100 U/ml penicillin, and 100 µg/ml streptomycin. LPS (InvivoGen, San Diego, United States) (100 ng/ml) was used as positive control for stimulated cells. Supernatants were harvested 20 hours after the addition of stimuli and cells were stained as described below.

Cytokine detection

The concentration of cytokines in culture supernatants was measured by ELISA according to the manufacturer's instructions. Mouse IL-12/IL-23 (p40), human IL-12/IL-23 (p40) and human IL-10 ELISA kits were purchased from Biolegend (ELISA MAX™ Standard Set; London, UK). Human IL-6 ELISA kit was purchased from Invitrogen (Thermo Fisher scientific, Merelbeke, Belgium). Microlon high binding 96 well plates (Greiner Bio-One International, Alphen a/d Rijn, The Netherlands) were used for the assays. Sample absorbance was measured using a Spectramax i3x (Molecular Devices, CA, USA) spectrometer. All samples were tested in duplicate. The concentration of TNF-α was determined using a MILLIPLEX MAP Human TH17 Magnetic Bead Panel (Merck, Darmstadt, Germany) according to manufacturer's protocol and also contained reagents for the quantification of IL-6, IFN-γ and GM-CSF. Milliplex data were measured using a Bio-Plex 200 system and analysed using Bio-Plex Manager software v6.2 (Bio-rad, Belgium).

Flow cytometric analysis of D1 cells

After 20 h stimulation, murine D1 cells were incubated for 30 minutes at 4 °C with the following dye-labelled antibodies: PE anti-mouse CD40 clone 3/23 (Biolegend, London, UK) and FITC anti-mouse CD86 clone B7-2 (eBioscience, San Diego, CA). Alternatively, cells were stained with Alexa-Fluor 647 anti-CLEC4E (clone 16E3; Novus Biologicals, Littleton, CO). Samples containing the stained cells were characterized on a BD FACSLyric™ flow cytometer and analysed using FlowJo v10 software (Treestar Inc).

Flow cytometric analysis of human moDCs

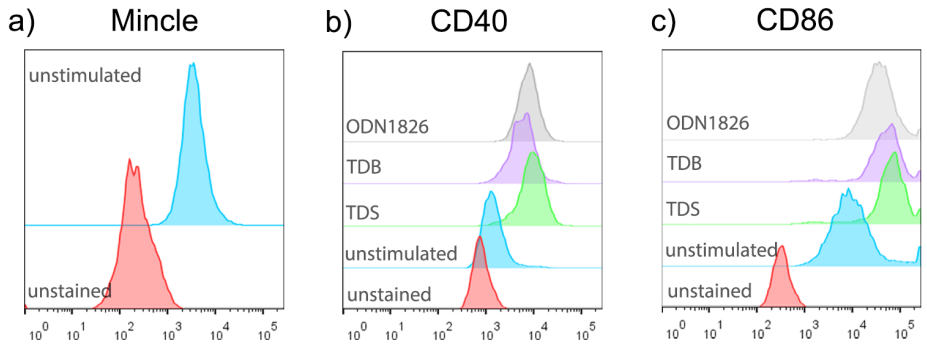
For quality check of the cells prior to stimulation, immature moDCs were stained for 30 minutes at 4°C with the following dye-labelled antibodies: Alexa Fluor 647 anti-human CD1a clone HI149 (Biolegend, London, UK), BB515 Mouse Anti-Human CD11b clone ICRF44 (BD Biosciences, San Diego, CA), PE anti-human CD163 Antibody clone GHI/61 (Biolegend, London, UK), Brilliant Violet 510 anti-human CD14 Antibody clone 63D3 (Biolegend, London, UK). Human moDCs were incubated for 10 minutes at room temperature with 5% human serum (Sigma, St.Louis, MO, USA) in PBS to prevent nonspecific binding of dye-labelled antibodies. Subsequently, cells were stained for 30 minutes at 4°C with the following dye-labelled antibodies: CD40 - APC (clone 5C3), CD80 - APC-R700 (clone L307.4), CD83 - PE (clone HB15e); all antibodies were purchased from BD Biosciences (San Diego, CA). Alternatively, cells were stained with Alexa-Fluor 488 mouse anti-human CD282 (clone 11G7; BD Pharmingen, San Diego, CA) and Alexa-Fluor 647 anti-CLEC4E (clone 16E3; Novus Biologicals, Littleton, CO). All samples were characterized on a BD FACSLyric™ flow cytometer and analysed using FlowJo v10 software (Treestar Inc).

T cell proliferation

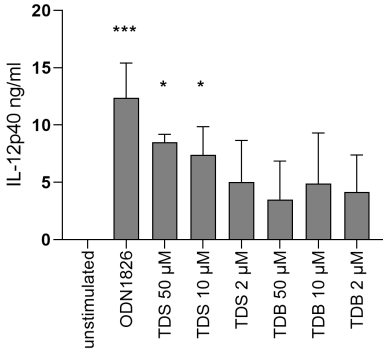
Thawed T cell clones, R2F10 (DR2 restricted, specific for p418-427 of hsp65 Mlep) and Rp15 1-1 (DR3 restricted, specific for p3-13 of hsp65 Mlep/Mtb), were cultured in triplicate in 96-

wells round-bottom plates (10.000 cells/well) together with HLA-DR-matched monocyte-derived dendritic cells (2500 cells/well) and cognate peptide (0.0001, 0.001, 0.01, 0.1, 1 and 10 μg/ml) in combination with TDS (0.8 and 4 μM) and/or UPam (0.2, 1, 5 μM) at 37◦C, 5% CO_2 in IMDM (Gibco, PAA, Linz, Austria) supplemented with GlutaMAX™ (Gibco, PAA, Linz, Austria) and 10% pooled human serum. After 72 hours, cells were pulsed for an additional 18 hours with [3H]thymidine (0.5 μCi/well; Thermo Fisher scientific, Merelbeke, Belgium), harvested with a TomTec cell harvester, and counted on a Microbetaplate counter (Wallac Turku).

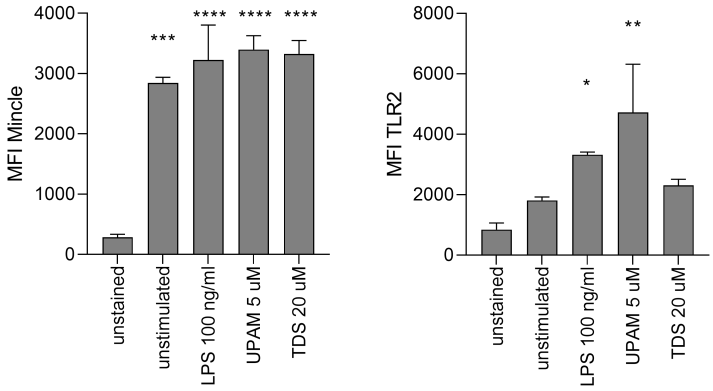
Supporting figures:



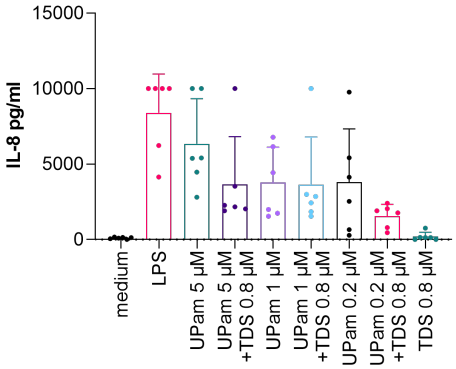
S1 Figure - Expression of surface proteins on D1 DC cells, as measured by flow cytometry. (A) Mincle expression. From front to back: in red unstained cells; in blue unstimulated stained cells. **(B)** CD40 expression and **(C)** CD86 expression. From front to back: in red unstained cells; in blue unstimulated stained cells; in green cells stimulated with TDS (50 μM); in purple cells stimulated with TDB (50 μM); in grey cells stimulated with CpG ODN1826 (1 μg/ml).



S2 Figure - Quantification of IL-12p40 produced by D1 DC cells, as measured by ELISA. Bar plot showing the amount of IL-12p40 cytokine detected in the supernatant of D1 DC cells as measured by ELISA after 20 hours stimulation with TDS and TDB. Error bars represent mean + SD of two independent experiments as calculated using GraphPad Prism. Statistical significance with reference to cells exposed to reference peptide was calculated through one-way ANOVA method (****p < 0.0001; ***p < 0.001; **p < 0.01; *p < 0.05). CpG ODN1826 (1 μg/ml) is used as positive control.



S3 Figure - Human moDC expression of pattern recognition receptors, as measured by flow cytometry. (A) Mincle expression; **(B)** TLR2 expression. Representative data from one human donor are shown. LPS is used as positive control. From front to back: in red unstained cells; in blue unstimulated stained cells; in orange cells stimulated with UPam (5 μM); in light green cells stimulated with TDS (20 μM); in dark green cells stimulated with LPS (100 ng/ml). **(A)** Median fluorescence intensity of Mincle receptor; **(B)** Median fluorescence intensity of TLR2 receptor. Bar plots represent mean value + SD (n = 2 donors) as calculated using GraphPad Prism. Statistical significance with reference to unstained cells exposed to medium + DMSO was calculated through one-way ANOVA method (***p < 0.001; **p < 0.01; *p < 0.05). LPS (100 ng/ml) was used as a positive control.



S4 Figure – IL-8 production profile of human moDCs stimulated for 20 hours using single adjuvants or mixtures thereof, as measured by milliplex. Dots shown in the scatter dot plots represent IL-8 data from 6 different donors, as measured by milliplex. Bars indicate mean value + SD.

References

1. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011 May;34(5):637–50.

2. Tan RS, Ho B, Leung BP, Ding JL. TLR cross-talk confers specificity to innate immunity. Int Rev Immunol. 2014 Jun;33(6):443–53.

3. Duthie MS, Windish HP, Fox CB, Reed SG. Use of defined TLR ligands as adjuvants within human vaccines. Immunol Rev. 2011 Jan;239(1):178–96.

4. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1–polarizing program in dendritic cells. Nat Immunol. 2005 Jul;6(8):769–76.

5. Tom JK, Albin TJ, Manna S, Moser BA, Steinhardt RC, Esser-Kahn AP. Applications of immunomodulatory immune synergies to adjuvant discovery and vaccine development. Trends Biotechnol. 2019 Apr;37(4):373–88.

6. Thakur A, Andrea A, Mikkelsen H, Woodworth JS, Andersen P, Jungersen G, et al. Targeting the Mincle and TLR3 receptor using the dual agonist cationic adjuvant formulation 9 (CAF09) induces humoral and polyfunctional memory T cell responses in calves. PloS One. 2018 Jul;13(7).

7. Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, et al. Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature. 2002 Nov;420(6913):324–9.

8. Schoenen H, Bodendorfer B, Hitchens K, Manzanero S, Werninghaus K, Nimmerjahn F, et al. Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. J Immunol. 2010 Mar;184(6):2756–60.

9. Ostrop J, Jozefowski K, Zimmermann S, Hofmann K, Strasser E, Lepenies B, et al. Contribution of MINCLE–SYK signaling to activation of primary human APCs by mycobacterial cord factor and the novel adjuvant TDB. J Immunol. 2015 Sep;195(5):2417–28.

10. Schick J, Etschel P, Bailo R, Ott L, Bhatt A, Lepenies B, et al. Toll-like receptor 2 and Mincle cooperatively sense corynebacterial cell wall glycolipids. Infect Immun. 2017 Jun;85(7):e00075-17.

11. Matsumura T, Ikebe T, Arikawa K, Hosokawa M, Aiko M, Iguchi A, et al. Sequential Sensing by TLR2 and Mincle Directs Immature Myeloid Cells to Protect against Invasive Group A Streptococcal Infection in Mice. Cell Rep. 2019 Apr;27(2):561–71.

12. Lee W-B, Kang J-S, Choi WY, Zhang Q, Kim CH, Choi UY, et al. Mincle-mediated translational regulation is required for strong nitric oxide production and inflammation resolution. Nat Commun. 2016 Apr;7(1):1–14.

13. Willems MM, Zom GG, Khan S, Meeuwenoord N, Melief CJ, van der Stelt M, et al. N-tetradecylcarbamyl lipopeptides as novel agonists for toll-like receptor 2. J Med Chem. 2014 Jul;57(15):6873–8.

14. Zom GG, Welters MJ, Loof NM, Goedemans R, Lougheed S, Valentijn RR, et al. TLR2 ligand-synthetic long peptide conjugates effectively stimulate tumor-draining lymph node T cells of cervical cancer patients. Oncotarget. 2016 Oct;7(41):67087.

15. Huber A, Kallerup RS, Korsholm KS, Franzyk H, Lepenies B, Christensen D, et al. Trehalose diester glycolipids are superior to the monoesters in binding to Mincle, activation of macrophages in vitro and adjuvant activity in vivo. Innate Immun. 2016 Aug;22(6):405–18.

16. Winzler C, Rovere P, Rescigno M, Granucci F, Penna G, Adorini L, et al. Maturation Stages of Mouse Dendritic Cells in Growth Factor–dependent Long-Term Cultures. J Exp Med. 1997 Jan;185(2):317–28.

17. Geluk A, van Meijgaarden KE, Janson A, Drijfhout J, Meloen R, de Vries R, et al. Functional analysis of DR17 (DR3)-restricted mycobacterial T cell epitopes reveals DR17-binding motif and enables the design of allele-specific competitor peptides. J Immunol. 1992 Nov;149(9):2864–71.

18. Verreck FA, de Boer T, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E, et al. Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco) bacteria. Proc Natl Acad Sci. 2004 Mar;101(13):4560–5.

19. Verreck FA, de Boer T, Langenberg DM, van der Zanden L, Ottenhoff TH. Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-γ-and CD40L-mediated costimulation. J Leukoc Biol. 2006 Feb;79(2):285–93.

20. Shi S, Zhu H, Xia X, Liang Z, Ma X, Sun B. Vaccine adjuvants: Understanding the structure and mechanism of adjuvanticity. Vaccine. 2019 May;37(24):3167–78.

21. Bachmann MF, Jennings GT. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. Nat Rev Immunol. 2010 Oct;10(11):787–96.

22. Mishra A, Akhtar S, Jagannath C, Khan A. Pattern recognition receptors and coordinated cellular pathways involved in tuberculosis immunopathogenesis: emerging concepts and perspectives. Mol Immunol. 2017 Jul;87:240–8.

23. Short KK, Miller SM, Walsh L, Cybulski V, Bazin H, Evans JT, et al. Co-encapsulation of synthetic lipidated TLR4 and TLR7/8 agonists in the liposomal bilayer results in a rapid, synergistic enhancement of vaccine-mediated humoral immunity. J Controlled Release. 2019 Dec;315:186–96.

24. Liu B, Liu Q, Yang L, Palaniappan SK, Bahar I, Thiagarajan P, et al. Innate immune memory and homeostasis may be conferred through crosstalk between the TLR3 and TLR7 pathways. Sci Signal. 2016 Jul;9(436):ra70–ra70.

25. Bayyurt B, Tincer G, Almacioglu K, Alpdundar E, Gursel M, Gursel I. Encapsulation of two different TLR ligands into liposomes confer protective immunity and prevent tumor development. J Controlled Release. 2017 Feb;247:134–44.

26. Surendran N, Simmons A, Pichichero ME. TLR agonist combinations that stimulate Th type I polarizing responses from human neonates. Innate Immun. 2018 Apr;24(4):240–51.

27. Blufstein A, Behm C, Gahn J, Uitz O, Naumovska I, Moritz A, et al. Synergistic effects triggered by simultaneous Toll-like receptor-2 and-3 activation in human periodontal ligament stem cells. J Periodontol. 2019 Oct;90(10):1190–201.

28. Hu Y, Cong X, Chen L, Qi J, Wu X, Zhou M, et al. Synergy of TLR3 and 7 ligands significantly enhances function of DCs to present inactivated PRRSV antigen through TRIF/MyD88-NF-κB signaling pathway. Sci Rep. 2016 Apr;6:23977.

29. Fischetti L, Zhong Z, Pinder CL, Tregoning JS, Shattock RJ. The synergistic effects of combining TLR ligand based adjuvants on the cytokine response are dependent upon p38/JNK signalling. Cytokine. 2017 Nov;99:287–96.

30. Nouri-Shirazi M, Tamjidi S, Nourishirazi E, Guinet E. TLR8 combined withTLR3 or TLR4 agonists enhances DC-NK driven effector Tc1 cells. Immunol Lett. 2018 Jan;193:58–66.

31. Pashenkov MV, Murugina NE, Budikhina AS, Pinegin BV. Synergistic interactions between NOD receptors and TLRs: Mechanisms and clinical implications. J Leukoc Biol. 2019 Apr;105(4):669–80.

32. Fritz JH, Girardin SE, Fitting C, Werts C, Mengin-Lecreulx D, Caroff M, et al. Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. Eur J Immunol. 2005 Aug;35(8):2459–70.

33. Tukhvatulin AI, Dzharullaeva AS, Erokhova AS, Scheblyakov DV, Naroditsky BS, Gintsburg AL, et al. NOD1/2 and the C-Type Lectin Receptors Dectin-1 and Mincle Synergistically Enhance Proinflammatory Reactions Both In Vitro and In Vivo. J Inflamm Res. 2020 Jul;13:357.

34. Toy R, Keenum MC, Pradhan P, Phang K, Chen P, Chukwu C, et al. TLR7 and RIG-I dual-adjuvant loaded nanoparticles drive broadened and synergistic responses in dendritic cells in vitro and generate unique cellular immune responses in influenza vaccination. J Controlled Release. 2021 Feb;

35. Van Haren SD, Dowling DJ, Foppen W, Christensen D, Andersen P, Reed SG, et al. Age-specific adjuvant synergy: dual TLR7/8 and mincle activation of human newborn dendritic cells enables Th1 polarization. J Immunol. 2016 Dec;197(11):4413–24.

36. Tukhvatulin AI, Gitlin II, Shcheblyakov DV, Artemicheva NM, Burdelya LG, Shmarov MM, et al. Combined stimulation of Toll-like receptor 5 and NOD1 strongly potentiates activity of NF-κB, resulting in enhanced innate immune reactions and resistance to Salmonella enterica serovar Typhimurium infection. Infect Immun. 2013 Jul;81(10):3855–64.

37. Decout A, Silva-Gomes S, Drocourt D, Barbe S, André I, Cueto FJ, et al. Rational design of adjuvants targeting the C-type lectin Mincle. Proc Natl Acad Sci. 2017 Mar;114(10):2675–80.

38. Smith AJ, Miller SM, Buhl C, Child R, Whitacre M, Schoener R, et al. Species-specific structural requirements of alpha-branched trehalose diester Mincle agonists. Front Immunol. 2019 Feb;10:338.

39. Kiyotake R, Oh-Hora M, Ishikawa E, Miyamoto T, Ishibashi T, Yamasaki S. Human Mincle Binds to Cholesterol Crystals and Triggers Innate Immune Responses. J Biol Chem. 2015 Oct;290(42):25322–32.

40. Hunter RL, Olsen MR, Jagannath C, Actor JK. Multiple roles of cord factor in the pathogenesis of primary, secondary, and cavitary tuberculosis, including a revised description of the pathology of secondary disease. Ann Clin Lab Sci. 2006;36(4):371–86.

41. Hawn TR, Misch EA, Dunstan SJ, Thwaites GE, Lan NT, Quy HT, et al. A common human TLR1 polymorphism regulates the innate immune response to lipopeptides. Eur J Immunol. 2007 Aug;37(8):2280–9.

42. Mikacenic C, Reiner AP, Holden TD, Nickerson DA, Wurfel MM. Variation in the TLR10/TLR1/TLR6 locus is the major genetic determinant of interindividual difference in TLR1/2-mediated responses. Genes Immun. 2013 Jan;14(1):52–7.

43. Dittrich N, Berrocal-Almanza LC, Thada S, Goyal S, Slevogt H, Sumanlatha G, et al. Toll-like receptor 1 variations influence susceptibility and immune response to *Mycobacterium tuberculosis*. Tuberculosis. 2015 May;95(3):328–35.

44. Kranzer K, Bauer M, Lipford G, Heeg K, Wagner H, Lang R. CpG-oligodeoxynucleotides enhance T-cell receptor-triggered interferon-γ production and up-regulation of CD69 via induction of antigen-presenting cell-derived interferon type I and interleukin-12. Immunology. 2001 Dec;99(2):170–8.

45. Gelman AE, LaRosa DF, Zhang J, Walsh PT, Choi Y, Sunyer JO, et al. The adaptor molecule MyD88 activates PI-3 kinase signaling in CD4+ T cells and enables CpG oligodeoxynucleotide-mediated costimulation. Immunity. 2006 Nov;25(5):783–93.

46. Komai-Koma M, Jones L, Ogg GS, Xu D, Liew FY. TLR2 is expressed on activated T cells as a costimulatory receptor. Proc Natl Acad Sci. 2004 Mar;101(9):3029–34.