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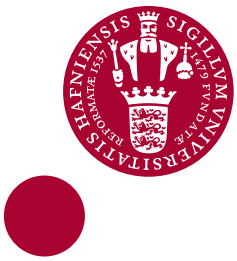

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Enhancement of ceramide formation increases endocytosis of *Lactobacillus acidophilus* and leads to increased IFN-β and IL-12 production in dendritic cells.

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Abstract

The sphingolipid ceramide plays a role in receptor clustering in the plasma membrane. Upon bacterial encounter, dendritic cells (DCs) initiate a bacteria-specific downstream signalling event. We hypothesized that conversion of sphingomyelin to ceramide by acid sphingomyelinase (SMase) is a key event in endocytosis of gram-positive *Lactobacillus acidophilus* and the subsequent induction of IFN-β in DCs. Conversely, endocytosis of the gram-negative *Escherichia coli*, which is not a potent IFN-β inducer would not be dependent on ceramide formation. SMase or an inhibitor of acid SMase and ceramidase, chlorpromazine (CPZ), was added to murine bone marrow (bm)DCs prior to stimulation with either of the bacteria. Simultaneous endocytosis of fluorescent-labelled bacteria and FITC-dextran measured by flow cytometry provided a method to distinguish between phagocytosis, constitutive macrocytosis, and induced macropinocytosis in the bmDCs. Addition of SMase increased the phagocytosis of *L. acidophilus* and *L. acidophilus*-induced IL-12/IFN-β but showed no effect on the uptake of *E. coli* nor on *E.coli* induced IL-12/IFN-β production. Also, SMase did not affect Pam3CSK4-induced macropinocytosis of FITC-dextran. Inhibition of both acid SMase and ceramidase by CPZ increased constitutive macropinocytosis of dextran and slightly increased *L.acidophilus* induced *Il12/Ifn-β* expression and *E.coli* induced *Ifnβ* expression. Our results confirm a role for ceramide in the *L.acidophilus* induced IL-12/IFN-β production but also enhancement of constitutive micropinocytosis by inhibiting sphingomyelin conversion may lead to enhanced IFN-β induction. Our data suggests that manipulation of the membrane sphingolipids provides a tool for manipulating the cytokine profiles in DCs, e.g. in vaccine development.

Keywords: Dendritic cells, *Lactobacillus acidophilus*, IFN-β induction, Ceramide, Sphingomyelin, Endocytosids, Acid sphingomyelinase

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Introduction

The activation of dendritic cells (DCs) by bacteria and viruses depends on ligation of various pattern recognition receptors (PPRs), which may facilitate endocytosis of the microbe (e.g. Dectin-1) or lead to the induction of cytokine responses. Cytokine production differs in both type and magnitude dependent on the type of microbial stimulation [1,2]. The type I interferons, interferon (IFN)-α and IFN-β are particularly induced upon activation of DCs by viruses [3-5], but certain bacteria have also shown to be potent inducers of IFN-β [6-10]. The induction of IFN-β in turn up-regulates a high number of viral defence genes, as well as the Th1 inducing cytokine interleukin (IL)-12 through ligation of the specific IFN type I receptor, IFNAR [11,12]. Hence, through IFN-β induction the bacterial activation may represent an important means to increase the anti-viral defence and cellular immunity, and an in-depth understanding of the mechanisms behind the bacterial induction of IFN-β in DCs may establish an improved basis for development of new anti-viral vaccines, virus-preventive drugs, and immune stimulatory food additives.

For some Gram negative bacteria, as well as for the Gram negative bacterial component lipopolysaccharide (LPS), IFN-β has been shown to be induced rapidly, but only weakly and very transiently in DCs upon stimulation [7]. The induction was shown to be dependent on the adaptor protein TRIF, which is recruited to ligated TLR4 in endosomes, and in turn stimulates phosphorylation of IRF3 and IRF7 [13,14]. The mechanisms behind induction of IFN-β from Gram positive bacteria are more diverse; while some Gram-positive bacteria, e.g. bifidobacteria, seem unable to induce IFN-β at all, others (e.g. *Streptococcus* ssp. and *Listeria* ssp.) induce a potent IFN-β response [6,9,10,15,16]. We have previously shown that also the Gram-positive bacterium *L. acidophilus*, along with a number of other *Lactobacilli*, is a strong inducer of IFN-β in murine bone marrow-derived DCs [7,17]. The mechanisms behind the induction of IFN-β through stimulation with these Gram positive bacteria, however, are only to some extend understood and seem to vary depending on the bacterial species/strain.

Common for all the IFN-β-inducing bacteria, as well as for LPS, it was demonstrated that endocytosis is a prerequisite for the induction of IFN-β [10,13,15,18-21]. We provided recently

evidence that the type of endocytosis employed for the uptake of the stimulating bacteria does not affect the resulting cytokine response as long as the stimulation does not involve stimulation of TLRs from the plasma membrane [19]. Phagocytic cells can take up particles and bacteria by two distinct ways; macropinocytosis and phagocytosis [22-24]. Macropinocytosis, in which the plasma membrane protrudes and engulf large volumes of the surrounding liquid with its content of particles, takes place constitutively in immature DCs and can be further increased upon stimulation of the cell's plasma membrane through Toll-like receptor (TLR) ligands [19,25]. In phagocytosis, internalization of the bacterium involves interaction between a high number of ligands and receptors, which is needed to trigger a zipper-like movement around the particle [23,26]. We have demonstrated that endocytosis of *L. acidophilus* is required for a strong induction of IFN-β and that stimulation of macropinocytosis with TLR2 or TLR4 ligands abrogated the IFN- β induction by *L. acidophilus* and accordingly most of the IL-12 [19]. In contrast, *E. coli* induced an increase in macropinocytosis, and only gave rise to weak induction of IFN-β. Nevertheless, *E. coli* was able to induce IL-12 induction through an IFN-β-independent pathway. On this background we hypothesised that *L. acidophilus* is taken up by DCs through phagocytosis and constitutive macropinocytosis, and that the endocytotic event is indispensable for the potent IFN-β response. The formation of ceramide in the plasma membrane (PM) has previously been linked to phagocytosis [27–29]. The most abundant sphingolipid in the PM is sphingomyelin (SM), which is hydrolysed to form ceramide by acid sphingomyelinase (ASMase). ASMase has been shown to be recruited from intracellular storage vesicles to the outer leaflet of the PM upon activation of FcγRII or DC-SIGN [27,28,30]. Ceramide formed in the PM can associate and give rise to ceramide micro-domains [31]. Such local changes in the membrane lipid composition can cause membrane-attached and membrane-associated proteins to co-localise, which is required for triggering of phagocytosis [26].

In this study, we studied the effect on IFN-β and IL-12 induction when adding acid SMase and blocking ceramide formation prior to activation of DCs by either the strong *IFN-β* inducer *L. acidophilus*, or by the weak *IFN-β* inducer *E. coli*. We found that the ceramide formation in plasma membrane was a key event in endocytosis of *L. acidophilus* and the consequent induction of a strong IFN-β response. In contrast, endocytosis of *E. coli* was unaffected by changes in the ceramide content, and did not induce a potent IFN-β response. The study reveals different routes of bacterial endocytosis that in different ways involve the sphingolipid classes in the plasma membrane and result in the induction of distinct cytokine profiles.

Materials and Methods

Ligands, inhibitors, and compounds

The following inhibitors, compounds and ligands were used at the final concentrations indicated: Chlorpromazine hydrochloride (CPZ, Sigma-Aldrich, St. Louis, MO) solubilised in AccuGENE H_2O (Lonza, Basel, Switzerland): 10 µM. Bacterial acid sphingomyelinase from *Staphylococcus aureus* (ASMase, Sigma-Aldrich): 0.1 U/ml. Pam3CSK4 (Invivogen, San Diego, CA) : 1 µg/ml. Cytochalasin D (Cyt. D, Sigma-Aldrich): 0.5 µg/m.l FITC-Dextran (Sigma-Aldrich): 500 µg/ml. Lysenin (Peptide Institute Inc., Osaka, Japan): 25 ng/ml.

Bacterial strains

The Gram-positive bacterium *Lactobacillus acidophilus* NCFM (Danisco, Copenhagen, Denmark) was grown anaerobically overnight at 37ºC in de Man Rogosa Sharp (MRS) broth (Merck, Darmstadt, Germany). The Gram-negative bacterium *Escherichia coli* Nissle 1917 O6:K5:H1 (Statens Serum Institut, Copenhagen, Denmark) was grown aerobically overnight at 37°C in Luria-Bertani (LB) broth (Merck). Bacteria were harvested by centrifugation at 2000 g for 15 min and washed twice in sterile PBS (Bio Whittaker, East Rutherford, NJ). The concentration was determined as the content of dry matter per ml upon lyophilisation, and the dry weight was corrected for buffer salt content. For experiments, both bacteria were used at a final concentration of 10 µg/ml.

For endocytosis experiments *L. acidophilus* NCFM were fluorescently labelled using Alexa-fluor conjugated succinimidyl-esters (SE-Alexa fluor 488 or Alexa fluor 647, Molecular Probes, Eugene, OR). Bacteria in DPBS were centrifuged for 2 min at 10.000 rpm and resuspended in sodium carbonate buffer (pH 8.5), 10 µl SE-AF647 or SE-AF488 was added per approximately 12 mg dry weight bacteria. Bacteria were incubated at room temperature with agitation for 1 h, washed 3 times in sodium carbonate buffer, and finally resuspended in original volume of DPBS.

Generation of murine dendritic cells

Bone marrow-derived dendritic cells (DCs) were prepared from 6-12 week old mice (C57BL/6, Taconic, Lille Skensved, Denmark). Cells were cultivated in RPMI 1640 with 10% heat-inactivated fetal calf serum in the presence of GM-CSF as described previously [23]. The percentage of CD11c expressing cells was determined by flow cytometric analysis using PE-conjugated anti-CD11c antibody (eBioscience, San Diego, CA) on a FacsCantoII (BD Biosciences, NJ, USA) using Diva Software (BD Biosciences). A purity of 75-90% was obtained.

5.4. Treatments and stimulations of DCs for endocytosis analysis

Immature DCs ($2 \cdot 10^6$ cells/mL) were seeded in 96-well tissue culture plates (Nunc, Roskilde, Denmark) and incubated with the pre-treatment(s) stated in the individual experiments (see Results) for 30 min or 1 h prior to another pre-treatment or incubation with fluorescently labelled *L. acidophilus* or *E. coli* in a final concentration of 10 µg/mL or with FITC-Dextran for 10 min to 1 h. All incubation steps were performed at 37°C in 5% CO_2. Finally, the uptake of dextran, fluorescently labelled

bacteria, or a mixture was analysed on a FACSCantoII flow cytometer using Diva Software (both from BD Biosciences).

5.5. Stimulation of DCs with bacteria, ligands, and inhibitors

Immature DCs (2 × 10^6 cells/mL) were seeded in 48-well tissue culture plates (Nunc) and incubated with the pre-treatment(s) stated in the individual experiment (see Results) for 1 h prior to stimulation with *L. acidophilus* or *E. coli* in a final concentration of 10 µg/mL. Cells were incubated at 37°C in 5% CO_2 in a humidified atmosphere. For cytokine quantification by enzyme-linked immunosorbent assay (ELISA), cells were incubated for 5 or 20 h, and for quantification of gene expression by qPCR, cells were incubated for 1, 3, 5 and 7 h.

5.6. Cytokine quantification by ELISA

After 5 and 20 h cell culture supernatants were harvested, and the amount of *IL-12* (p70), *IL-10*, (R&D systems, Minneapolis, MN), and *IFN-β* (PBL Assay Science, Piscataway, NJ) was analysed using commercially available ELISA kits according to the manufacturer's instructions.

5.7. RNA isolation, cDNA synthesis and gene expression analysis by qPCR

Total RNA was extracted by MagMAX Express (Applied Biosystem, Foster City, CA) using the MagMAX-96 RNA Isolation Kit (Ambion, Austin, TX) following the suppliers protocol. For all samples cDNA was produced from ~500 ng total RNA by using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems) according to the manufacturers' instructions. The expression of the genes encoding IFN-β, IL-12p40, and β-actin was detected using primers and probes as previously described [20], and for the genes encoding IL12p35: Il12a (Mm00434165_m1, Applied Biosystems).

For each sample, 2 µL cDNA (3 ng/µL) was amplified in triplicates on a StepOnePlus by using universal fast thermal cycling parameters, and TaqMan Fast universal PCR Mastermix (both from Applied Biosystems) in a total reaction volume of 10 µL. Fold changes in gene expression were calculated by the comparative cycle threshold (CT) method [24]. The expression of target genes were normalized to beta actin as a reference gene [ΔCT=CT(target) – CT(reference)]. Fold change in gene expression was calculated as 2-ΔΔCT where ΔΔCT=ΔCT (sample) – ΔCT (calibrator), where the average ΔCT of samples from controls at 0 h of stimulation was used as calibrator.

5.8. Statistical analysis

Data represents mean of measurements from triplicate cultures and are representative of at least three independent experiments. Error bars indicate standard deviation. Statistical calculations were performed using the software GraphPad Prism 5 (GraphPad Software, San Diego, CA). Results were analysed by ANOVA with Dunnet's post-test (compared to unstimulated sample). P-values of <0.05 were considered significant and indicated by asterisks.

6. Results

6.1. E. coli but not L. acidophilus induce enhanced macropinocytosis in DCs

The extent of macropinocytosis induced by *L. acidophilus* or *E. coli* in DCs was compared by stimulation with either *L. acidophilus* or *E. coli* for 60 min followed by incubation with FITC-dextran for 10 min before uptake of FITC-dextran was measured by flow cytometry (Figure 1A). Addition of FITC-dextran to immature DCs gave rise to around 4.5% cells with a high uptake of FITC-dextran indicative of macropinocytosis. Stimulation of DCs with *E. coli* prior to addition of FITC-dextran increased the number of DCs with a high uptake of dextran by 5% to 9.5% while *L. acidophilus* stimulation did not increase the number of these FITC-dextran positive cells.

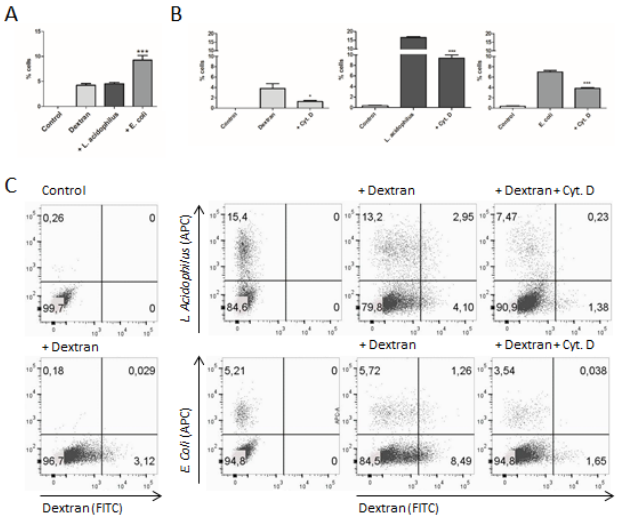

Figure 1. *E. coli* but not *L. acidophilus* induce increased actin-dependent uptake of dextran in DCs. A) DCs were stimulated with *L. acidophilus* or *E. coli* (10 µg/ml) for 1 h and incubated with FITC-Dextran (500 µg/ml) for 10 min. Uptake of bacteria and/or dextran was analysed by flow cytometry. B and C) DCs were treated with complete medium (control) or Cytochalasin D (Cyt. D, 0,5 □g/ml) for 1 h, then stimulated with Alexa Fluor 647-coupled *E. coli* or *L. acidophilus* (10 µg/ml) for 1 h and finally incubated with FITC-dextran (500 µg/ml) for 10 min.

Simultaneous stimulation with APC-labelled *L. acidophilus* or *E. coli* and FITC-dextran with or without pretreatment with the actin polymerization inhibitor Cytochalasin D demonstrated that the cells with the highest FITC intensity was particularly reduced, indicative of an inhibition of macropinocytic uptake of dextran (Figures 1B and 1C). In addition, the proportion of DCs positive for uptake of *L. acidophilus* and *E. coli*, respectively, after Cytochalasin D treatment was around 7.5% and 3.5%, which corresponds to DCs where bacteria had adhered to the surface and were not internalised. Hence, the proportion of DCs that had taken up *L. acidophilus* was around 8% and 3.5% for *E. coli* (Figures 1B and 1C). These results demonstrated that only cells with a high uptake of dextran have

increased macropinocytosis and, even though both bacterial species were endocytosed by the DCs only stimulation with *E. coli* led to increased macropinocytic activity in the cells. Thus, *E. coli* and *L. acidophilus* differed in their way of activating endocytosis.

6.2. Simultaneous inhibition of ceramide formation and ceramide degradation enhances uptake of dextran but does not significantly affect the uptake of bacteria

Chlorpromazine (CPZ) is an inhibitor of acid SMase and acid ceramidase [32], which upon addition to cells may lead to accumulation of both spingomyelin and ceramide in the plasma membrane and a reduction in sphingosine content (Figure 2A). To assess the effect of blocking the ceramide and spingosine formation on bacterial stimulation on uptake of bacteria and dextran, CPZ was added to the DCs prior to stimulation with *L. acidophilus* or *E. coli* (Figures 2B and 2C).

An increased number of cells with high dextran uptake was observed in non-stimulated DCs where CPZ and dextran were added, which was most likely due to enhanced constitutive macropinocytosis. Also a slight increase in the number of cells with both bacteria and high dextran uptake (from 2.1 to 2.6%), indicative of micropinocytosis was seen (Figures 2B and 2C). Neither the total uptake of bacteria nor the proportion of cells with bacteria and a high dextran uptake was however significantly increased. Thus, although addition of CPZ without microbial stimulation led to an increase in the number of cells with high dextran uptake, only minor and non-significant effects on the bacterial uptake was seen.

The TLR2-ligand Pam3CSK4 induces a potent increase in macropinocytosis [19,25], and stimulation with Pam3CSK4 prior to addition of *L. acidophilus* or dextran accordingly increases the uptake of both bacteria and dextran particles.

As for stimulation with bacteria the effect of blocking ceramide and sphingosine formation by CPZ on Pam3CSK4 induced macropinocytosis of bacteria was not significant (Supplementary Figure 1). Together, these results indicated that CPZ in itself makes the membrane in immature cells more prone to do macropinocytosis, but if microbially stimulated the effect on the uptake of bacteria is minor.

6.3. Inhibition of ceramide formation and ceramide degradation enhances bacteria-induced gene expression in DCs

We have previously shown that intact *L. acidophilus* and to a minor degree *E. coli* induce a potent *Ifnβ* and *Il-12* expression upon endocytosis [7,33]. Hence, to assess how the slightly increased uptake of *L. acidophilus* induced by CPZ affected the induction of *Ifnβ* and *Il-12* expression, DCs were treated with CPZ prior to stimulation with *L. acidophilus* or *E. coli* and gene expression was measured by Q-PCR. As shown in Figure 3, addition of CPZ led to an increase in *L. acidophilus*-induced Ifn-β expression at 3 to 5 h post stimulation and in *E. coli*-induced Ifnβ expression at 1 h. Expression of *Il-12 and Il-10* induced by *L. acidophilus* was also enhanced by CPZ pretreatment. *Il-10* expression following stimulation with *E. coli* was likewise enhanced by CPZ addition, but *Il-12* expression was not. Of note, the expression profile of *Ifnβ* induced by *L. acidophilus* showed an expression that was later, but more prolonged compared to *E. coli*-induced *Ifnβ* expression, which was induced very rapidly and diminished again within the first hours after stimulation. Also the *Il-12* expression was induced faster upon *E. coli* stimulation, than after *L. acidophilus* stimulation. To sum up, treating DCs with CPZ to inhibit ceramide and sphingosine formation in the plasma membrane, led to increase constitutive macropinocytosis and a slight increase in the uptake of *L. acidophilus*, and enhanced the gene expression induced by *L. acidophilus* more but showed only minor effects on *E. coli* stimulation.

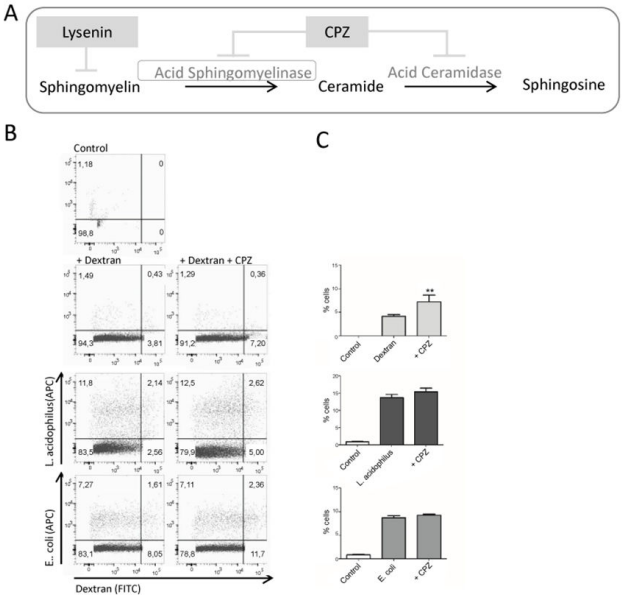

Figure 2. Inhibition of ceramide and sphingosine formation increases macropinocytic uptake of dextran and *E. coli* but not of *L. acidophilus*. A) Ceramide metabolism of focus and the inhibitors and enzyme (squared) used in this study. B, C) DCs were pre-treated with culture medium (control) or Chlorpromazine (CPZ, 10 □M) for 30 min, then stimulated with Alexa Fluor 647-coupled *E. coli* or *L. acidophilus* (10 μg/ml) for 30 min and finally incubated with FITC-dextran (500 μg/ml) for 30 min. Uptake of bacteria and/or dextran was analysed by flow cytometry.

6.4. Ceramide formation is required for enhanced uptake of **L. acidophilus** *and enhanced IFN-β production*

Ceramide formation in the outer plasma membrane is facilitated by acid sphingomyelinase (SMase), which is recruited to the membrane from intracellular vesicles upon receipt of for example a microbial stimulus [28,34]. Addition of exogenous SMase to the cells may also lead to a higher ceramide concentration in the outer plasma membrane as shown in an artificial membrane system by Nurminen et al. [30]. To directly investigate if an increase in the ceramide content in the membrane affects the uptake of bacteria, SMase was added to DCs prior to stimulation with *L. acidophilus* and

E. coli to enhance the formation of ceramide in the DC membrane at the time of stimulation.

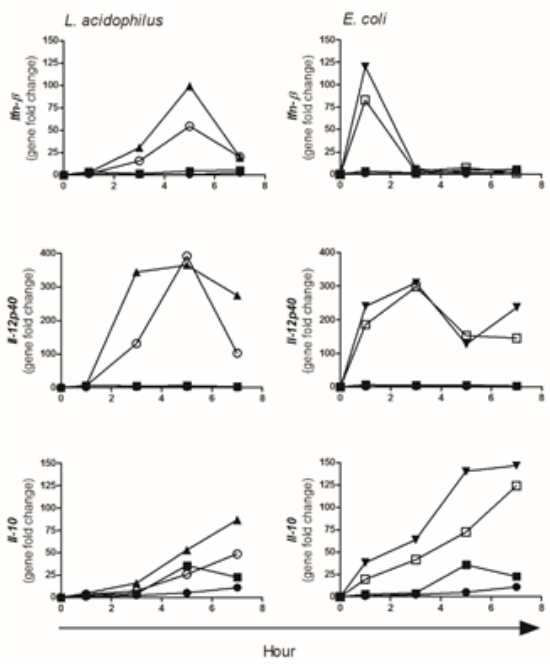

Figure 3. Inhibition of ceramide and sphingosine formation increases *L.acidophilus*-induced gene expression of *Ifnβ* in DCs. DCs were pre-treated with Chlorpromazine (CPZ, 10 µM) for 1 h. and stimulated with *L. acidophilus* or *E. coli* (10 µg/ml). Expression of *Ifn-β*, *Il-12* and *Il-10* was analysed by RT-PCR and normalized to β-actin after 1, 3, 5 and 7 h of stimulation. ● control (culture medium), ○ *L. acidophilus*, □ *E.. co* , ■ CPZ, ▲CPZ + *L. acidophilus*, ▼ CPZ + *E. coli.*

As anticipated, addition of SMase enhanced phagocytosis of *L. acidophilus* (Figure 4). In contrast, SMase did not affect the uptake of *E. coli*, indicative of a ceramide-independent uptake of this bacterium.

To address the effect of SMase treatment on the ability of the DCs to induce IFN-β and IL-12 upon microbial stimulation we assessed the cytokine response by ELISA (Figure 5). In the presence of SMase, *L. acidophilus* induced IFN-β production more rapidly as seen by the concentration in the supernatant at 5 h after stimulation, which was almost three times the level in supernatants of untreated *L. acidophilus* stimulated cells. At 20 h after stimulation, however, no difference in the IFN-β concentration was seen. Also the IL-12 concentration increased more rapidly in SMase treated cells, but was also significantly higher at 20 h, which corresponds well with a stimulating effect of the increased IFN-β levels on the IL-12 production.

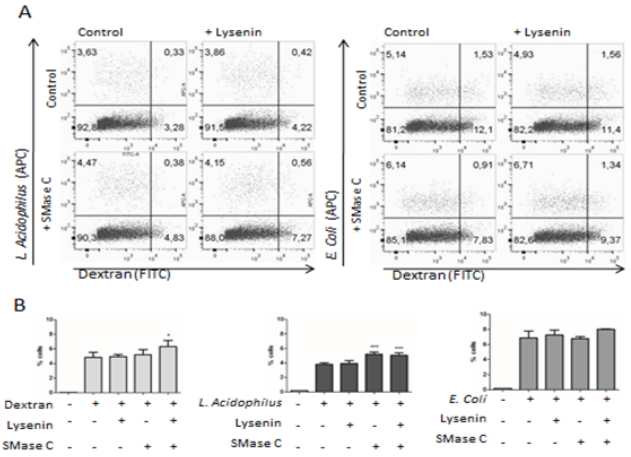

Figure 4. Addition of SMase to enhance ceramide formation in the plasma membrane increases phagocytosis of *L. acdiophilus*, but does not affect *E. coli* stimulated macropinocytosis. DCs were pre-treated with lysenin (25 ng/ml) for 1h, then treated with SMase C for 1 h, stimulated with Alexa Fluor 647-coupled *E. coli* or *L. acidophilus* (10 µg/ml) for 30 min. and finally incubated with FITC-dextran (500 µg/ml) for 30 min. A) Uptake of bacteria and dextran was analysed by flow cytometry. B) Data showed as percent cells positive as depicted in A.

There was no effect of SMase treatment on the *L. acidophilus* stimulated IL-10 production. In contrast, only a modest effect of SMase treatment on cytokine production was seen on DCs stimulated with *E. coli* after 5 h and this effect disappeared at 20 h of stimulation.

Treatment with lysenin alone or together with SMase prior to stimulation with bacteria demonstrated that lysenin did not affect the *L. acidophilus*-induced IL-12 level at 20 h but, independently of SMase, reduced the level of IL-10 in the supernatant (Figure 6). Lysenin increased IL-12 levels induced by *E. coli*, while decreasing the production of IL-10. Taken together, SMase treatment had a pronounced effect both on *L. acidophilus* induced endocytosis and induction of IFN-β and IL-12, but did not increase the uptake of *E. coli* nor cause major changes in cytokine production. This indicates that the two bacteria are taken up by different mechanisms, where only the mechanism involved in uptake of *L. acidophilus* is dependent on formation of ceramide in the plasma membrane.

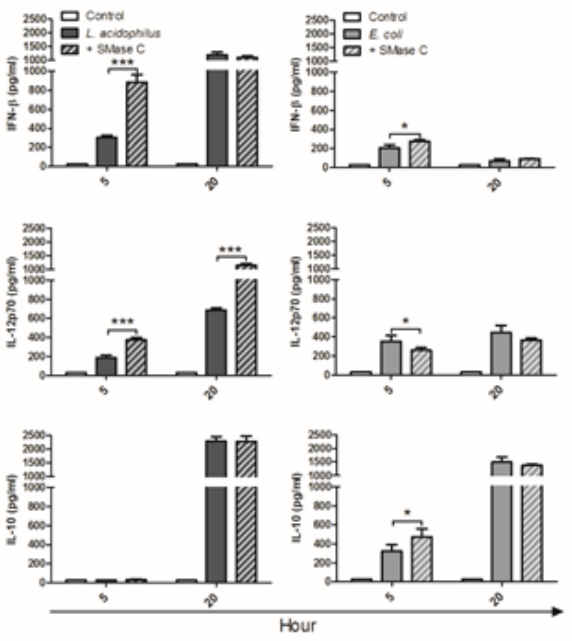

Figure 5. SMase addition to increase formation of ceramide in the plasma membrane enhances *L. acidophilus*-induced IFN-β and IL-12 response in DCs. DCs were pre-treated with SMase C (0.1 U/ml) for 1 h and stimulated with *L. acidophilus* or *E. coli* (10 µg/ml). Production of IFN-β, IL-12 and IL-10 was measured by ELISA in supernatants after 5 and 20 h of stimulation.

Stimulation with Pam3CSK4 to induce increased macropinocytosis led to more than doubling the uptake of high dextran amounts compared to unstimulated DCs. Treatment of DCs with SMase prior to addition of Pam3CSK4 did not influence the effect of Pam3CSK4 (Figure 7).

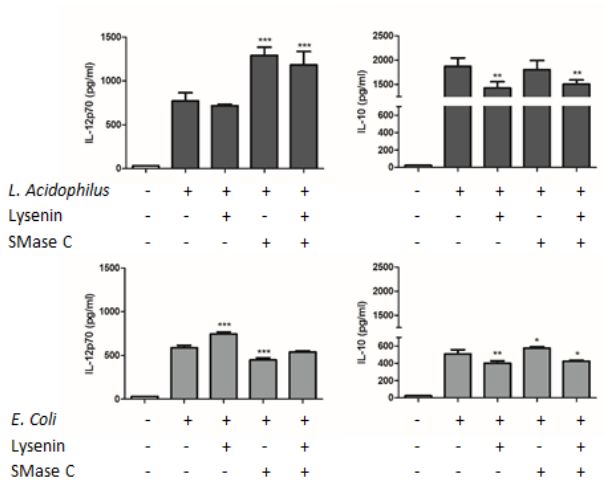

Figure 6. Lysenin enhances IL-12 response towards *E.coli* but not towards *L. acidophilus*. DCs were pre-treated with Lysenin (25 ng/ml) for 1 h followed with treatment with SMase C (0.1 U/ml) for 1 h and stimulated with *L. acidophilus* or *E. coli* (10 µg/ml). Production of IL-12 and IL-10 was measured by ELISA in supernatants after 20 h of stimulation.

Uptake of *L. acidophilus* following stimulation with Pam3CSK4 was increased, as was the number of cells internalising bacteria following SMase treatment. When both SMase and Pam3CSK4 were added together the effect of adding the compounds separately was enhanced, suggesting that macropinocytosis and ceramide-dependent phagocytosis were both triggered, possibly in distinct groups of cells.

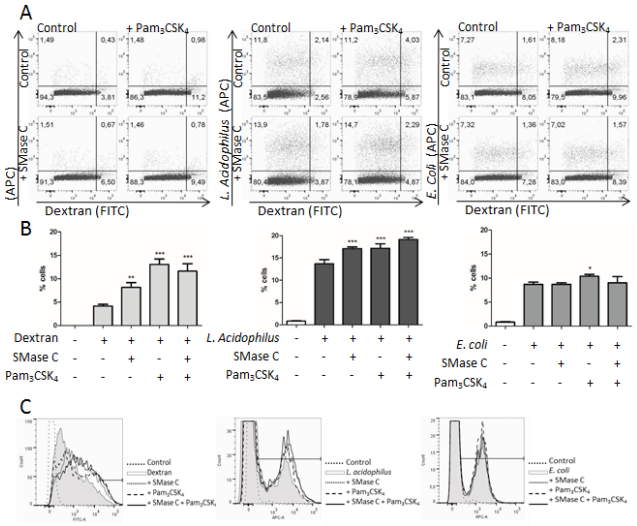

Figure 7. Increased formation of ceramide in the plasma membrane enhances uptake of *L. acidophilus* but does not affect induction of macropinocytosis. DCs were pre-treated with culture medium (control) or SMase C (0.1 U/ml) for 30 min, then treated with Pam3CSK4 (1 ng/ml) for 30 min, followed by stimulation with Alexa Fluor 647-coupled *E. coli* or *L. acidophilus* (10 µg/ml) for 30 min and finally incubated with FITC-dextran (500 µg/ml) for 30 min. Uptake of bacteria or dextran was analysed by flow cytometry.

7. Discussion

The plasma membrane is highly dynamic and movements as well as intracellular signalling in response to microbial stimuli are highly dependent on the sphingolipids that, through the activity of enzymes, readily change their respective proportions in the membrane. These changes lead to significant changes in endocytic activities and signal transduction [31,34-37]. The formation of ceramide-rich domains is considered an important factor in assembly of signalling platforms in the plasma membrane [27–29,38,39]. Here, we investigated the importance of ceramide formation in the plasma membrane in DCs for induction of IFN-β and IL-12 by *L. acidophilus*. We found that the ability of the cell to form ceramide, and in turn, to endocytose the bacteria was a prerequisite for the induction of IFN-β in response to *L. acidophilus*, but not for induction of macropinocytosis induced by *E. coli* or Pam3CSK4.

DCs and other myeloid cells capable of endocytosis of macromolecules employ two distinct mechanisms to internalise large molecules and particles; macropinocytosis and phagocytosis [22,24]. The two methods are experimentally difficult to distinguish and most studies do not differentiate between them. We aimed to investigate the role of plasma membrane changes in sphingolipids for the endocytosis of *L. acidophilus* and *E. coli*, respectively. The two bacteria seem to affect the plasma membrane differently [19].

In order to distinguish between the two types of macromolecular endocytosis, we developed a flow cytometric method, in which we by assessment of the uptake of fluorescence labelled bacteria and fluorescence-labelled dextran could make some distinction between macropinocytosis and phagocytosis. Dextran is often used as a marker of macropinocytosis. This is demonstrated by an increased uptake of the dextran molecules upon stimulation with the Pam3CSK4, which induces a potent increase in macropinocytosis [19,25]. This uptake can be inhibited by Cytochalasin D, an inhibitor of actin rearrangement in the cell. In this way, we could demonstrate that only cells with the highest uptake of dextran had increased the macropinocytic activity. By comparing cells, which had taken up bacteria, a high amount of dextran only, or bacteria and a high amount of dextran we were able to make some distinction between cells that employ macropinocytosis and phagocytosis, respectively, to internalise bacteria.

By addition of acid SMase, to enhance the concentration of ceramide in the plasma membrane, we could demonstrate an increase in the proportion of cells that had phagocytosed *L. acidophilus* and this coincided with a transient increase in the IFN-β concentration in the supernatant of DCs treated with SMase and *L. acidophilus*. This in turn led to almost a doubling in the concentrations of IL-12 after 20 h of incubation. For comparison, we used stimulation with *E. coli*, a Gram negative bacteria which we previously demonstrated was a potent inducer of macropinocytosis and therefore primarily taken up by DCs through this mechanism. In contrast, Pam3CSK4 also increased uptake of *L. acidophilus* but also led to high uptake of FITC-dextran, indicative of increased micropinocytosis. We have previously shown that this is due to stimulation of TLR2 in the plasma membrane and that this stimulation abrogates the IFN-□induction [19]. Even though SMase treatment of DCs prior to stimulation with *E. coli* did not increase the total number of cells that took up bacteria, we observed a slight shift the number of cells employing phagocytosis instead of macropinocytosis, which coincided with a slight transient increase in IFN-β and a slight drop in IL-12 concentration at 5 h of incubation. In this regard, it is worth noticing that IL-12 can be induced by an IFN-β-dependent and an IFN-β-independent pathway, and that the IFN-β-independent pathway more rapidly induces production of IL-12 [7]. Thus, the transient increase in IFN-β and decrease in IL-12 is like to be due to the observed shift from macropinocytosis to phagocytosis of *E. coli* due to an increased level of ceramide in the plasma membrane after SMase treatment.

Conversely, to inhibit the formation of ceramide we treated the cells with CPZ. However, CPZ also inhibits the further conversion of ceramide into sphingosine, and thereby the concentration of both spingomyelin and ceramide may be held constant by this treatment [32]. Pam3CSK4-induced macropinocytosis was not inhibited by CPZ treatment indicating, as anticipated, that increased ceramide formation is not involved in macropinocytosis since receptor clustering is not required. In immature DCs, however, an increase in macropinocytic uptake of dextran was observed. This may indicate that changing the equilibrium towards more spingomyelin in the steady state enhanced constitutive macropinocytic activity in the immature cells. The slightly increased uptake of *L. acidophilus* led to increased expression of *Ifn-β* as well as increased and more rapid upregulation of the *Il-12* expression, thus supporting that more *L. acidophilus* was endocytosed by the increase constitutive micropinocytosis activity.

Lysenin is a protein of microbial origin that binds to spingomyelin in plasma membranes and induces cytolysis of erythrocytes [40-43]. Here we used lysenin in an attempt to inhibit conversion of spingomyelin to ceramide as we expected less spingomyelin substrate to be available for the SMase. While we did not observe any major change in endocytosis of bacteria, we found that lysenin increased the macropinocytic uptake of dextran in immature cells, indicative of some effect on the steady state equilibrium between spingomyelin and ceramide in the plasma membrane, which is overruled by a microbial signal. At the cytokine level, however, we found that lysenin increased IL-12 production in *E. coli* stimulated DC and had a tendency to counteract the IL-12-decreasing effect of SMase treatment, which corresponds well with the presumption that the cell membrane become less prone to sphingomyelin to ceramide conversion. Induction of IL-10 is usually induced by a signalling pathway independent of the IFN-β and IL-12 inducing pathways [19,44]. For both *L. acidophilus* and *E. coli* we found that production of IL-10 was decreased by lysenin, and this was unaffected by simultaneous treatment with SMase. This may indicate that a third signalling pathway, perhaps independent of endocytosis, or dependent on an endocytic pathway that by our method is indistinguishable from either of the types of endocytosis discussed here. Of note, in the absence of a microbial signal, addition of CPZ but not SMase affected the constitutive endocytosis of FITC-dextran. Most important, however, is the signal from the bacteria or TLR ligand, which induces the specific event in the plasma membrane and subsequently in the cellular signalling pathways. Such stimuli seem to overrule or direct the effect of SMase and CPZ. Thus, manipulation of the sphingolipid content in the plasma membrane may support but not to a major extend counteract the effects of the microbial signals. Also, the effects were more pronounced on the cytokine response than on the induction of macropinocytosis/ phagocytosis. This may, at least in part, be due the method; as we demonstrated, a significant part of the signal obtained in the flow cytometric measurement of cells is due to adherence of the bacteria outside the cells. These bacteria have accordingly not been internalised and should be excluded from the results. However, we can only assess the contribution of the adherent bacteria when we add cytochalasin D, which will inhibit endocytosis. Therefore we can only assume that the number of bacteria that are not internalized is the same independent on the prior treatment.

In conclusion, we have provided evidence that formation of ceramide in the plasma membrane of DCs is a key event in the endocytosis of *L. acidophilus* and in the subsequent production of IFN-β and IL-12. This is in contrast to an increase in macropinocytosis triggered by *E. coli* or the TLR2 ligand

Pam3CSK4, which was not affected by enhancement or inhibition of ceramide formation. These data show the importance of distinguishing between the types of endocytosis induced by bacteria and may point towards new ways to boost the immune system, for example in relation to vaccine development.

8. References

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