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FINAL REPORT - MONDAY, 26 OCTOBER 2015
A NEW CLASS OF CATIONIC LIPIDS THAT ACTIVATE INNATE IMMUNE RECEPTORS TLR4-CAT

ATTACHMENT: RESULTS & REFERENCES

Our project focused on the pro-inflammatory properties of a cationic lipid synthesized in SFMB laboratory (ULB, Brussels), diC14-amidine (1-6) *(see Figure A1)*. We previously showed that this lipid induces pro-inflammatory cytokine secretion in immune cells through a Toll-like receptor 4 (TLR4)-dependent mechanism (7). Toll-like receptors are involved in recognition of pathogen-associated molecular patterns and activation of innate immune system. Natural ligands of TLR4 are the bacterial lipopolysaccharides (LPS), major components of outer membrane of Gram-negative bacteria (8) *(see Figure A1)*.

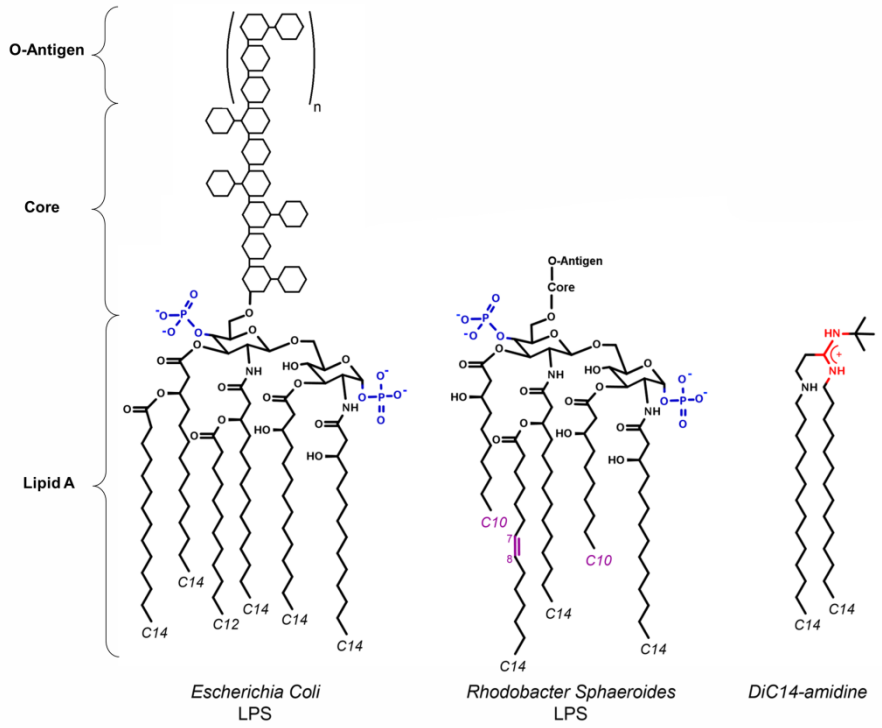

***Figure A1*: E. coli LPS (EC-LPS), Rhodobacter sphaeroides Lipopolysaccharide (RS-LPS) and diC14-amidine structures.** *In their general architecture, LPS molecules consist of a hydrophobic part named 'lipid A' covalently attached to a polysaccharide region made of a rather well-conserved 'core' oligosaccharide backbone, and an highly variable outer chain ('O-antigen') consisting of a complex polymer of oligosaccharides.*

Low toxicity LPS structures are used as vaccine adjuvants. The observation that cellular pathways are similarly activated by LPS and diC14-amidine (7) suggest that the latter may be a good candidate for vaccine development (9-13). This molecule, structurally different from all existing LPS derivatives, is obtained by chemical synthesis, and can be modified easily to improve adjuvanticity, purity and safety, at reduced costs. My main objective in my research work is to determine the molecular basis of the interaction between diC14-amidine and the components of the TLR4 ligand recognition system (TLR4 and its co-receptors MD-2 and CD14).

The **aim of this project** was to study the interaction between diC14-amidine and TLR-4/MD-2 at the structural level, building on the available structural data(14) on ligand-bound TLR-4/ MD-2 complexes.

In the **publication Cell Mol Life Sci. (2015) 72(20):3971-82** we showed that diC14-amidine, a molecule initially designed to be used as a transfection agent in gene therapy, activates TLR-4/MD-2 signalling through **a novel mechanism of TLR4 activation**. The subsequent cell signalling activated by diC14-amidine is analogous to the novel mechanism proposed for TLR4 activation by nickel ions.

In non-stimulated cells, TLR4 is thought to exist as a monomer linked to MD2. After ligand recognition TLR4/MD2 dimerizes to form a homodimer that activates the signalling pathways. This dimerization process is generally induced by TLR4 agonists, after binding into the hydrophobic pocket of the co-receptor MD2, a key step for induction of signalling. Interestingly, our data suggests that diC14-amidine interacts with the amino acid residues located in the TLR4 dimerization interface, rather than at the TLR4/MD2 binding site, forcing dimerization without binding to MD2 bringing both TLR4s into close proximity. Despite the different mechanisms that diC14-amidine used to interact with TLR4, compared to LPS, it was still able to induce signalling in a very similar manner to LPS through both MyD88-dependent and the TRIF-dependent pathways. It is thought that the first cascade to be induced upon LPS recognition by TLR4 at the plasma membrane, is the MyD88-dependent signalling leading to NF-κB activation. The second cascade (TRIF-dependent) is initiated after endocytosis of the whole TLR4/MD2/LPS complex, and initiates the TRIF-signalling from the endosome (15). This step was further demonstrated to be dependent on the presence of the co-receptor CD14 (16).

LPS is only able to activate TLR4/MD-2 low levels of signalling in the absence of CD14, we found **diC14-amidine's TLR4 agonist activity is not affected by the lack of CD14** (*Figure A2*).

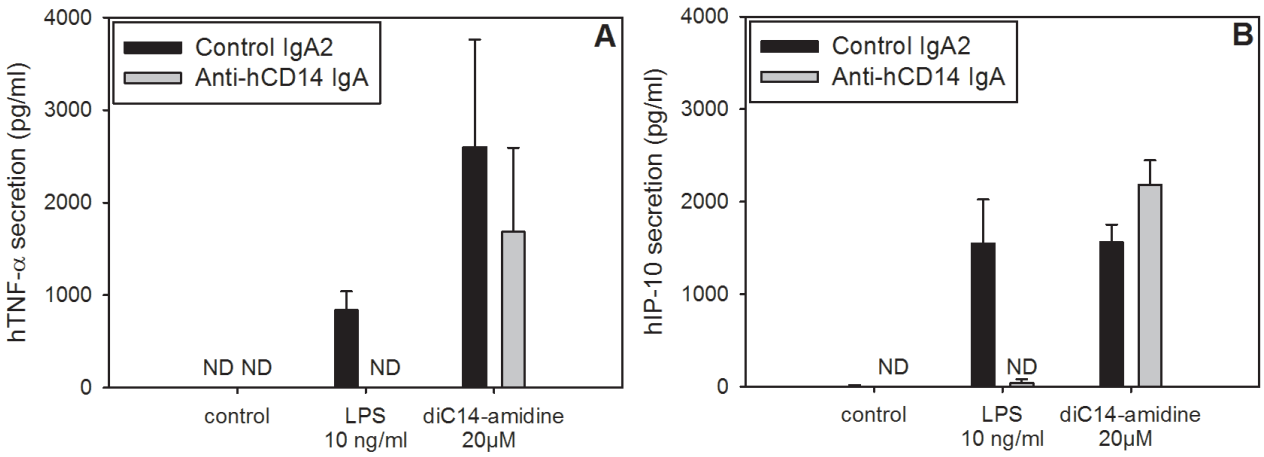

Figure A2. ***Effect of CD14 neutralizing antibodies on the MyD88-dependent and TRIF-dependent cell response of primed THP1 cells after stimulation with EC-LPS or diC14-amidine.*** *After priming for 24 hours with PMA followed by 4 hours in complete medium the cells to be treated with the control antibody (Control-IgA2) or blocking antibody against CD14 (Anti-hCD14-IgA) were incubated with 20 µg/ mL antibody in RPMI for the control and for the cells to be stimulated with diC14-amidine and with the same concentration of antibodies in complete medium in the case of subsequent EC-LPS stimulation. After 1 hour of incubation concentrated stimulants were added to the cells to reach final stimulant concentrations of 10 ng/ mL for EC-LPS and 20 µM for diC14-amidine. After 4 hours of stimulation the supernatant was recovered and the secretion of hTNF-α was quantified by ELISA. n = 3, means ± s.d.; ND = Not detected, i.e. at the minimum reporting level.*

Similar CD14-independency has been reported for the activation of the MyD88-dependent pathway by two synthetic lipid A derivatives: MPL (Monophosphoryl Lipid A) and CRX-527 and for the rough form of LPS (LPS lacking the full-length O-chains)(17-20). These LPS derivatives, like diC14-amidine, share a small headgroup in comparison to LPS which suggests that CD14 is dispensable for the more hydrophobic TLR4 ligands. In contrast it is generally accepted that CD14 is required for the activation of the TRIF-dependent pathway (15,16), but we found that, unexpectedly diC14-amidine is still able to trigger TRIF-dependent pathway in the absence of CD14. This result is surprising and questions the previously reported requirement of CD14 for TRIF-dependent signalling. CD14 is thought to be critical for the endocytosis of TLR4/MD2 (16) and hence required for activation of the TRIF signalling (15), so we also compared diC14-amidine and LPS mechanisms of activation in the presence of two endocytosis blockers, Dynasore (DYN) and Bafilomycin A1 (BAF) (*Figure A3*). The results show that Dynasore blocks both MyD88- and TRIF-dependent pathways induced by diC14-amidine, while Bafilomycin A1 only blocks the TRIF-dependent signalling. Dynasore blocks the endocytosis process at an earlier stage (pinching off of the formed clathrin-coated vesicles) than Bafilomycin A1, preventing the acidification and the maturation of the endosome, our results suggest that **the**

cationic lipid diC14-amidine may induce MyD88-dependent pathway from the early endosome and its TRIF-dependent pathway from the late endosome (see *Figure A6*).

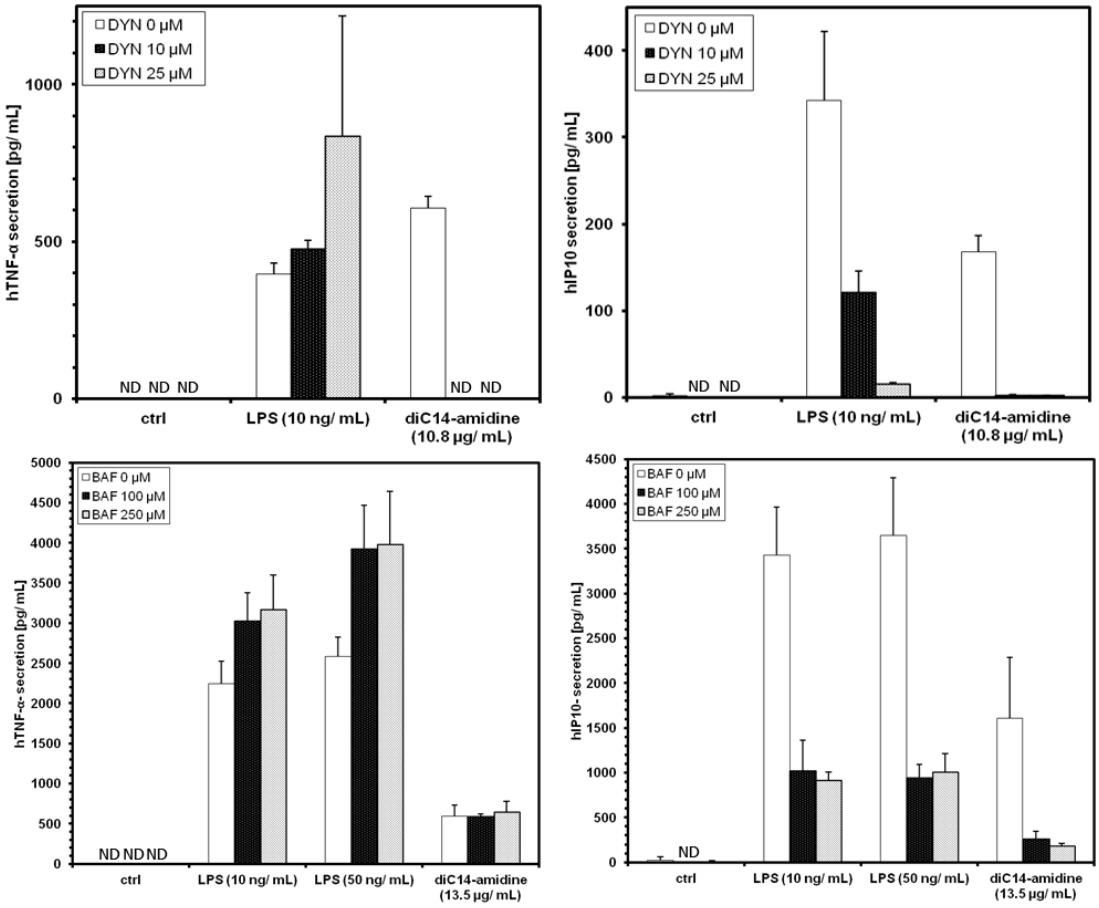

Figure A3*: *Effect of endocytosis blockers on the MyD88-and TRIF-dependent cell response of primed THP1 cells after stimulation with LPS or diC14-amidine. After priming for 24 hours with PMA followed by 4 hours in complete medium the cells to be treated with endocytosis blockers were incubated with the indicated concentrations of the endocytosis blockers (0 µM, 10 µM, 25 µM for Dynasore, and 0 µM, 100 µM, 250 µM for Bafilomycin A1) in RPMI for the control and for the cells to be stimulated with diC14-amidine and with the same concentration of blocker in complete medium in the case of subsequent LPS stimulation. After 1 hour of incubation concentrated stimulants were added to the cells to reach final stimulant concentrations of 10 ng/mL for LPS (corresponding to 0.5-1.5 nM) and 10.8 µg/mL for diC14-amidine (corresponding to 20 µM) or 13.5 µg/mL (corresponding to 25 µM) with a total volume of 100 µL per condition. After 4 hours of stimulation the supernatant was recovered and the secretion of hTNF-α was quantified by ELISA. n = 3, means ± s.d.; ND = Not detected, i.e. at the minimum reporting level.

Although our data suggests that CD14 is not required for diC14-amidine signalling, it does modulate the cellular response to this ligand. This suggests a possible interaction of diC14-amidine liposomes with this co-receptor. We therefore studied the interaction between CD14 (expressed as recombinant protein) and diC14-amidine using two physico-chemical methods: Fourier-transform Infrared Spectroscopy (*Figure A4*) and Mass Spectrometry (*Figure A5*) which suggested that diC14-amidine might activate another CD14-dependent signalling pathway independently of TLR4. Future work will focus on trying to understand the functional consequences for cells of this interaction.

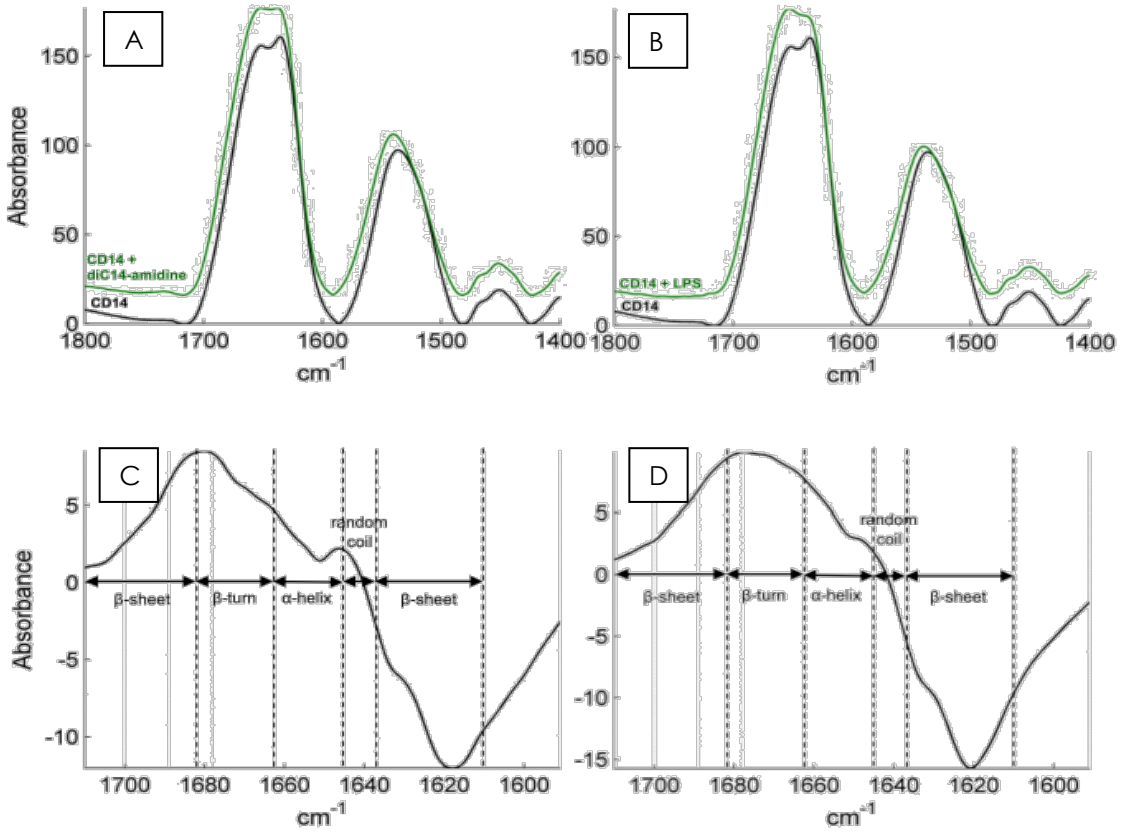

Figure A4. ***Comparison of the secondary structure of cluster of differentiation 14 (CD14) with the secondary structure of CD14 incubated with diC14-amidine (A and C) or LPS (B and D) by ATR-FTIR (non-deuterated spectra, spectrum of ligand substracted).*** **A**: Infrared spectrum of CD14 alone (black) and CD14 incubated with diC14-amidine (green), **B**: Difference spectrum of the spectra shown in (A) in the range between 1710 cm^{-1} and 1591 cm^{-1} (*Amide I* region) with individual secondary structure contributions. **C**: Infrared spectrum of CD14 alone (black) and CD14 incubated with LPS (green). **D**: Difference spectrum of the spectra shown in (A) in the range between 1710 cm^{-1} and 1591 cm^{-1} (*Amide I* region) with individual secondary structure contributions.

The difference infrared spectrum for CD14 in the presence of LPS (see Fig. A4) essentially matches the findings from the CD14 alone/ CD14 in the presence of diC14-amidine difference spectrum: a decrease in the β-sheet region around 1620 cm^{-1}, an increase for β-turn and β-sheet (1690 cm^{-1} to 1660 cm^{-1} region) and a minor additional peak in the α-helix/ random coil region - demonstrating similar structural changes into CD14 secondary structure in the presence of diC14-amidine or LPS. These structural changes are slightly more pronounced in the LPS-CD14 than for CD14 in the presence diC14-amidine. These results highly suggest that diC14-amidine, similar to LPS, does bind CD14 although this co-receptor is not required for its induction of TLR4 activity.

The spectrum of CD14 alone analysed by mass spectrometry, in the non-denaturing condition, (Figure A5-A) showed a large peak characteristic of differently glycosylated species in the sample. Other peaks (indicated by **) are also present and were identified as a contamination of the protein sample with phospholipids which resisted buffer exchange, suggesting they were bound to the protein. When KdO2-LipidA (a lipid A derived from E.Coli LPS)(B) or diC14-amidine (C) were added, no more lipids were identified suggesting both ligands have replaced the native phospholipids in the CD14 binding pocket. In the case of diC14-amidine, the presence of free cationic lipids is also shown by the presence of its characteristic peak (see * in the figure) while in the case of KdO2-lipidA, no free ligand can be visualized. The presence of diC14-amidine in the protein peak was then further shown by MS/MS selection of the peak at 3500 m/z, demonstrating the binding of diC14-amidine to CD14.

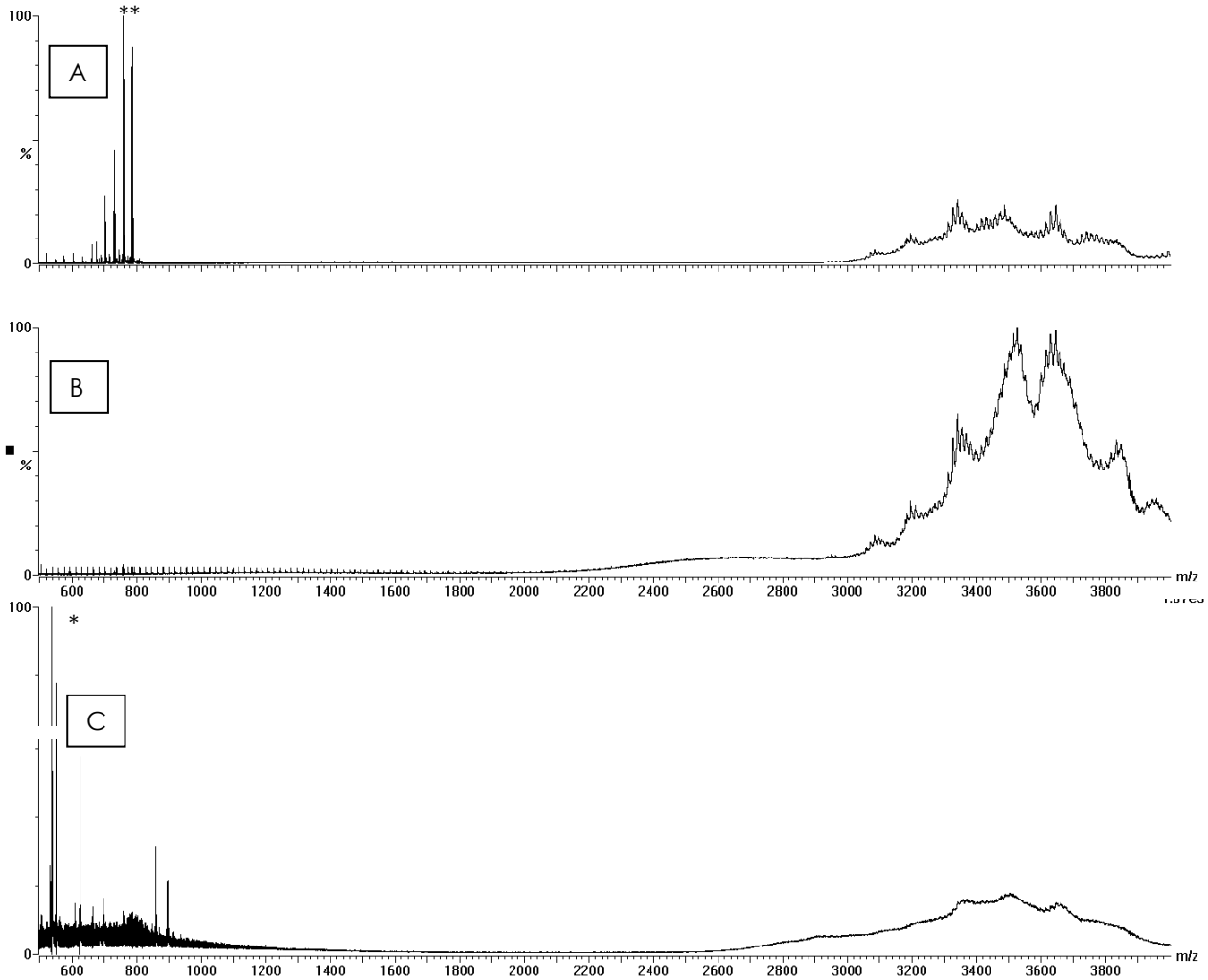

Figure A5. Mass spectrometry spectra of CD14 in Ammonium acetate (10 mM) alone (A) or after incubation with diC14-amidine © or KdO2-LipidA (B). **Spectrum were obtained with a mass spectrometer** Q-Tof Ultima (Waters/Micromass) equipped with a nanoelectrospray Z-spray source using MassLynx 4.0 as thecquisition data software.

In conclusion, we have demonstrated that diC14-amidine, a molecule initially designed to be used as a transfection agent in gene therapy, can **cause immunostimulatory activity via a novel mechanism of TLR4 stimulation.** Our species-specific chimeric receptor and TLR4 mutagenesis experiments show that diC14-amidine binds at the N- and C-terminal edges of the TLR4/TLR4* dimerization interface, and induces its dimerization via cross-linking of two receptors. The subsequent cell signalling is likely to occur in a manner analogous to the mechanism proposed for TLR4 activation by nickel ions (21).

This new binding region of TLR4 should, therefore, be further investigated as a therapeutic target. The last decade of research has demonstrated that the innate immune system, even though generally activated during infection, may be stimulated by different sterile stimuli ranging from crystals (e.g. monosodium urate and cholesterol crystals) or chemicals (e.g. taxol) to lipids (e.g. gangliosides, fatty acids), inducing the so-called "sterile inflammation"(22). Some of these stimuli are caused by endogenous lipids such as cholesterol (23) or oxidized phospholipids (24), that might activate TLR4 through interaction with the diC14-amidine binding site, expanding the potential ligands of TLR4 to non-MD-2- binding lipids. Other data obtained with other cationic lipids support the idea that non-canonical ligands could bind TLR4 at a different binding site than the one already described.

Based on our results with endocytosis blockers, we propose a model in which diC14-amidine initiates its own internalization after fusion with the plasma membrane. Once internalized, diC14-amidine activates both the MyD88- and the TRIF-dependent signalling cascades from inside endosomal vesicles, but at different stages of the endocytosis process (*Figure A6*).

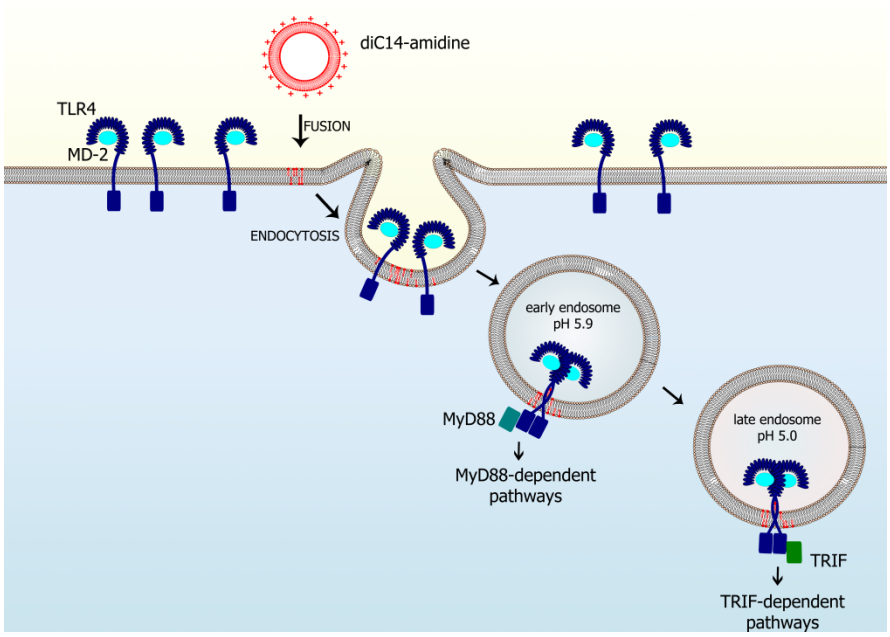

Figure A6. ***Proposed model for the activation of the TLR4-dependent signalling cascades by diC14 amidine.*** DiC14-amidine fuses with the cell membrane and induces its own internalization. Inside the endosomes it interacts with TLR4-MD2, initiating the MyD88-dependent pathway from inside early and the TRIF-dependent pathway from inside late endosomes.

This hypothesis has to be confirmed using other endocytosis blockers but also by studying the **intracellular fate of diC14-amidine molecules** and TLR4/MD2 complexes.

Finally, our binding results made on diC14-amidine/CD14 complexes demonstrate that diC14-amidine binds this co-receptor although it is not required for diC14-amidine's TLR4 agonist activity, suggesting that a possible CD14-dependent signalling pathway might be activated by diC14-amidine independently of TLR4.

1) References

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