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Final Report

Scientific output of my research internship at the Division for Infectious Diseases and Immunology, University of Massachusetts Medical School (UMass), Worcester, USA

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Project title:

Pro-atherogenic oxLDL stimulation inhibits TLR9 agonist induced plasmacytoid dendritic cell activation

Background:

Dendritic cells (DCs) are specialized antigen-presenting cells that are thought to be involved in atherosclerosis adaptive immune responses. Maturation and activation of DCs induced by danger signals derived from pathogenic agents or damaged tissue via pattern-recognition receptors such as Toll-like receptors, is a crucial step in DC innate and adaptive immune actions. Hypercholesterolemia, a major risk factor in atherosclerosis development, may influence signaling pathways relevant to DC immune functions, thereby screwing host immune responses against invading pathogens. Indeed, hyperlipidemia has been shown to abrogate the activation of certain murine DC subsets, due to uncoupling of Toll-like receptor-mediated signaling, thus affecting host resistance to microbial infections.

Hypothesis:

We hypothesize that a hyperlipidemic microenvironment inhibits TLR9-induced activation of pDC .

Aim of the planned experiment and objectives:

In this project I investigated the influence of a hyperlipidemic environment on the phenotype of human pDC, their activation status, and functionality. In particular, I was interested in lipid uptake mechanisms by pDC, intracellular lipid storage, and whether lipid storage impairs the activation of intracellular pathways that regulate pDC functions.

Methods/ project design:

- Lipoprotein uptake and intracellular storage/ influence on intracellular signaling pathways -

To investigate, how lipoproteins (LDL or oxLDL) are taken up (f.e. caveolin-/ clathrin-mediated uptake), how they are stored intracellularly (cytosol versus lysoendosomes) and whether they impair ligand binding to TLR9, I performed several *in vitro* experiments, using confocal microscopy. Therefore, human pDC were isolated from human peripheral blood mononuclear cells (PBMC) by BDCA-4 magnetic beads and incubated with TLR9 agonist CpG-ODN -/+ the lipoproteins for several hours. To follow CpG-ODN and lipoprotein uptake and intracellular trafficking, the components were labeled with a fluorescent dye. Additionally, fluorescently-labeled dyes (lysotracker), able to detect the lysosomal compartment(s), were used to examine a co-localization of lipoproteins and/ or CpG-ODN within the lyso-endosomes.

Furthermore, I addressed the question whether (modified) lipoproteins particularly impair the activation of TLR9-induced upstream signaling pathways. To answer this question, pDC were

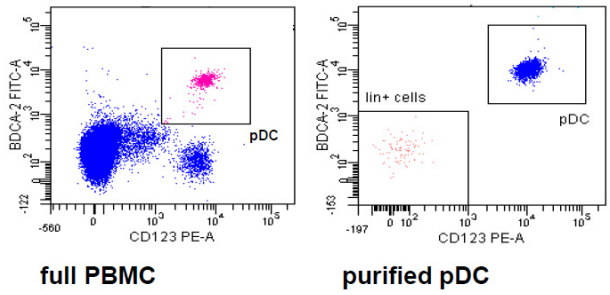
incubated with TLR9 agonist CpG-ODN -/+ the lipoproteins for 6-24 hours. Intracellular stainings were subsequently performed to analyze translocation of the p65-subunit from the cytoplasm into the nucleus. pDC functionality was as well determined by measuring cytokine levels (TNF-α, IL-6) in the cell supernatant (by ELISA).

Results:

a) Lipoprotein uptake capability, intracellular storage and TLR-binding

First I examined whether pDC, freshly isolated from human PBMC by BDCA-4 magnetic beads, are able to take up oxLDL (and LDL). Enrichment of isolated immature pDC was confirmed by flow cytometry according to the surface marker expression of BDCA-2 and CD123 and reached between 90-95%, represented by flow cytometry (**Figure 1A**). Overnight incubation of immature pDC with oxLDL led to a slightly increased cell death (**Figure 1B**). LDL did not markedly influence cell viability (**Figure 1B**). Average cell viability amounted 70-90% (**Figure 1B**). Immature, as well as CpG-matured pDC were incubated for 24 hours in the absence or presence of DiI-labeled oxLDL (10 and 50µg/ml). DiI-oxLDL exposed pDC exhibited modest lipoprotein uptake, as assessed by flow cytometry ($p<0.05$; **Figure 1C, D)**. CpG-stimulated pDC showed markedly increased DiI-oxLDL uptake ($p<0.001$; **Figure 1C, D**). OxLDL (as well as LDL) accumulation by pDC was confirmed by confocal microscopy (**Figure 1E**).

(A)



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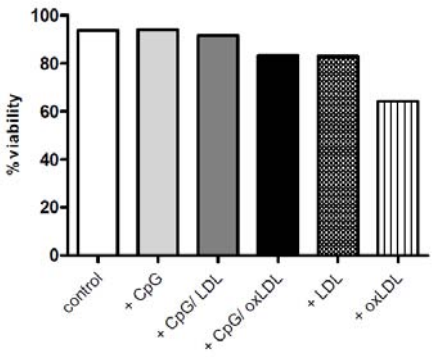


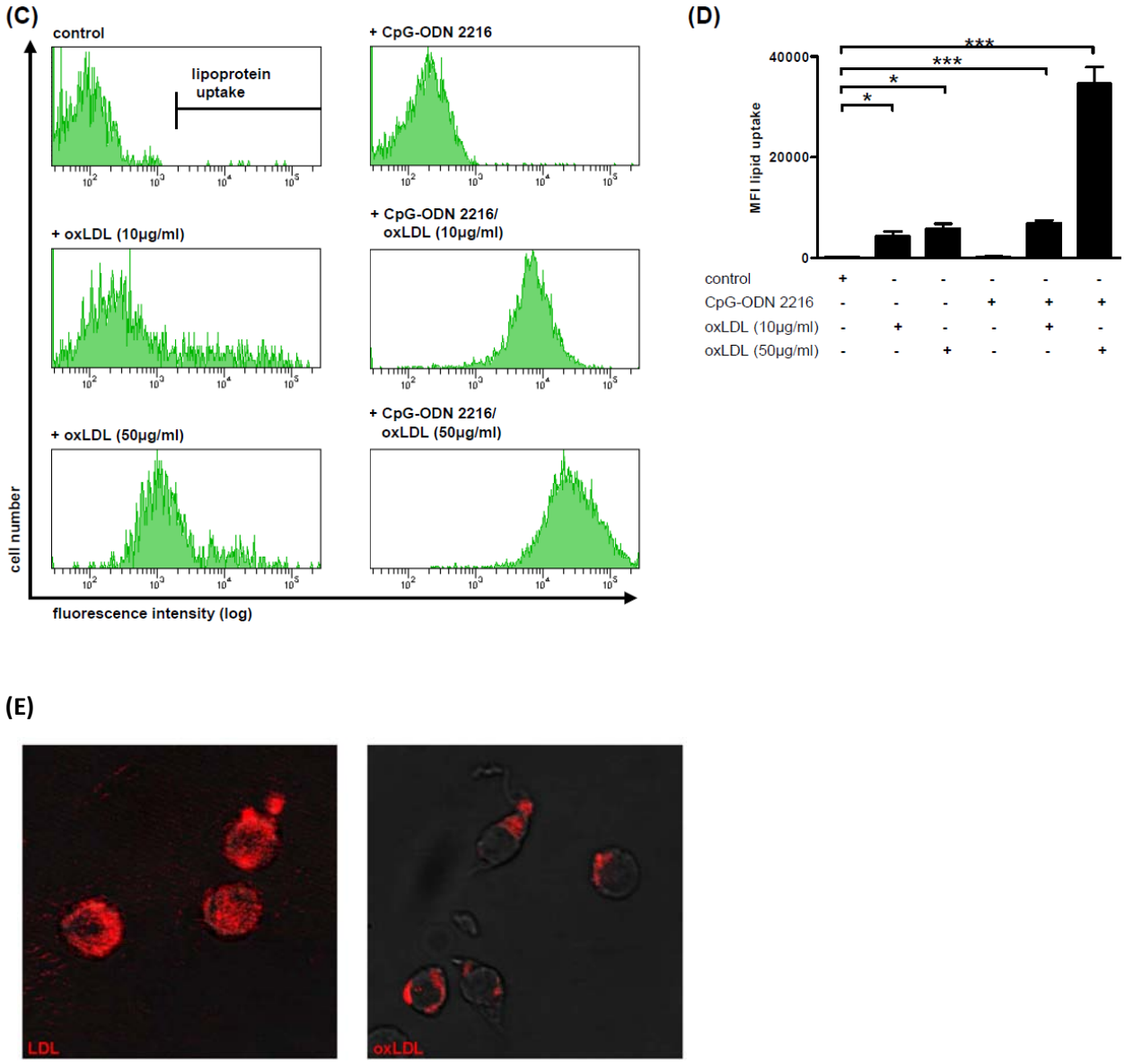
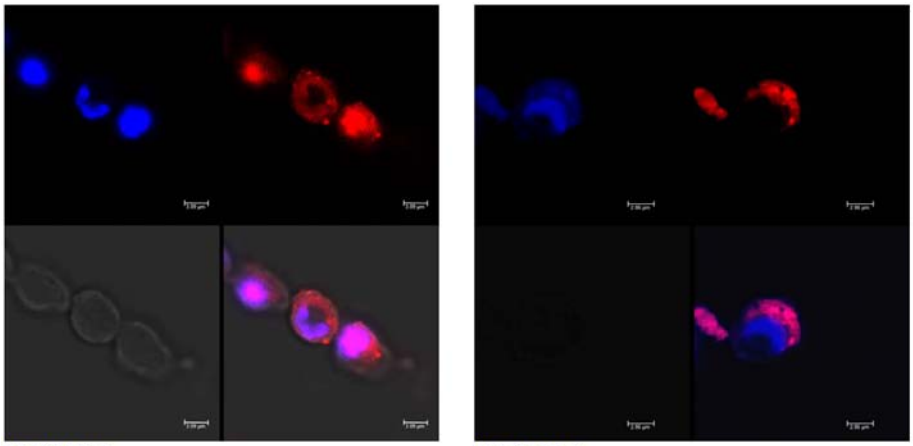


Figure 1. Lipoprotein uptake capability of human pDC. pDC, isolated from human PBMC, were cultured for 24 hours with CpG-ODN 2216 (10µg/ml), CpG-ODN 2216 (10µg/ml) plus (DiI-labeled) lipoproteins (LDL or oxLDL), or (DiI-labeled) lipoproteins alone. Non-supplemented medium was used as control**. (A)** Enrichment of $CD123^+$ $BDCA\text{-}2^+$ pDC from isolated PBMCs. Purity was determined by flow cytometry. **(B)** Percentage of viable pDC after 24 hour stimulation. Cells were stained with 7-AAD for the presence of dead cells. **(C)** FACS plots demonstrating oxLDL uptake (10 ug/ml, 50 ug/ml) in the presence or absence of CpG-ODN by pDC. **(D)** Mean fluorescence intensity (MFI) of DiI-oxLDL exposed pDC. **(E)** DiI fluorescence (red signal) determination by confocal microscopy as a measure of LDL, or respectively oxLDL uptake. *p< 0.05, **p< 0.01, ***p<0.001

Fluorescent staining of the lysosomal compartment by use of the fluorescently-labeled dye lysotracker demonstrated the co-localization of DiI-labeled LDL as well as DiI-labeled oxLDL within the endo-lysosomal compartment (**Figure 2A**). As a next step, we examined the intracellular lipoprotein and CpG-ODN storage. DiI-labeled LDL and CpG were thoroughly co-localizing within the same lysosomal compartment (**Figure 2B**), even after long-term exposure (24 hours; data not shown). As against, DiI-labeled oxLDL and CpG showed only partial intracellular overlap after co-incubation, indicating that oxLDL is probably stored in another intracellular compartment (**Figure 2C**).

(A)



(B) **(C)**

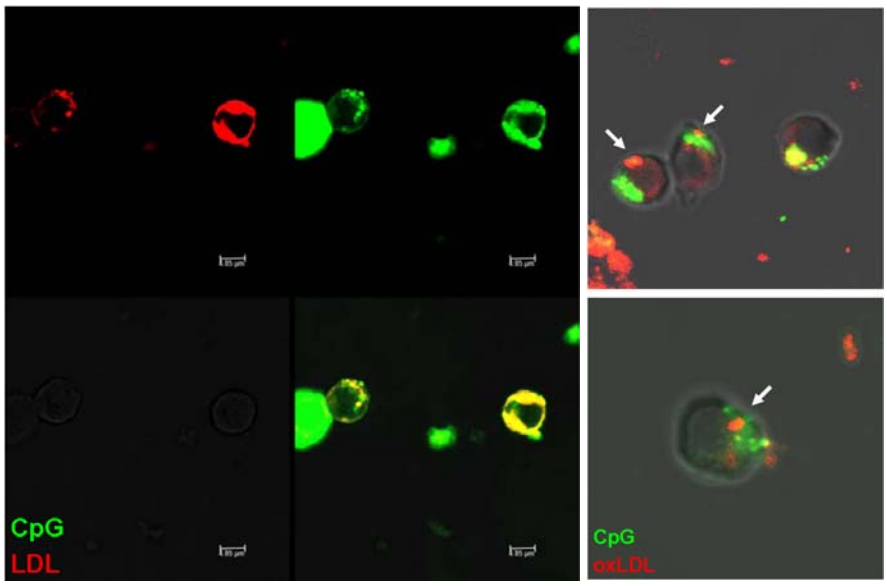


Figure 2. Intracellular lipoprotein storage in human pDC. pDC, isolated from human PBMC, were cultured overnight with DiI-LDL or DiI-oxLDL (50µg/ml). **(A)** Co-localization of LDL or oxLDL with the intracellular endo-lysosomal compartment, analyzed by confocal microscopy. The fluorescent dye lysotracker (blue signal) was used to label the intracellular lysosomal compartments. **(B + C)** (Co-) localization of LDL (red signal), and accordingly oxLDL (red signal) with CpG-ODN (green signal) in the same lysosomal compartment, assessed by confocal microscopy; LDL and CpG are thoroughly co-localizing, whereas oxLDL and CpG only partly overlap in the same lysosomal compartment.

b) Human pDC activation in the presence of lipoproteins

In previous experiments we have already shown that oxLDL accumulation in pDC inhibited the CpG-ODN induced cell maturation and activation (data not shown here). In contrast, co-treatment of pDC with LDL and CpG did not influence pDC maturation, as it did not abrogate CpG-induced pDC maturation, indicated by the significant up-regulation of co-stimulatory markers (data not shown here).

The oxLDL-associated inhibition of CpG-ODN induced pDC maturation led us to test their impact on pDC cytokine production. In the context of systemic viral or bacterial infections pDC rapidly release large amounts of type I interferons (IFN), as well as other pro-inflammatory cyto- and chemokines, which promote lymphocyte recruitment to sites of infection. Here, simultaneous treatment of immature pDC with oxLDL (50µg/ml) and CpG-ODN for 24 hours completely prevented the CpG-ODN induced production of the pro-inflammatory cytokine IFN-α (**Figure 3A**), as well as TNF-α and IL-6 (data not shown). In line, IFN-α gene expression levels were almost completely blunted when pDC were simultaneously treated with oxLDL and CpG-ODN (**Figure 3B**). In contrast, co-treatment of pDC with LDL (50µg/ml) and CpG-ODN did not quench the CpG-ODN induced production of IFN-α (**Figure 3A, B**). Consistently with these data, LDL and CpG-ODN/ LDL-stimulated pDC exhibited a considerable nuclear translocation of the NF-κB/ p65-subunit (**Figure 3C**), assessed by confocal microscopy. However, CpG-ODN induced NF-κB/ p65-subunit translocation from the cytoplasm into the nucleus was abated in the presence of oxLDL, and completely abolished when oxLDL was administered solely (**Figure 3C**).

Altogether, these data reveal that the CpG-induced pDC activation is abrogated in the presence of oxLDL.

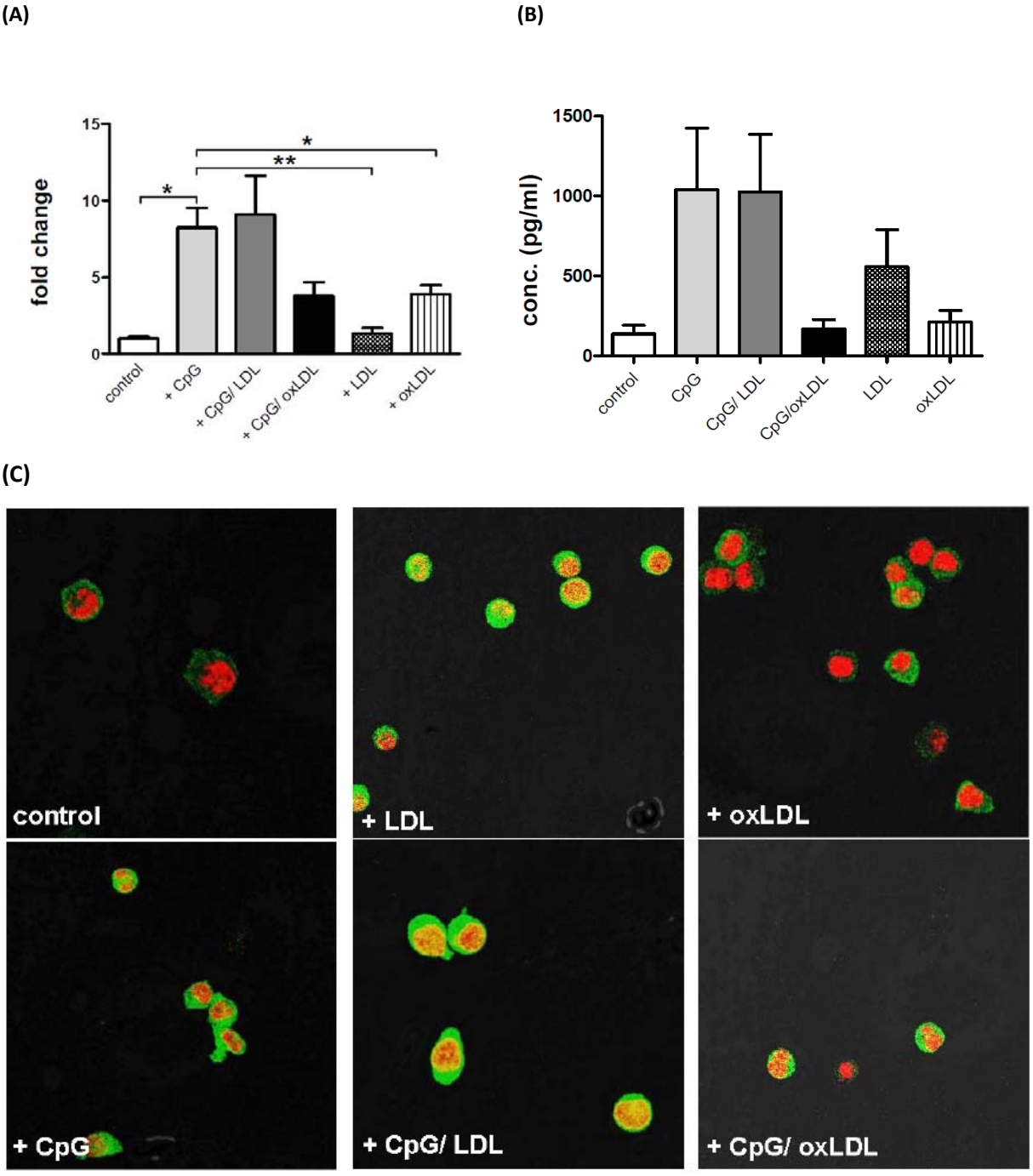


Figure 3. OxLDL inhibits the CpG-ODN induced pDC activation. Freshly isolated human pDC were stimulated with CpG-ODN 2216 (10µg/ml) either with or without LDL or oxLDL (50µg/ml) for 24 hours. Non-stimulated pDC were used as control. **(A)** IFN-α gene expression levels (fold change) in cultured pDC measured by quantitative RT-PCR. **(B)** Measurement of IFN-α levels in the cell supernatant of cultured pDC. **(C)** NF-κB/ p65-subunit translocation in cultured human pDC (red signal: cell nucleus staining, green signal: anti-p65). Data are shown as mean ± SEM of three independent experiments using different donor pDC. *p< 0.05, **p< 0.01, ***p<0.001.

Resume:

My short-term stay at UMass positively contributed to my scientific career, as it established the opportunity to broaden my networks and collaborations. Besides, I personally benefitted from this stay, as I was able to make social contacts and built up new friendships. I appreciated and enjoyed my time in the US and would recommend such a stay abroad to all my fellow PhD students.

I would like to thank Prof. Golenbock for accepting me as an exchange graduate student in his lab, my PI for supporting my short-term stay abroad, and the ESC for funding part of my internship.