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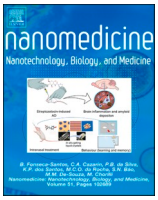

Liposomes - Human phagocytes interplay in whole blood: effect of liposome design

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ABSTRACT

Nanomedicine holds immense potential for therapeutic manipulation of phagocytic immune cells. However, *in vitro* studies often fail to accurately translate to the complex *in vivo* environment. To address this gap, we employed an *ex vivo* human whole-blood assay to evaluate liposome interactions with immune cells. We systematically varied liposome size, PEG-surface densities and sphingomyelin and ganglioside content. We observed differential uptake patterns of the assessed liposomes by neutrophils and monocytes, emphasizing the importance of liposome design. Interestingly, our results aligned closely with published *in vivo* observations in mice and patients. Moreover, liposome exposure induced changes in cytokine release and cellular responses, highlighting the potential modulation of immune system. Our study highlights the utility of human whole-blood models in assessing nanoparticle-immune cell interactions and provides insights into liposome design for modulating immune responses.

Backround

To increase interactions between drug-loaded nanoparticles and their target tissue and cells, nanomedicines have traditionally been designed to minimize their extensive clearance by the mononuclear phagocyte system. When the encapsulated drug is to be delivered into for example cancer cells, this is a sensible strategy. However, enormous research effort in the nanomedicine field during the last decades has demonstrated that it is extremely challenging to avoid this extensive nanomedicine uptake by phagocytic immune cells. Interestingly, although long considered as bystanders in immunological processes, phagocytes have appeared to be important regulators of immune responses in numerous pathological conditions.[1] Since phagocytes extensively engage administered nanoparticles, nanomedicine has been recognized as a potentially powerful gateway for the therapeutic manipulation of these cells.

To successfully develop nanomedicine targeting specific innate immune cells, a mechanistic understanding of the interaction between nanoparticles and leukocytes and their effect on biological responses is needed. Although these interactions have been extensively studied[2–4] most of those studies have focused on preventing nanoparticle uptake by these cells. Moreover, primary isolated cells or immortalized cell lines are typically used in these studies, disregarding the effects of different cell types and blood/tissue components.[5–8] Consequently, observations in single-cell *in vitro* setups often don't translate to *in vivo* conditions.[9–11] As a result, in depth mechanistic understanding for *in vivo* nanoparticle

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behaviour remains limited and the dominating strategy for the development of novel nanomedicines is based on costly nanoparticle library screening approaches in mouse models of human disease.[12–14]

Although these *in vivo* experiments are critically important, there is a translational gap from animal studies to application in humans[15,16] due to a lack of understanding of how species differences influence the behaviour and functionality of nanomedicines in the body.[17] It has been demonstrated that nanoparticles accumulate in phagocytes in both animal models and patients.[18–20] However, to our knowledge no data exists on the extent of this interaction in patients as a function of nanoparticle design parameters like size and surface properties. To address these issues, we systematically assessed interactions between liposomes and immune cells in human whole blood.

Human whole blood has been utilized previously to evaluate nanoparticle interactions with immune cells[21–23] and to determine nanoparticle toxicity.[24,25] The human whole blood model encompasses physiological qualities allowing crosstalk between blood soluble proteins and leukocytes, and it has proven suitable in evaluating biological effects like inflammatory response, biocompatibility, and tolerability of various nanomaterials.[24,25] The model requires that the blood used is fresh and anticoagulated with compounds that do not inhibit immune activation pathways. The immune status of healthy donors is usually unknown, which can introduce significant variation between samples. Furthermore, effects of pharmacokinetics and biodistribution are typically absent in *ex vivo/in vitro* models. Despite these limitations, the whole blood model offers cost-effective evaluation of diverse nanomaterials under physiological relevant conditions.[21–25]

Here, we comprised liposome libraries with variations in liposome size, PEG surface density, lipid composition and ligand decoration and assessed their uptake by and immunological effects on phagocytic myeloid cells in human whole blood. We observed that the human whole blood model provides results that differ from single-cell experiments and resemble various aspects of published *in vivo* results. We anticipate that integration of human whole blood models in nanomedicine development pipelines can aid the interpretation of results obtained in animals and contribute to overcoming the translational gap from mouse to man.

Methods

For more details, see “Supplementary information”.

Liposome preparation and characterization

Liposomes were prepared using a solvent injection method. The appropriate amounts of lipid stock solutions (in chloroform:methanol 9:1) were mixed to achieve the desired ratios (Table S1, S2, and S3). 0.2 mol% of the fluorescent lipid dye DiD was added to each formulation. The lipid mixture was slowly dripped into preheated DPBS (pH 7.4) at 70 °C while vigorously stirring at 700 rpm. The suspension was stirred at 70 °C for 5 min to allow solvent evaporation. Final lipid concentration was 5 mM. Liposomes were downsized by extrusion and stored at 4 °C. Liposome diameter (z-average, nm), polydispersity index (PDI, Đ), and ζ potential (mV) were determined by dynamic light scattering (DLS) using Malvern Zetasizer Nano—ZS (Malvern, Oxford, UK).

Whole blood model

Peripheral blood was drawn by venipuncture from healthy volunteers (approval by the Regional Committee for Medical and Health Research Ethics REC Central, Norway, No. 2009/2245) into 6 ml vacutainer heparin tubes (#367886, BD) and gently inverted several times after sampling.

Flow cytometry

To prepare blood samples for flow cytometry, red cells were lysed at room temperature for 5 min. The suspension was centrifuged, supernatant was aspirated out, and the lysis process was repeated. The obtained immune cell pellet was resuspended in FACS buffer and blocked at RT. Then, cells were incubated for 30 min at RT in the dark with the respective antibodies (see below). Cells were stained with Zombie Aqua (BioLegend, #423101) in a serum-free medium following the manufacturer’s instructions. Finally, cells were washed twice with FACS buffer and analyzed on BD LSR II flow cytometer. Single staining and fluorescence-minus-one (FMO) staining were performed. Data was analyzed using FlowJo™ v10.

Liposome uptake

To assess the uptake of liposomes by leukocytes in whole blood, heparinized human blood was incubated for 30 min or 1 h at 37 °C with DiD-labeled liposomes at 0.5 mM lipid concentration (one blood sample per donor per liposome). Then, red cells were lysed, and samples were prepared for flow cytometry as described above. Cells were stained with CD45-PE-efluor610, CD3-FITC, CD14-PerCP-efluor710, CD16-SB600 from eBioscience, ThermoFisher. After incubation, Zombie Aqua was added, followed by 2 washes, and the sample was analyzed on a BD LSR II flow cytometer.

Multiplexed cytokine profiling

Heparinised human blood was incubated with liposomes at 0.5 mM lipid concentration for 6 h or 1 h followed by 5 h stimulation with lipopolysaccharides (LPS) (100 ng/ml) at 37 °C (one blood sample per donor per liposome). Sample were centrifugated at 340 x g for 5 min at 4 °C. Supernatants were collected and frozen at −80 °C until analyses. Supernatants were diluted 1:4 and analyzed according to the manufacturer’s protocol using the Inflammation 20-Plex Human ProcartaPlex™ Panel (#EPX200-12185-901, Invitrogen, ThermoFisher) on a Bio-Plex 200 instrument (BioRad).

Intracellular reactive oxygen species (ROS) levels

The respiratory burst assay was performed according to the manufacturer’s instructions (Abcam, ab236210). Briefly, heparinized human blood was incubated with the dihydrorhodamine 123 (DHR) assay reagent for 15 min at 37 °C, followed by incubation with liposomes at 0.5 mM lipid concentration for 2 h or 1 h followed by 1 h stimulation with phorbol 12-myristate 13-acetate (PMA) (200 ng/ml) at 37 °C (one blood sample per donor per liposome). Then, red cells were lysed, and the samples were prepared for flow cytometry, as explained above. Cells were stained with CD45-PE-efluor610 and Zombie Aqua, washed twice with FACS buffer and analyzed on BD LSR II flow cytometer.

E. coli bioparticles phagocytosis

pHrodo™ red *E. coli* BioParticles™ (Invitrogen, Thermo Fisher, #P35361) were diluted to 2 mg/ml in PBS. 200 μl heparinised human blood was incubated for 1 h with liposomes at 0.5 mM lipid followed by addition of 22 μl of pHrodo Red E coli bioparticles and incubated for 1 h at 37 °C (one blood sample per donor per liposome). Red cells were lysed, and samples were prepared for flow cytometry, as explained above. Cells were incubated with CD45-FITC and Zombie Aqua, and analyzed on a BD LSR II flow cytometer.

Neutrophil cell surface markers

Heparinized whole human blood was incubated for 2 h with liposomes at 0.5 mM lipid or 1 h with liposomes, followed by 1 h stimulation with LPS (100 ng/ ml) at 37 °C (one blood sample per donor per liposome). Then, red cells were lysed, and samples were prepared for flow cytometry, as explained above. Cells were stained with the following

fluorescent antibodies: CD45-FITC, CD16-BV605, CD11b-PE, CD62L-BV785 and CD66b-BV421 from BioLegend. Cells were stained with Zombie Aqua, washed twice with FACS buffer, and analyzed on a BD LSR II flow cytometer.

Statistical analysis

Statistical analysis was performed using GraphPad Prism. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparisons tests. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. The reported N (number of donors) corresponds to one blood sample per donor incubated with one liposome formulation.

Results

Impact of liposome size and surface PEG density on uptake in phagocytes

To assess the effects of liposome size and PEG surface density on their interaction with human phagocytic immune cells, we created a library of 25 DSPC:Chol:DSPE-PEG2000 liposome formulations (PL1-25) and varied the DSPE-PEG2000 (PEG-DSPE) content (Library A, Fig. 1, Table S1).

Using flow cytometry, we quantified liposome uptake by phagocytic granulocytes (primarily neutrophils), monocytes and lymphocytes in heparinized fresh human whole blood. Less than 0.3 % of lymphocytes were positive for any of the formulations (Fig. S1), which aligns with these cells' lack of phagocytic activity. Liposome uptake was significantly higher in neutrophils and monocytes, with monocytes exhibiting the highest liposome uptake (Fig. 1D and E). As expected, liposome uptake by neutrophils and monocytes increased as a function of incubation time. Uptake levels in monocytes increased faster from 30 to 60 min than in neutrophils, most notable for the larger liposomes with 1 and 2.5 mol% PEG-DSPE. Interestingly, while monocyte uptake generally decreased as a function of PEG-DSPE content, uptake by neutrophils was hardly affected by PEG-DSPE content between 1 and 5 mol%. Furthermore, monocytes had the highest preference for liposomes around 200 nm, while neutrophil engagement of liposomes generally increased with increasing liposome size.

To evaluate whether the assessed liposome design parameters resulted in differential liposome uptake by the phagocytes, we calculated the ratio between % liposome positive neutrophils and monocytes (N/M ratio; Fig. 1F). The highest N/M ratios were observed for liposomes larger than 200 nm, which agrees with the higher affinity of neutrophils for phagocytosis of larger particles.[5,26]

Impact of stealth lipids on liposome uptake in phagocytes

PEG-lipids have been widely and successfully applied to reduce nanoparticle contact with plasma proteins and the formation of a protein corona. However, it is now well known that PEG can induce immunogenic responses, and anti-PEG antibodies can cause accelerated blood clearance after repeated dosing of liposomes.[27] To address this, we replaced the PEG-lipid with the alternative stealth sphingolipids sphingomyelin (SL1-6) or ganglioside (GL1-6) in Library B (Fig. 2, Table S2, for plasma stability and stability under storage conditions see Figs. S2 and S3A).

Liposome uptake in lymphocytes was negligible (1.5 %) (Fig. S3B). In accordance with the uptake of Library A liposomes, monocytes took up more liposomes than neutrophils, however, the trends in uptake as a function of time and liposome characteristics was similar for both cell types (Fig. 2D–E). Less than 2 % of neutrophils and 6 % of monocytes were positive for liposomes containing ganglioside and cholesterol (GL1, GL2, GL3). Uptake in both cell types increased threefold when liposomes containing 10 mol% ganglioside lacked cholesterol (GL6). Liposomes containing 1 mol% sphingomyelin (SL1) showed relatively high uptake by both neutrophils (5 %) and monocytes (12 %), and the uptake decreased with the increase in sphingomyelin content, reaching 3 % of neutrophils and 4 % of monocytes in liposomes containing 10 mol% sphingomyelin (SL3). Exchanging DSPC by DMPC (GL2 and SL2 *vs.* GML and SML, respectively) did not significantly affect liposome uptake, indicating that membrane fluidity did not have a significant effect on uptake by phagocytes.

In Library B, higher N/M ratios were observed in formulations with highest mol% of sphingomyelin. Increased sphingomyelin content resulted in decreased liposome uptake by both neutrophils and monocytes, with a stronger inhibitory effect observed in monocytes (Fig. 2F).

Impact of peptide decoration on liposome uptake

So far, we have shown that liposome size and composition significantly affect their uptake by phagocytes in our human whole blood model. In Library C we assessed the effects of the well-known nanoparticle ligands cRGD [cyclo (Arg-Gly-Asp-d-Tyr-Lys)] (a_vb_3-integrin receptor ligand) and cFLFLF [cinnamoyl-Phe-(D)Leu-Phe-(D)Leu-Phe], (formyl peptide receptor-1 (FPR1) antagonist) on liposome uptake by neutrophils and monocytes. To allow for comparison of these results to our published *in vivo* work[28,29] we selected PL12, which is a Doxil-like liposome formulation. We included the following liposomes as well: PL14 (same lipid composition, larger size), PL2 (same size as PL12, 1 mol% PEG), PL4 (same size as PL14, 1 mol% PEG), SL3 (high N/M ratio) and GL6 (high monocyte uptake). We incorporated 1 mol% of either cRGD-PEG-lipid or cFLFLF-PEG-lipid, using cRAD-PEG-lipid as control for non-specific binding (Fig. 3, Table S3).

Liposome uptake in lymphocytes was, again, negligible (2 %) (Fig. S4). For the PEG and sphingomyelin formulations both cRGD and cFLFLF decoration resulted in a significant increase in liposome uptake by neutrophils and monocytes as compared with non-decorated or cRAD-decorated analogues (Fig. 3C–D). Monocytes showed similar uptake of cRGD or cFLFLF-decorated liposomes, which did not increase over time, except for PL12. Neutrophils showed higher uptake of the cFLFLF-decorated liposomes compared with cRGD formulations, and uptake increased as a function of incubation time. In contrast, for GL6, cRGD decoration did not increase uptake. Like PL12, uptake of cRAD- and cRGD-decorated GL6 in neutrophils and monocytes increased from 30 to 60 min of incubation compared with non-decorated liposomes. Effects of size and PEG surface density in Library A were conserved in Library C, with liposome uptake being higher when liposomes were larger and contained less PEG-lipid. A significant effect of the ligands on N/M ratios was observed only for PL14, where cFLFLF caused a significant increase (Fig. 3E).

A summary of the observed trends in uptake of Library A-C liposomes by monocytes and neutrophils is provided in Table 1. Liposome size, DSPE-PEG content and ligand introduction were the design parameters with the most predominant effects on liposome uptake by these cells.

Impact of the liposomes on unstimulated leukocytes in whole blood

The interaction of liposomes with leukocytes can modify cell function and induce activation.[30–32] We selected PL12 and SL3 from Library C to evaluate the effect of liposomes on immune responses. PL12 was selected as it is very similar to approved liposomal formulations (*e.g.* Doxil, Lipodox) and SL3 because sphingomyelin is used in clinically approved Marqibo (albeit at higher mol% than in our formulation) and since sphingomyelin has various known biological functions that may correlate with therapeutic effects.[33,34] We performed multiplex cytokines profiling on plasma after incubation of heparinized human whole blood with liposomes. Whole blood without liposomes was used as a control, and stimulation with LPS was used to estimate the physiological relevance of the cytokine levels. The levels of IL1a, IL6, CXCL8, CXCL10, TNFa, CCL2 (MCP1), CCL3 (MIP1a), and CCL4 (MIP1b) were significantly increased (Fig. 4A). The levels of the remaining 12 cytokines were unaffected (Fig. S5). IL1a, CXCL8, and CCL2 levels were increased after

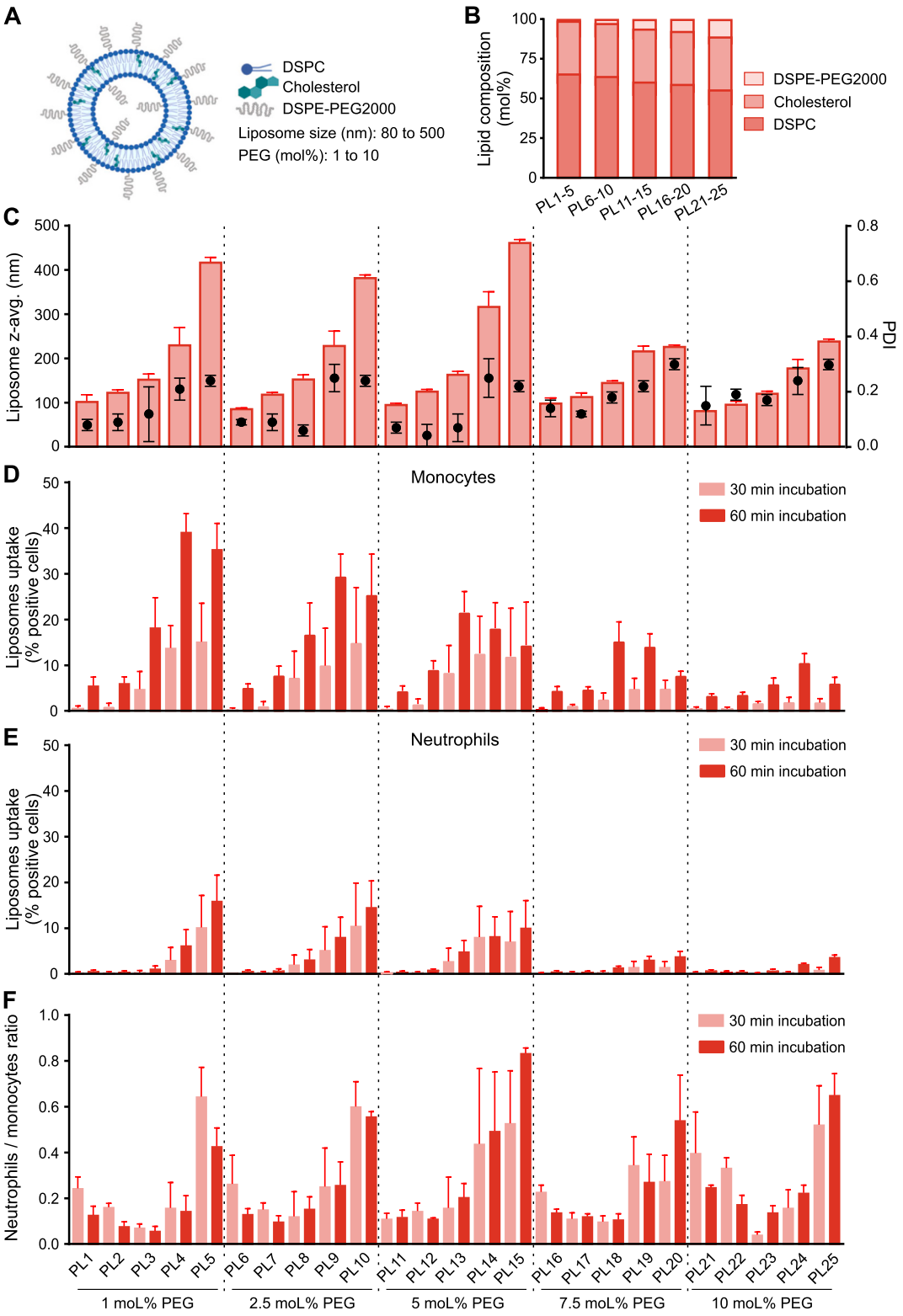

Fig. 1. Library A – PEGylated liposomes
A) Schematic representation of Library A liposomes. B) Variation of DSPE-PEG2000 content in PEGylated liposomes (PL) C) Diameter (z-average) and polydispersity index (PDI), bars represent mean ± SD, N = 3. D–E) Quantification of liposome uptake as % of liposome-positive cells. Bars show % liposome-positive monocytes (D) or neutrophils (E) after 30 or 60 min incubation. F) Ratio between % of liposome-positive neutrophils and monocytes. The vertical dotted lines separate liposomes into 5 groups, based on DSPE-PEG2000 mol%. Graphs in D–F show mean ± SEM, N = 4.

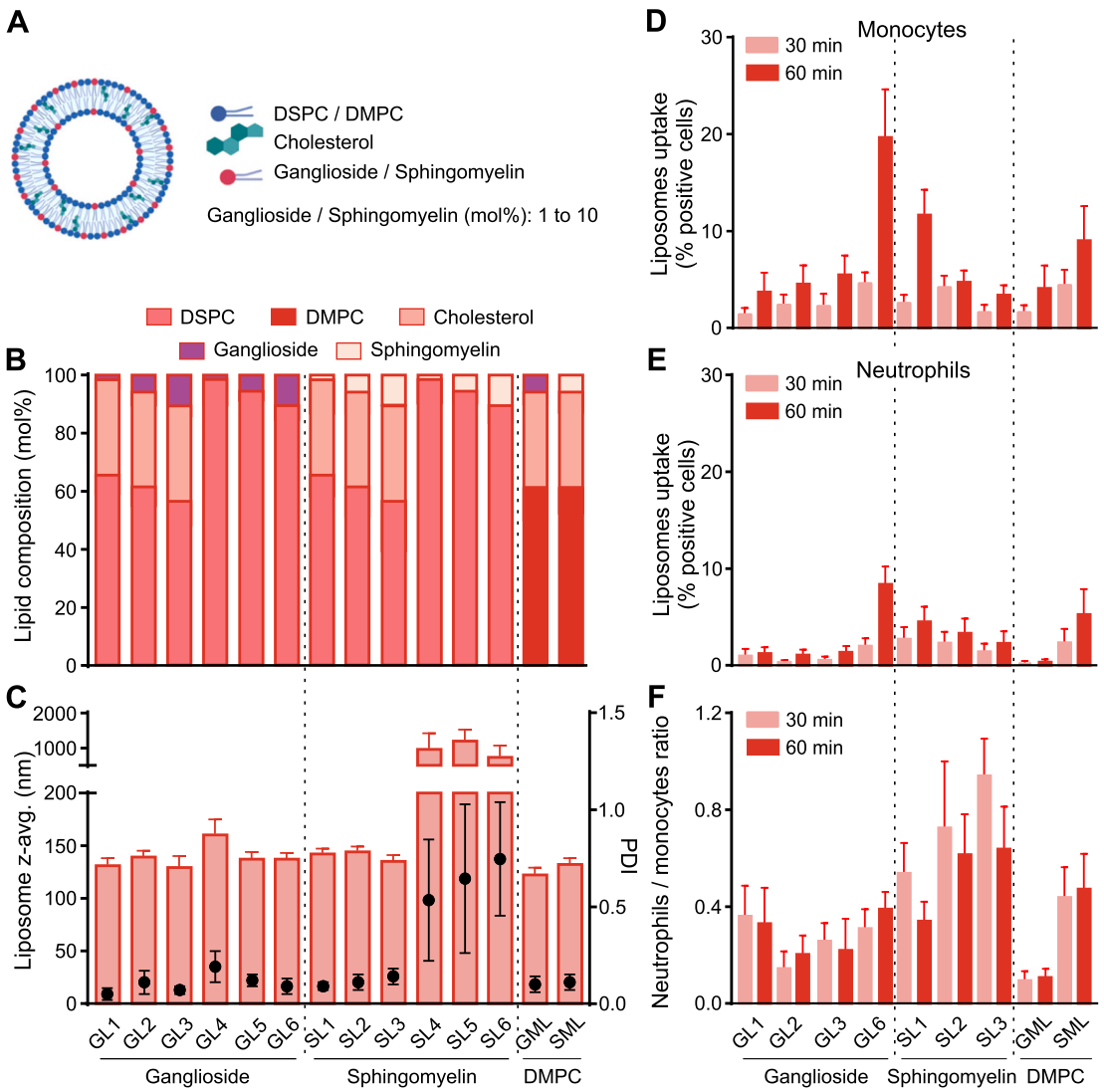

Fig. 2. Library B – sphingolipid liposomes.
A) Schematic representation of Library B liposomes. B) Lipid composition. C) Diameter (z-average) and polydispersity index (PDI), bars represent mean ± SD, N = 3. D–E) Quantification of liposome uptake as % of liposome-positive cells. Bars show % liposome-positive monocytes (D) or neutrophils (E) after 30 or 60 min incubation. F) Ratio between % of liposome-positive neutrophils and monocytes. Graphs D–F show mean ± SEM, N = 5.

incubation with all liposome formulations except SL3-cRAD. However, only CXCL8 and CCL2 levels were comparable to those induced by LPS, reaching the highest levels upon incubation with cFLFLF-decorated liposomes. All liposome formulations induced low CCL3 secretion, except PL12, SL3-cRAD and SL3-cFLFLF, while only PL12-cRGD and PL12-cFLFLF induced a small secretion of TNFa and IL10. Interestingly, only cFLFLF-decorated liposomes slightly induced CCL4 and IL6 secretion.

In neutrophils and monocytes, phagocytosis of microorganisms is followed by ROS production and destruction of the pathogen. To investigate if the uptake of liposomes could induce ROS production, we incubated heparinized blood with liposomes and measured ROS levels in neutrophils and monocytes using flow cytometry. Only SL3-cFLFLF significantly increased the ROS levels in these cells. (Figs. 4B and S6A).

To further evaluate the effect of liposomes on unstimulated neutrophils, we measured the expression of activation markers on neutrophils. Upon neutrophil activation, CD11b and CD66b surface expression increases, while CD62L expression decreases by shedding. We incubated heparinized blood with liposomes and quantified the activation markers on neutrophils by flow cytometry. A statistically significant increase of CD11b and CD66b occurred only after incubation with PL12-cFLFLF, SL3, SL3-cRGD, and SL3-cFLFLF liposomes (Figs. 4C and S6B). CD62L decrease was statistically significant after incubation with all liposome formulations, except PL12-cRGD. These results suggest that sphingomyelin containing liposomes have a strong effect on activation markers which could be associated with the priming of the neutrophils by the liposomes more than with their activation since ROS levels were only slightly increased after incubation with cFLFLF-decorated liposomes.

Impact of the liposomes on leukocytes' immune response in whole blood

Our results show that the presence of liposomes affects the leukocyte's basal activity. However, it is unknown how this would affect the cell's function when classical agonists activate them. To study the effect of the liposome's presence on the immune response induced by LPS *in vitro*, we incubated heparinized blood with liposomes for 1 h, followed by 5 h stimulation with LPS. We measured cytokine release and found that 16 increased upon LPS stimulation (Fig. S7). Only CXCL8, TNFa, IP10, and CCL2 (MCP1) showed significant differences compared with LPS (Fig. 5A). cFLFLF-decorated liposomes increased CXCL8 levels, while in all formulations except SL3-cRAD increased TNFa levels. IP10 was significantly decreased with SL3-cFLFLF liposomes, and CCL2 was increased in the presence of PL2-cFLFLF liposomes.

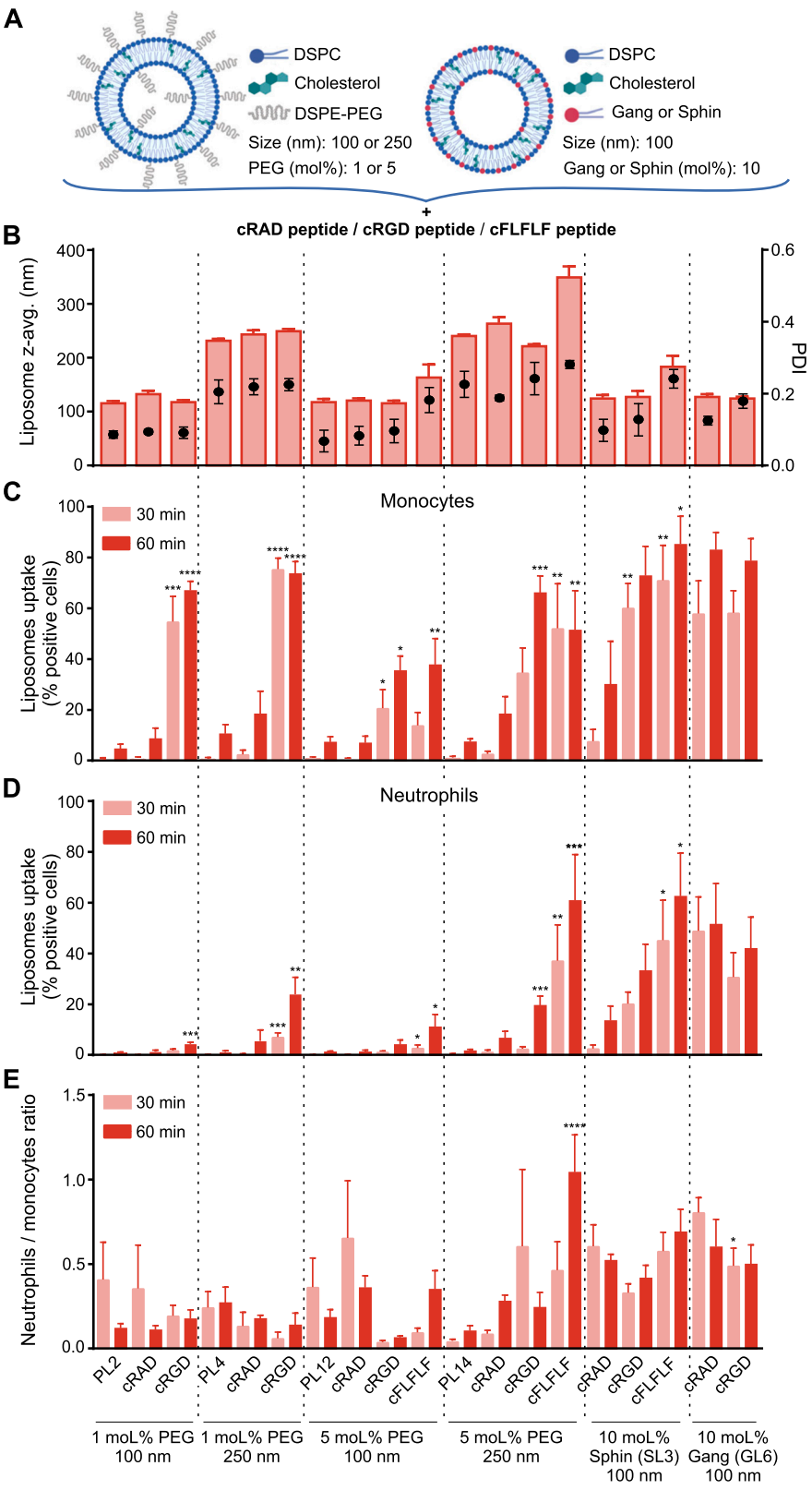

Fig. 3. Library C – peptide decorated liposomes.
A) Schematic representation of Library C liposomes. B) Diameter (z-average) and polydispersity index (PDI), bars represent mean ± SD, N = 3. C–D) Quantification of liposome uptake as % of liposome-positive cells. Bars show % liposome-positive monocytes (C) or neutrophils (D) after 30 or 60 min incubation. E) Ratio between the % of liposome-positive neutrophils and monocytes. The uptake of surface-decorated liposomes was compared with uptake of its undecorated analogue at the same time point. Graps C–E show mean ± SEM, N = 6.

Table 1
Summary of the observed general trends in liposome uptake by monocytes and neutrophils.

Liposome design parameter	Effect on uptake by monocytes[a]	Effect on uptake by neutrophils[a]
Size ↑	Maximal around 200 nm	Uptake ↑↑
DSPE-PEG content ↑	Uptake ↓↓	Uptake ↓↓ (but stable up to 2.5 mol%)
Ganglioside content ↑	Uptake ↑	No effect
Removal of cholesterol from ganglioside liposomes	Uptake ↑	Uptake ↑
Sphingomyelin content ↑	Uptake ↓	Uptake ↓
Introduction of cRGD	Uptake ↑↑	Uptake ↑↑
Introduction of cFLFLF	Uptake ↑↑	Uptake ↑↑

[a] Double arrows indicate the strongest trends.

To study the effect of liposomes on phagocyte functions, we assessed the ROS production induced by PMA and phagocytosis of *E. coli* particles using flow cytometry. ROS levels were significantly increased upon PMA stimulation in both monocytes and neutrophils. Monocyte ROS levels were unaffected by liposomes, but significantly increased in neutrophils incubated with cFLFLF-decorated liposomes (Figs. 5B and S8A). *E. coli* phagocytosis was significantly reduced in the presence of cFLFLF-decorated liposomes in neutrophils and monocytes (Figs. 5C and S8B).

To further evaluate the effect of liposomes on neutrophil activation, we measured CD11b, CD66b, and CD62L expression after 1 h stimulation with LPS in the presence or absence of liposomes. After stimulation, CD11b and CD62L were not affected by the presence of the liposomes, reaching the same value as LPS alone. In contrast, CD66b expression was significantly higher than LPS alone when neutrophils were incubated with PL12-cRGD, PL12-cFLFLF, SL3 or SL3-cFLFLF liposomes (Figs. 5D and S8C).

Discussion

Nanomedicine is increasingly recognized as a potentially powerful gateway for therapeutic manipulation of phagocytic immune cells. With the increasing significance of therapeutically targeting differential phagocytic immune cell sub-sets, it is extremely useful to have physiologically relevant cellular models in which nanoparticle interactions with different cell types can be directly compared. Therefore, we assessed liposome interactions with immune cells using an *ex vivo* human whole blood assay and evaluated how our observations correspond to published data on liposome phagocyte interactions. We chose to work with liposomes as 60 % of clinically approved nanomedicines are liposomal formulations.[35] We used fresh human blood from healthy donors as a model since it is a physiologically relevant system that preserves interactions between liposomes, blood soluble components, and cells. It allowed us to directly evaluate and compare the effect of the liposome design on the uptake by different immune cells simultaneously, which cannot be done using immortalized cell lines or isolated primary immune cells.

It is well established that PEGylation reduces nanoparticle recognition and uptake by cells of the mononuclear phagocyte system.[36,37] However, to our knowledge, no data exist on the extent of nanoparticle interactions with phagocytes as a function of nanoparticle size and PEG-surface density in human blood or patients. Therefore, we systematically varied liposome size and PEG-surface densities in Library A. We observed significant but varying liposome uptake by neutrophils and monocytes in whole human blood, while uptake by lymphocytes was negligible. For all formulations in Library A (including the Doxil like formulation PL12) monocytes took up more liposomes than neutrophils. Although hardly any studies exist on this direct comparison, Yang and collaborators showed indications towards higher *ex vivo* nanoparticle interactions with primary human granulocytes than monocytes, which contradicts our findings.[6] Interestingly, in both healthy and tumor-bearing mice,[29,38,39] as well as in human cancer patients[40,41] we and others have observed that monocytes exhibit a higher liposome-affinity than neutrophils. This demonstrates that our approach resembles the *in vivo* situation.

In our study, monocytes showed highest uptake of liposomes around 200 nm. Although not directly comparable, the existence of optimal sizes for nanoparticle uptake have been reported before.[26] Neutrophils, in contrast, engaged increasing amounts of material with increasing liposome size, which agrees with data on isolated neutrophils,[5] but to our knowledge no studies have addressed this *in vivo*.

As expected, increasing the PEG-surface density reduced monocyte uptake (more pronounced with increasing size). However, in neutrophils the liposome uptake was not affected by PEG-surface densities up to 5 mol% PEG-DSPE, but it decreased as well at higher PEG-surface densities. Conversely, PEGylation of polymeric nanoparticles has been shown to increase uptake in isolated primary human neutrophils *ex vivo*.[8] However, in human primary cells, it was less effective in reducing uptake in granulocytes than in monocytes.[6] *In vivo* in mice, on the other hand, PEGylated liposomes were shown to associate to a lower extent with neutrophils than their non-PEGylated counterparts.[38] This limited literature suggests that the impact of PEG-surface density on neutrophil and monocyte nanoparticle uptake may differ, challenging the assumption that more PEG always reduces cellular uptake. Finally, we observed that neutrophils associated faster with the liposomes than monocytes, which was expected,[1] further indicating how our whole blood model recapitulates natural phagocyte behaviour. Although published data is scarce, taken together our observations in human whole blood differ from *in vitro* observations in isolated primary cells or cell lines, and are in close resemblance with *in vivo* observations in mice and patients.

Although highly successful in improving pharmacokinetic profiles, PEGylation of nanoparticles comes with immunogenic responses and antibody production *in vivo*.[42–44] Among the stealth-lipid alternatives for PEG-lipids, the sphingolipids sphingomyelin and ganglioside are established biocompatible substitutes.[45–48] Incorporating sphingomyelin or ganglioside into liposomes has increased their circulation half-live in pre-clinical models[49,50] and reduced immunogenic responses.[43,51] However, as is the case for PEG-lipids, very little is known about how these lipids affect the preference of differential immune cell subsets for particles containing these lipids.

Comparing 100 nm liposomes from Library A and B, trends in neutrophil and monocyte liposome uptake as a function of time and stealth lipid content were similar for liposomes with PEG-lipid or ganglioside. This is not unexpected considering ganglioside and PEG-lipids both increase liposome circulation times, in part, by reducing blood protein binding.[46,49,52] In contrast, sphingomyelin liposomes showed higher uptake than PEGylated ones, which decreased with increased sphingomyelin concentrations. Sphingomyelins' inhibitory effect on uptake was more pronounced in monocytes than in neutrophils, suggesting that sphingomyelin could be introduced in liposomes to increase their relative neutrophil affinity. These results suggest that even though sphingomyelin could be used to stabilize and increase the circulations times of liposomes,[45,50] it does not fully mimic the interaction of PEG with soluble molecules and cells.

We also assessed the effects of well-known nanoparticle ligands on cellular uptake. We used cRGD-decorated liposomes to evaluate if our approach could reproduce observations from mouse studies where cRGD-conjugated liposomes are more extensively taken up by circulating neutrophils than monocytes in mice, with which these agents hitchhike to cancerous[29] and inflammatory[28] lesions. While cRGD increased uptake in both monocytes and neutrophils in our study, monocytes engaged significantly more cRGD-liposomes than neutrophils. We also decorated liposomes with cFLFLF, a ligand for FPR1,

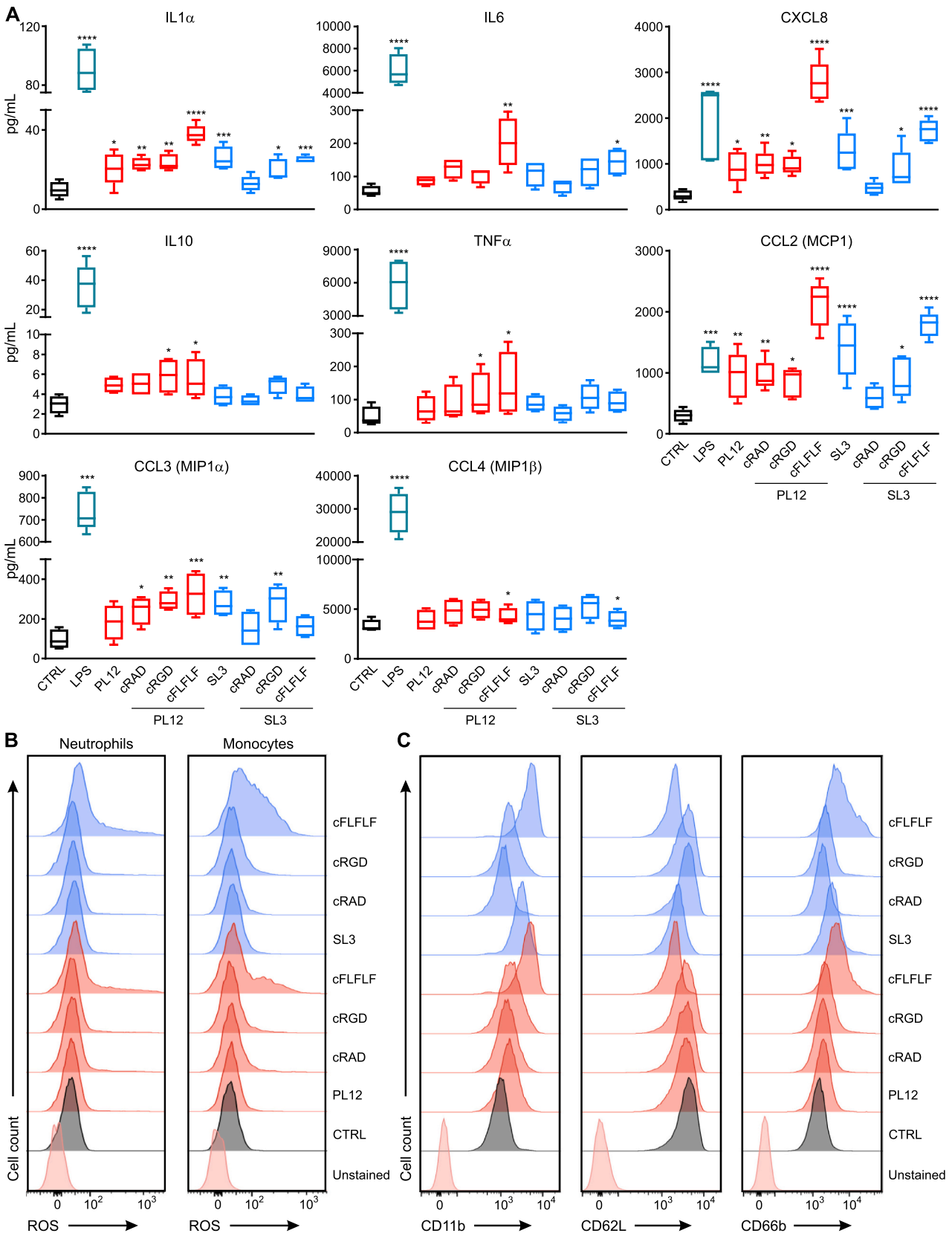

Fig. 4. Immune response induced by the presence of the liposomes.
A) Box plots of cytokine levels that significantly increased by the presence of liposomes (levels of 12 cytokines that were not affected are reported in Fig. S5). Whole blood without liposomes was used as control, while LPS-stimulated blood was used to estimate the cytokine levels' physiological relevance. Cytokine levels in the presence of liposomes or LPS were compared with the control. N = 5. B–C) Flow cytometry histograms showing ROS levels in neutrophils and monocytes (B) or CD11b, CD62L, and CD66b expression on neutrophils (C) after incubation of human blood with liposomes. Representative histograms of at least five independent experiments are shown.

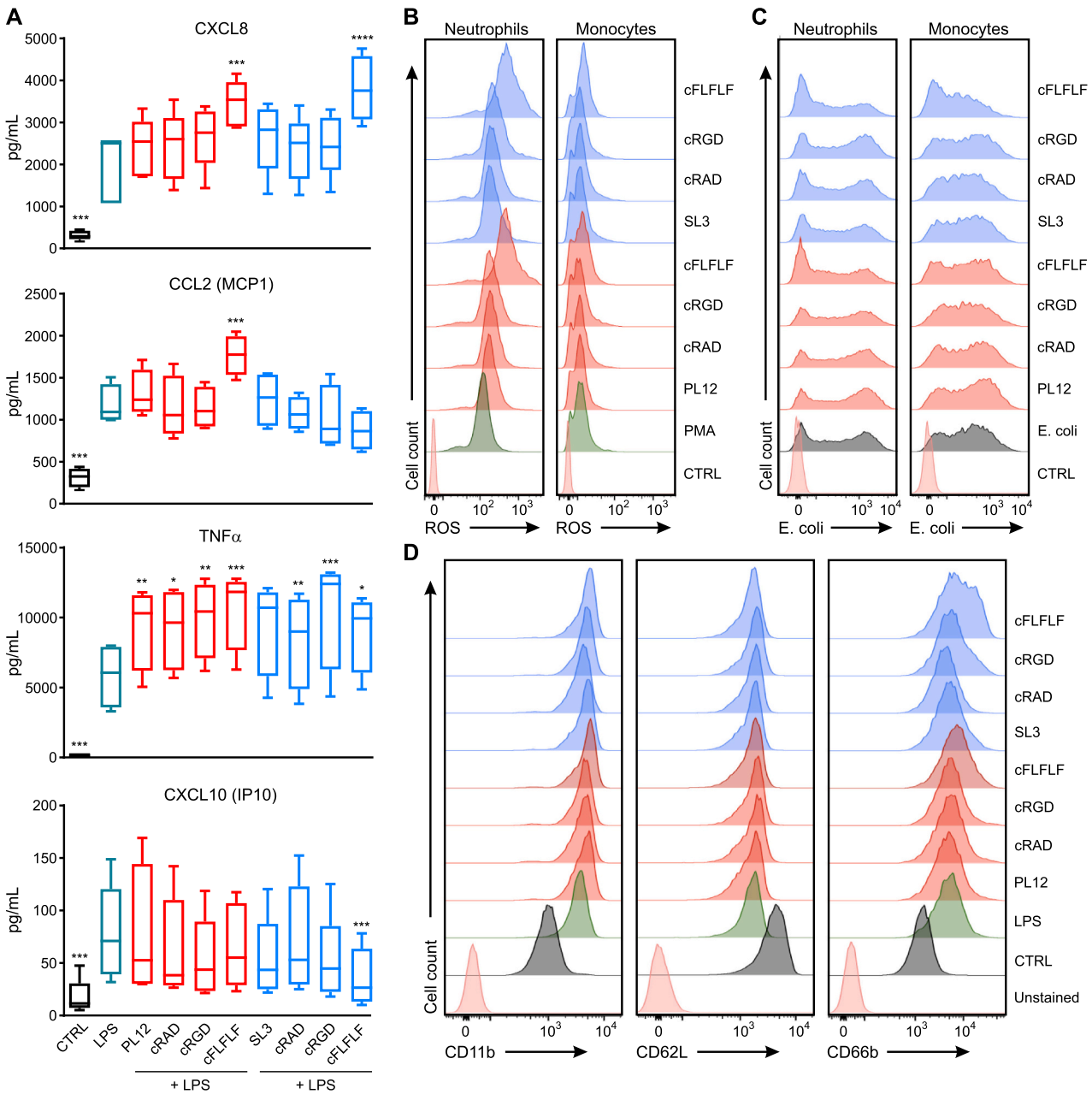

Fig. 5. Liposome presence affects the immune responses induced by classical agonists.
A) Box plot visualizing distribution of differential cytokine levels after incubation of human blood with liposomes, followed by LPS stimulation. Whole blood without liposomes, as well as LPS-stimulated whole blood, were used as control. Cytokine levels induced in all the conditions were compared with LPS alone. N = 5. B–C) Representative histograms showing ROS levels (B) or *E. coli* phagocytosis (C) in neutrophils and monocytes after incubation with liposomes followed by PMA stimulation (B) or incubation with *E. coli* beads (C). D) Representative histograms showing CD11b, CD62L, and CD66b expression on neutrophils after incubation of human blood with liposomes followed by LPS stimulation. Representative histograms of at least five independent experiments are shown.

expressed by both neutrophils and monocytes. In our study, cFLFLF-liposomes were taken up to higher extents by monocytes than neutrophils.[53,54] Direct comparisons of monocyte and neutrophil uptake of these ligands are limited, but reported targeting neutrophils using cFLFLF may be promising simply due to their abundance in circulation.[54] We observed that cFLFLF-liposomes, like cRGD-liposomes, were taken up to higher extents by monocytes than by neutrophils. Our whole blood model allowed us to assess the effects of targeting ligands on cellular uptake, but it did not fully replicate *in vivo* findings in mice. Although it is not known how these agents would behave *in vivo* in humans, one explanation for the observed discrepancies could be that the mice used in the studies mentioned were disease models (cancer/inflammation). This may affect the activation status and phagocytic activity of the cells as compared to the cells in blood from healthy human donors. Hence, an obvious improvement to the human whole blood assay will be the use of blood from patient donors.

It has been proposed that the interaction of nanomaterials with phagocytic cells could be used to regulate trained immunity by delivering drugs to myeloid cells.[55] The trained/primed phenotype enables innate immune cells to respond faster and stronger to secondary challenges.[55–57] This state of enhanced immune responsiveness leads to increased protection against infectious or tumoral challenges but could also exacerbate chronic inflammatory conditions.[56,58] Here we showed that the incubation of liposomes with whole blood induced cytokine

release and changes in biological responses but found no explicit pattern linking them to specific liposome features. Nevertheless, cFLFLF-decorated and sphingomyelin liposomes induced the most significant changes in biological responses. We observed that cFLFLF-decorated liposomes induce the release of several cytokines, along with a slight expression of activation markers on neutrophils, in basal conditions. However, upon LPS stimulation, higher levels of proinflammatory cytokines CXCL8, MCP1, and TNFa and low levels of IP10 (CXCL10) were released. Interestingly, these liposomes induced a higher basal expression of CD11b and CD66b and higher CD66b expression and ROS production upon neutrophil stimulation, a characteristic hallmark of primed/trained neutrophils.[59,60] This suggest that cFLFLF-decorated liposomes could increase neutrophil responses or induce a trained phenotype. In contrast, we did not observe the same effect on monocytes, indicating that cFLFLF-liposomes' trained/primed effect is cell and function-specific. Moreover, cFLFLF-liposomes reduced neutrophils and monocytes' capacity for phagocytosis, which could result from decreased presence of FPR1 receptors on the cell's surface after liposome uptake. More studies will be needed to evaluate the *in vivo* effects of cFLFLF-decorated liposomes on neutrophil function, inflammatory responses, and their differential effect on phagocytic cells.

To summarise, we observed that liposome size and PEGylation had significant differential effects on neutrophil and monocyte uptake. Hence, a thorough optimization of size and PEG-surface density seems an important aspect in the development of nanoparticles targeting differential innate immune cell subsets. Incorporation of sphingolipids also induced changes in cellular uptake, however these were not as pronounced as the effects of PEG-surface density and liposome size (see Table 1). The most significant effects on liposome uptake and immune cell function were observed for liposomes decorated with the ligands cRGD and cFLFLF. This shows that nanoparticle targeting towards $\alpha_v\beta_3$-integrin receptor or FPR1 may be a potentially powerful approach to tune nanoparticle uptake by and modify function of human monocytes and neutrophils.

In conclusion, our model reproduces various aspects of *in vivo* nanoparticle behaviour significantly better than simple *in vitro* experiments. We don't advocate for a replacement of *in vivo* experiments, but we do hypothesize that integration of human whole blood assays in nanomedicine development pipelines can potentially reduce the number of *in vivo* experiments needed and contribute to overcoming the translational gap from mouse to man.

CRediT authorship contribution statement

Miriam Giambelluca: Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing, Visualization. **Elena Markova:** Conceptualization, Investigation, Formal analysis, Writing – original draft, Visualization, Writing – review & editing. **Claire Louet:** Investigation. **Bjørg Steinkjer:** Investigation. **Rune Sundset:** Resources, Supervision, Funding acquisition. **Nataša Škalko-Basnet:** Conceptualization, Resources, Supervision, Funding acquisition. **Sjoerd Hak:** Conceptualization, Resources, Supervision, Funding acquisition, Writing – review & editing, Project administration.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2023.102712.

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