Lipid Nanocrystal Delivery of siRNA:

Dynamics of Uptake in Innate Immune Cells in Human Blood

and

Visualization of Small Oligonucleotide Delivery in Cell Culture

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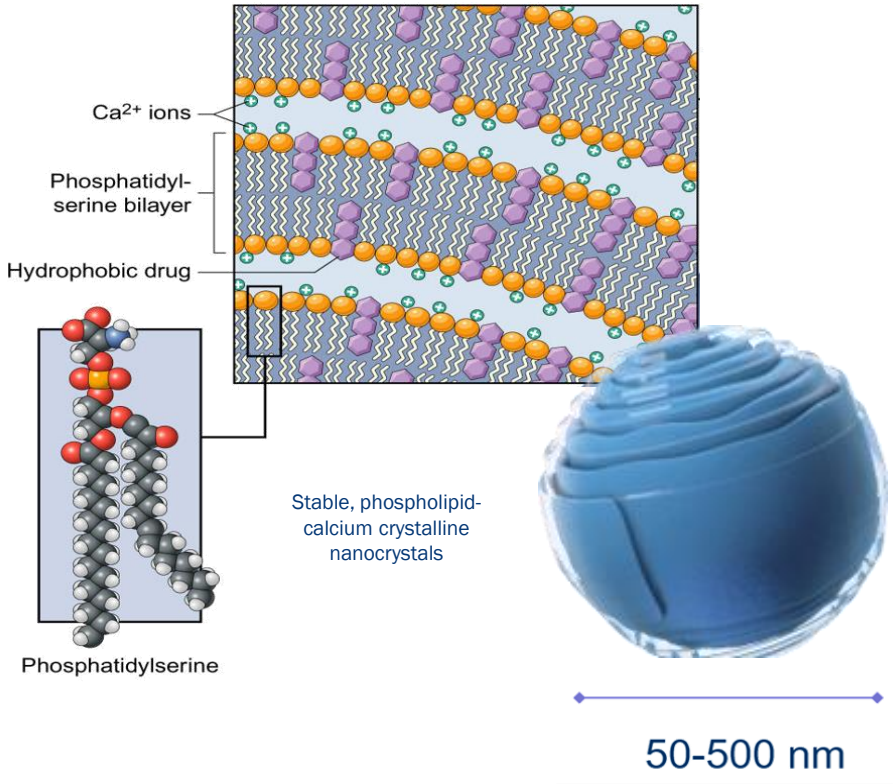
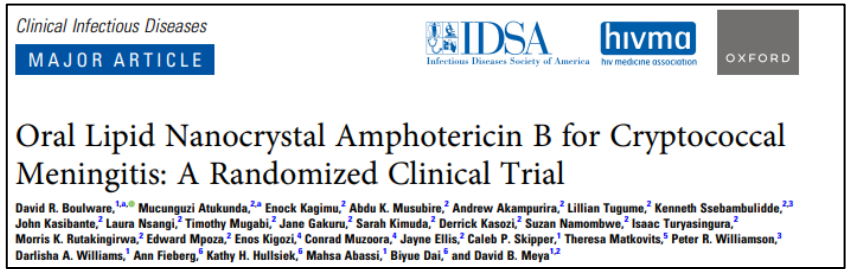
May 10, 2024
Baltimore, Maryland

Lipid Nanocrystals (LNCs) – Successful Oral Intracellular Delivery of Therapeutic Cargos



- Highly stable nanoparticles that self-assemble when phosphatidylserine-containing liposomes and Ca^{++} are combined
- **Structure** - anhydrous crystalline particles comprised of concentrically-wrapped lipid bilayers with embedded cargo
- Outside of cells, normal extra-cellular high Ca^{++} levels maintain the original crystalline LNC structure
- Inside cells, the much lower Ca^{++} concentrations alter the LNC structure, and they release their cargo
- PS has a key role in both targeting and intracellular delivery


Clinical Infectious Diseases

MAJOR ARTICLE

IDSA Infectious Diseases Society of America · hivma hiv medicine association · OXFORD

Oral Lipid Nanocrystal Amphotericin B for Cryptococcal Meningitis: A Randomized Clinical Trial

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Boulware DR *et al.* ***Clin Inf Disease*** 2023; 77 (12): 1659-67
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The most advanced clinical application of this platform is **MAT2203**, an investigational **oral** LNC formulation of Amphotericin-B

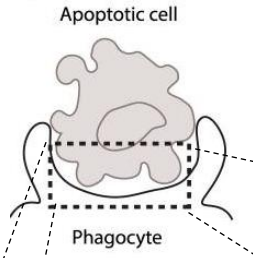
Key role of phosphatidylserine (PS) in cellular targeting and delivery

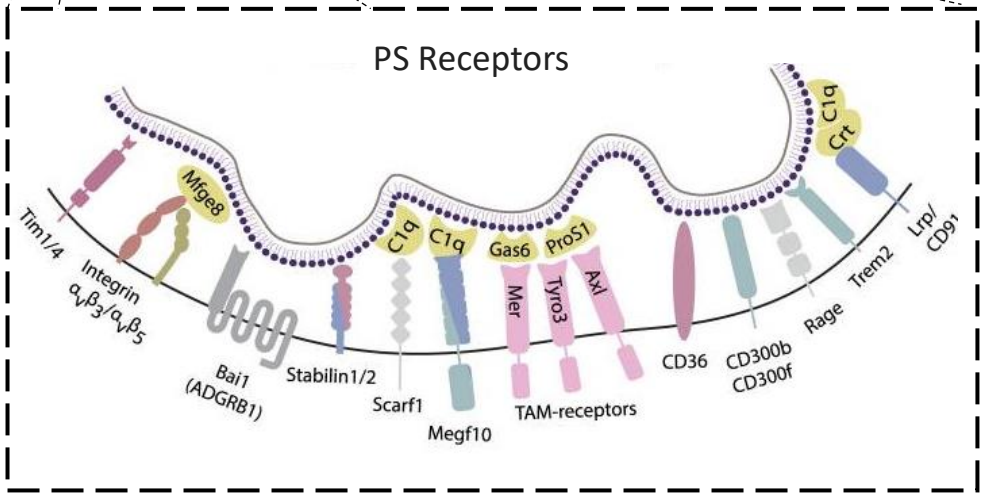
PS-Recognizing Cells

Endocytosis

Phagocytotic

Professional Phagocytes



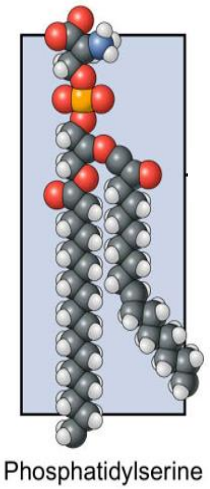


Non-Phagocytotic

Other Immune Cells
Other cells ?

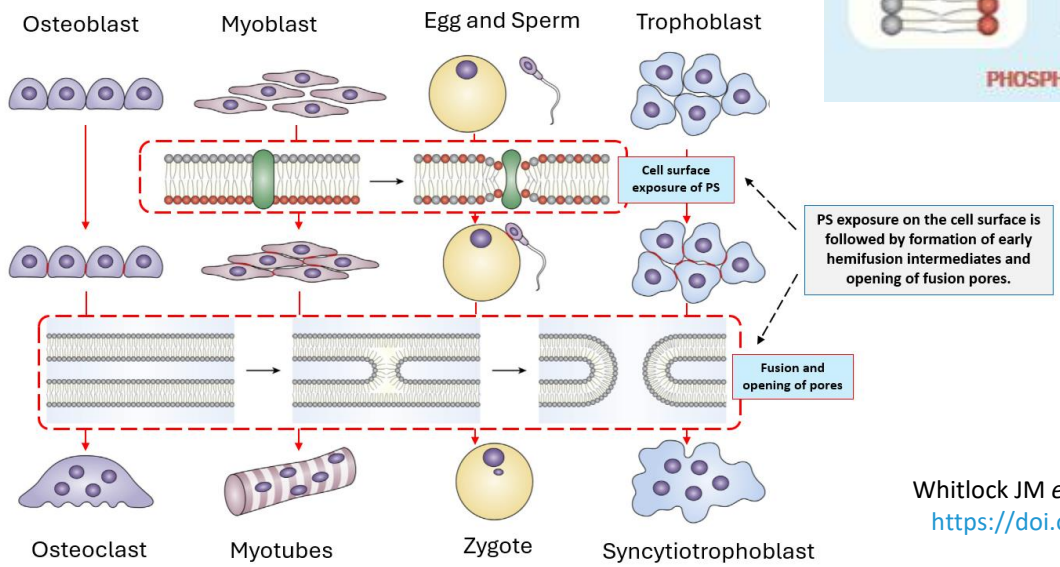
Dysregulation of TAM signaling has been linked to the pathogenesis of **autoimmune**, **inflammatory**, and **infectious** diseases.

TAM receptors have also been associated with **cancer** development and progression.



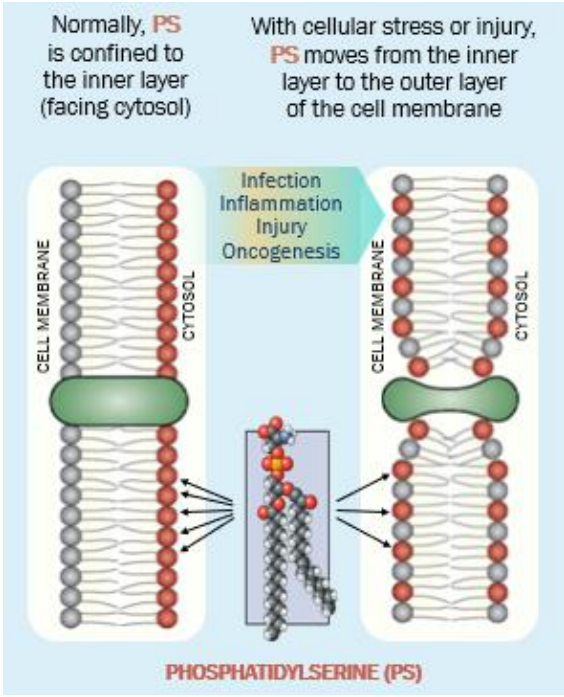
Membrane-to-membrane Fusion

(delivery directly to the cell interior)



PS-Expressing Cells

Stressed Cells Externalize PS



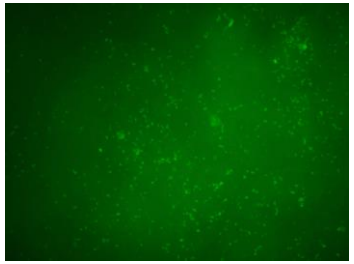
Whitlock JM *et al.* ***J Biol Chem*** 2021; 296: 1-16
https://doi.org/10.1016/j.jbc.2021.100411

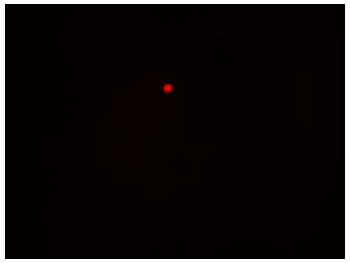
LNCs protect cargo and deliver it at low [Ca^{++}]

LNC FITC

siRNA Cy5

Before EDTA



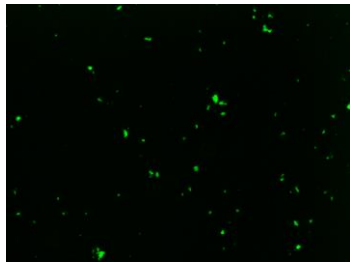


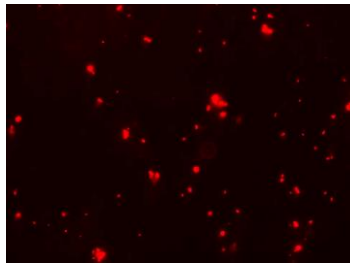
Cargo encapsulation and protection with physiologic Ca^{++} levels

EDTA

Virtually no Cy5 observed, confirming that cargo is encapsulated within unopened LNCs

After EDTA



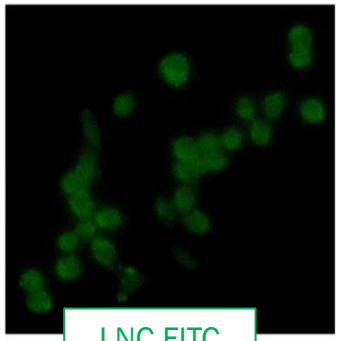


With EDTA-induced Ca++ reductions LNCs lose their crystalline structure and release their cargo

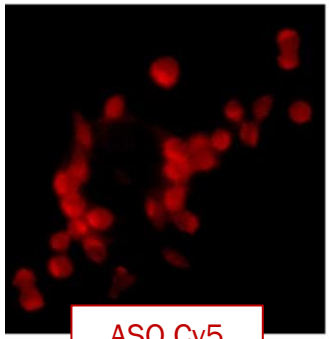
Appearance of significant amounts of Cy5 indicates that the LNCs have opened and released their siRNA cargo

In Vitro

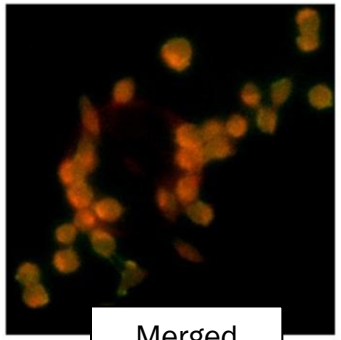
MCF7 Cells
1 hour incubation with FITC-labeled LNCs carrying a Cy5-labeled ASO cargo
(20 nM)



LNC FITC



ASO Cy5



Merged

Similarly, after uptake, in the much lower calcium environment of the cytosol LNCs release their cargo to the cell interior

Purpose and Approach

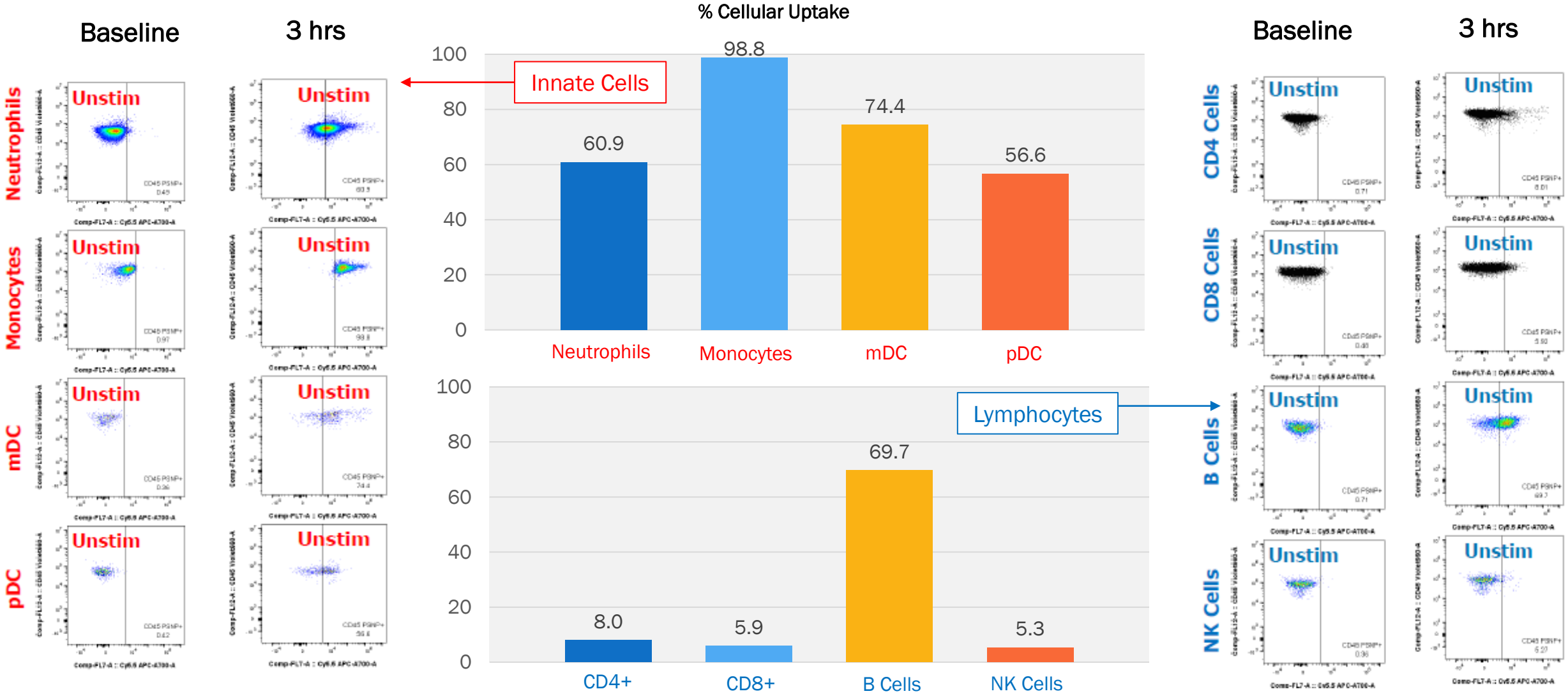
In the present work, we sought to extend our mechanistic understanding of LNCs and examine the uptake and delivery of LNCs with small oligonucleotide cargo in human blood and in different types of cells.

Two Primary Questions	Two series of experiments
What cells take up LNCs in blood ? →	Ex vivo uptake on LNCs in human blood with and without siRNA cargo (flow cytometry)
What are the mechanisms (fusion and/or endocytosis) and dynamics of cellular uptake in different cell types ? →	Live cell imaging with fluorescently-labeled siRNA-carrying and ASO-carrying LNCs **HEK** (siRNA) (low PS expressing somatic human embryonal kidney cells) **SKBR3** (ASO) (higher PS expressing $HER2^+$ human breast cancer cells)

Empty LNC Uptake

Human Whole Blood Flow Cytometry (3 hr incubation)

80 ug/mL Cy5-labeled LNC

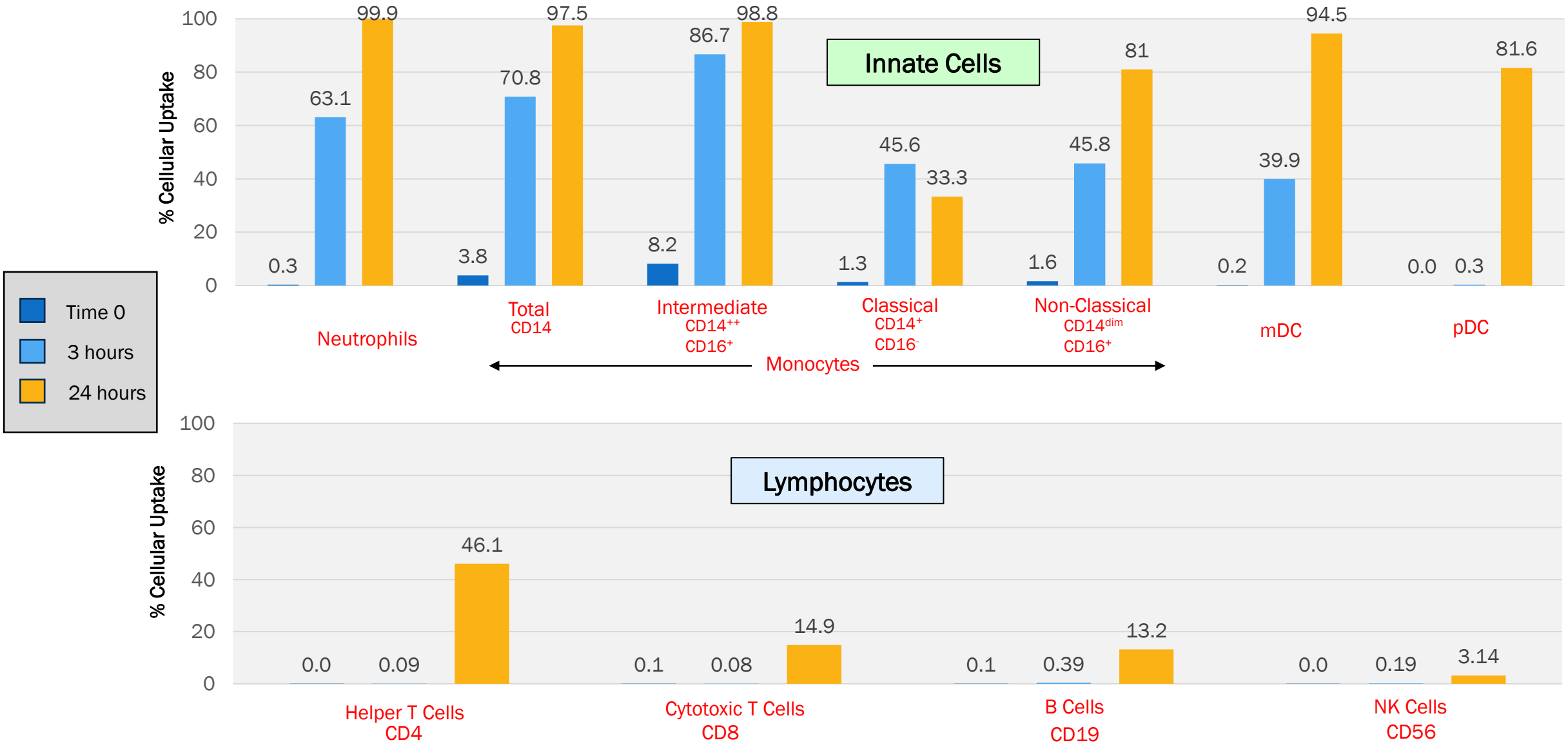


Stimulation with either phytohaemagglutinin (5 ug/mL) or Staphylococcal enterotoxin B (0.1 ug/mL) did not appreciably affect uptake.

LNC-formulated siRNA Delivery

Human Whole Blood Flow Cytometry (0, 3, 24 hr incubation)

80 ug/mL LNC (not labeled)
512 ng/mL Cy5-labeled siRNA cargo



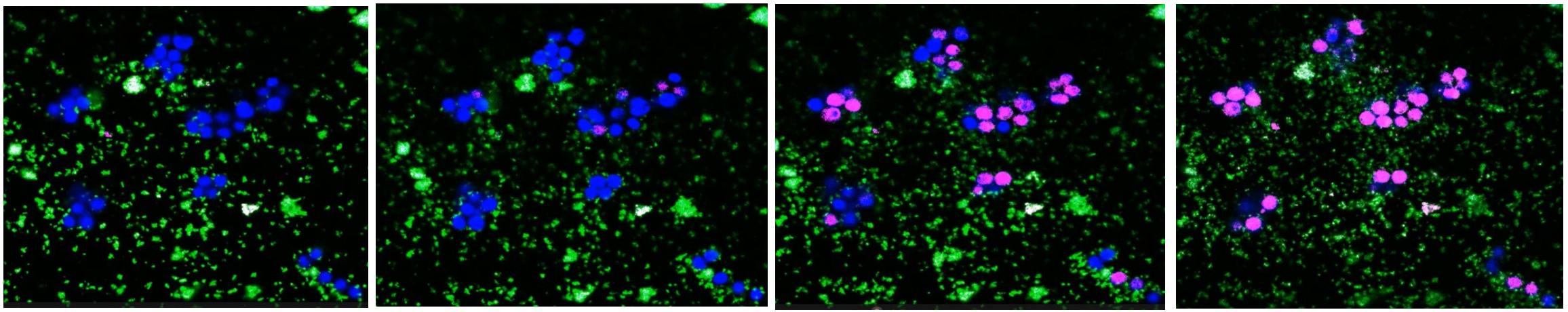
Key Observations - Ex vivo uptake of LNCs in human blood (Flow cytometry)

- Empty LNCs were avidly taken up by innate immune cells and B cells, with greater uptake at higher concentrations.
- There was no uptake in RBCs
- Dual-labeled siRNA cargo-carrying LNCs showed a generally similar uptake pattern
- siRNA cargo delivery was also noted relatively early in innate immune cells, though less notable in T cells, B cells and NK cells
- Stimulating healthy immune cells did not noticeably affect uptake or delivery.
- LNC delivery of siRNA cargo was more efficient than delivery of naked siRNA in innate immune cells

In vitro Dynamic Cell Imaging with Dual-labeled siRNA LNCs in HEK293 cells

LNCs surround **cells** → Initial siRNA release in some cells → Continued intracellular release of siRNA

Time 0 | 6 hr | 10 hr | 21 hr



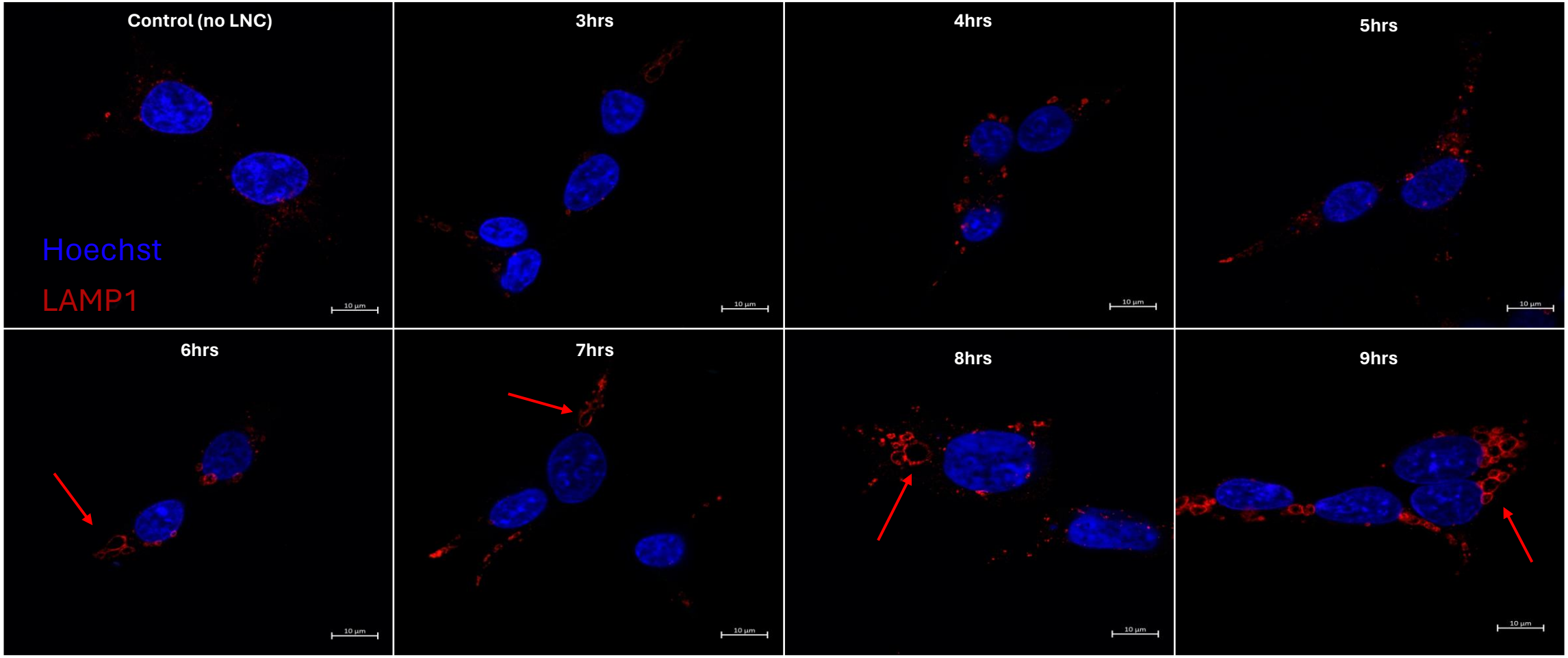
Live cell imaging corroborated LNC uptake and delivery of labeled siRNA cargo into the cytosol and provided visual confirmation that LNCs encapsulate and protect the siRNA cargo until intracellular release.

Green: LNC FITC
Pink: siRNA Cy5
Blue: nuclear Hoechst

80 ug/mL FITC-labeled LNCs
512 ng/mL Cy5-labeled siRNA cargo

LAMP1 (Membrane) Imaging in HEK293 Cells

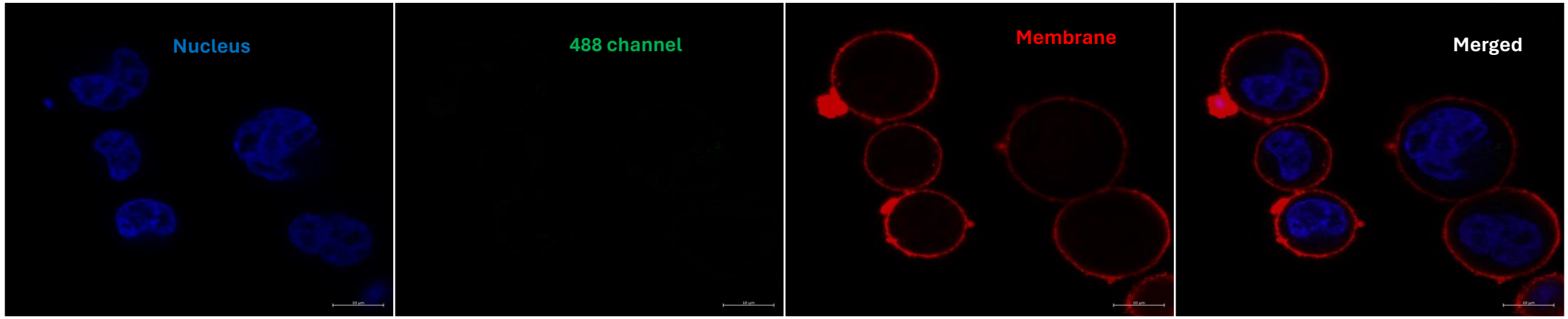
Unfortunately, the preparation protocols for higher-resolution simultaneous endosome imaging proved to be incompatible with the LNC/fluorophore formulation, and details of endosomal escape could not be ascertained.



However, LAMP1 imaging did show a prominent increase in endosome/lysosome formation following incubation with LNCs, peaking at 9 hours with large structures visible in all cells, strongly suggesting an endocytotic uptake mechanism.

LNC Uptake in SKBR3 Cells

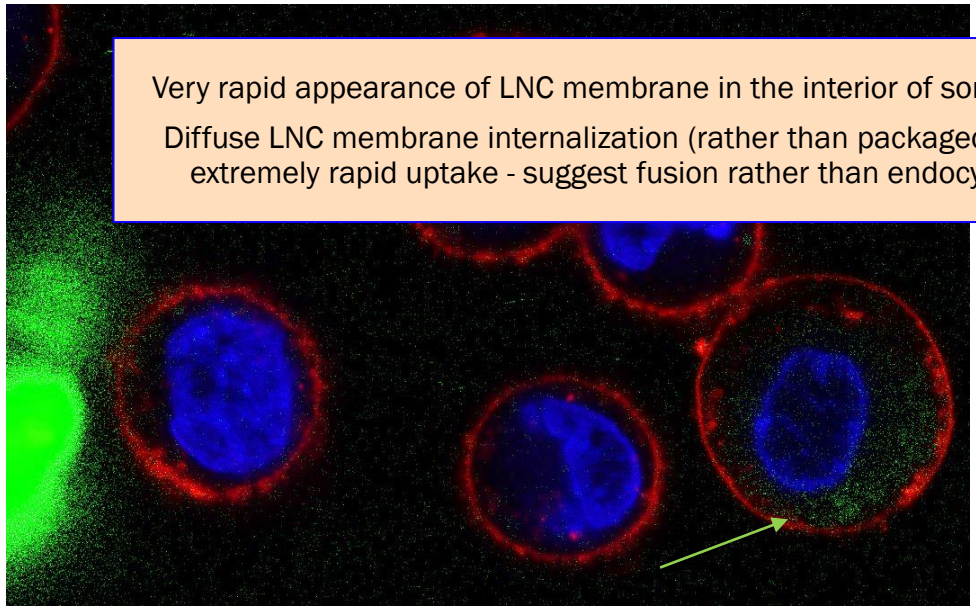
Membrane LAMP1-labeled cells (no LNCs)



+ FITC-labeled LNC (Cy5-labeled ASO cargo)

Rapid incorporation of LNCs into cell surface ruffles (yellow)

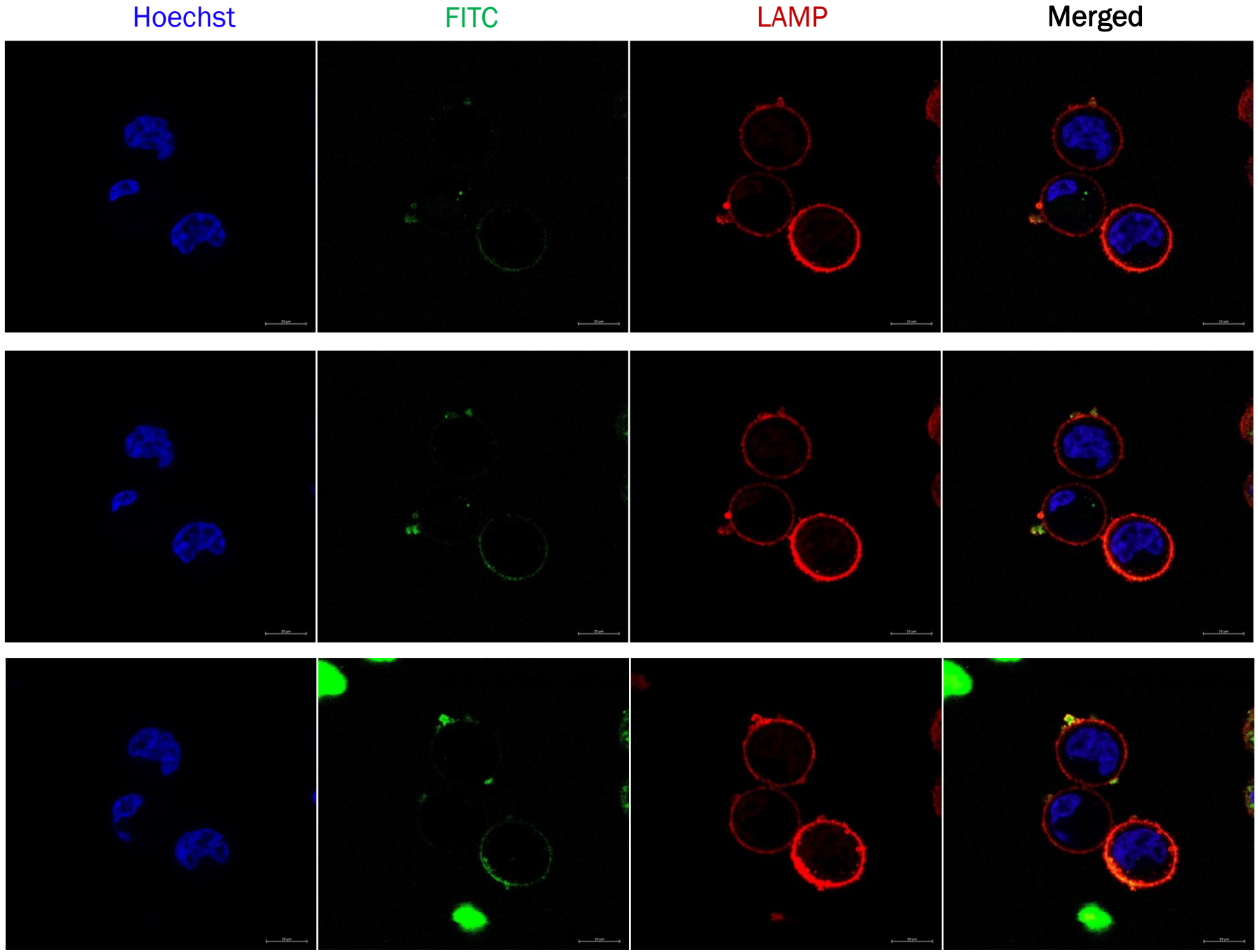
[within 10 minutes]



Very rapid appearance of LNC membrane in the interior of some cells

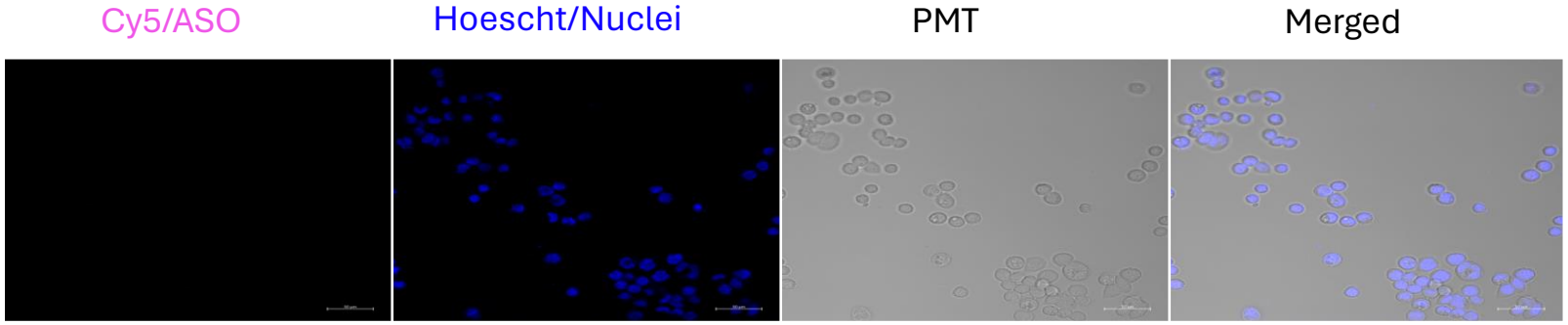
Diffuse LNC membrane internalization (rather than packaged) - and extremely rapid uptake - suggest fusion rather than endocytosis

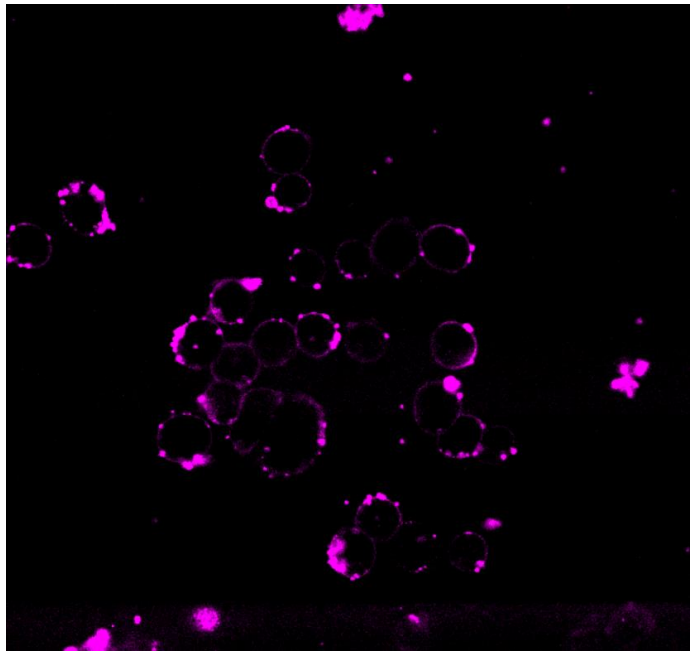
LNC Uptake in SKBR3 Cells



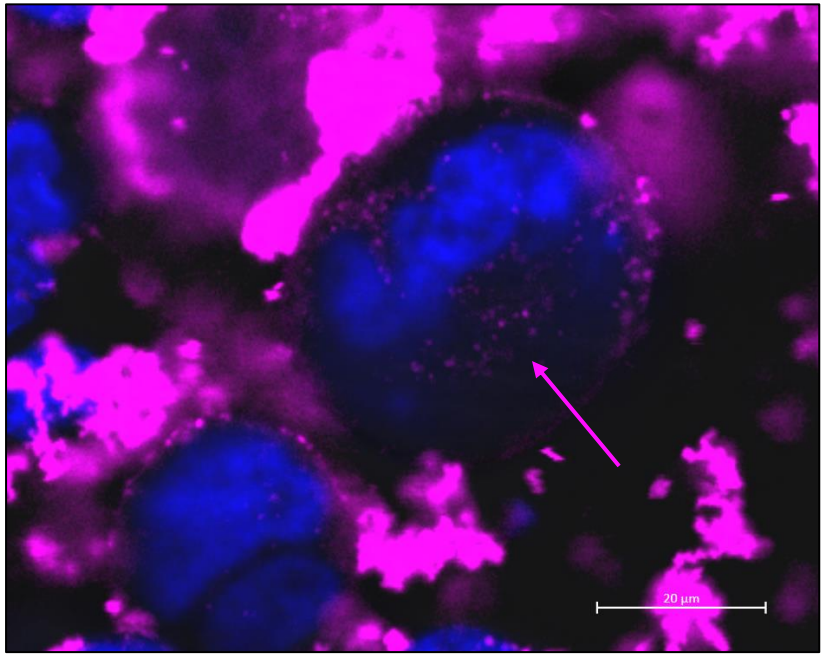
Rapid appearance of labelled LNCs into cell surface ruffles (yellow color in merged images)

Dynamic cell imaging of LNC siRNA delivery in SKBR3 Cells





By 12 minutes ASO cargo strongly evident in membrane

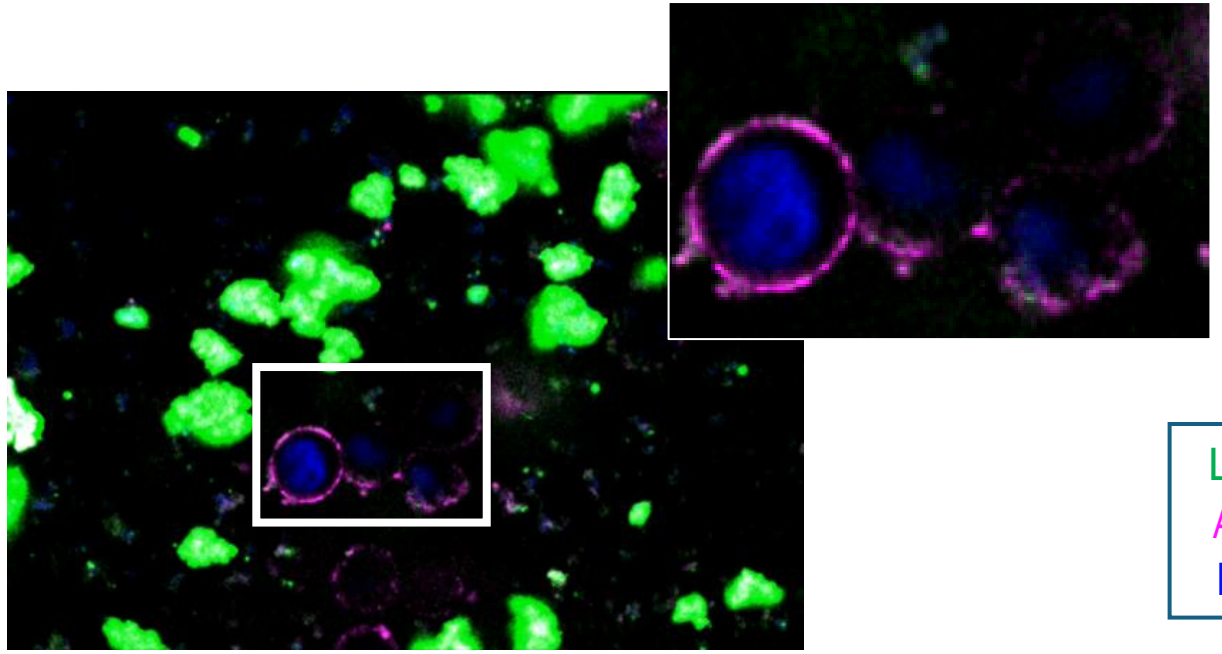


At 145 minutes, on magnified view, ASO cargo clearly visible inside cell

Concerns that over-saturation might be interfering with images

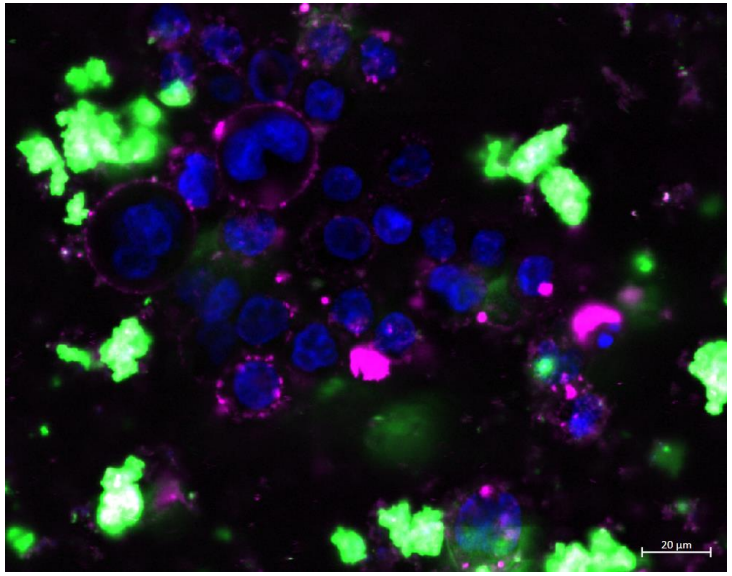
Reduced LNC concentration by ½
Dropped Cy5 Gain

45 minutes

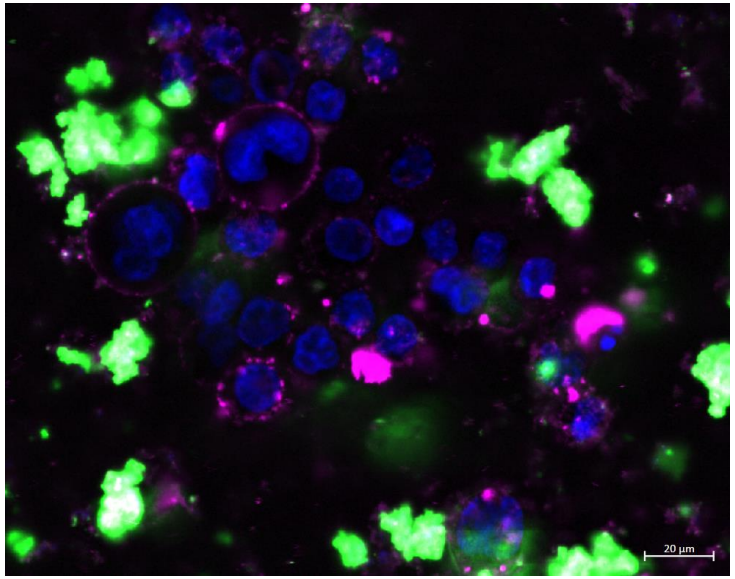


LNC FITC
ASO Cy5
Hoechst

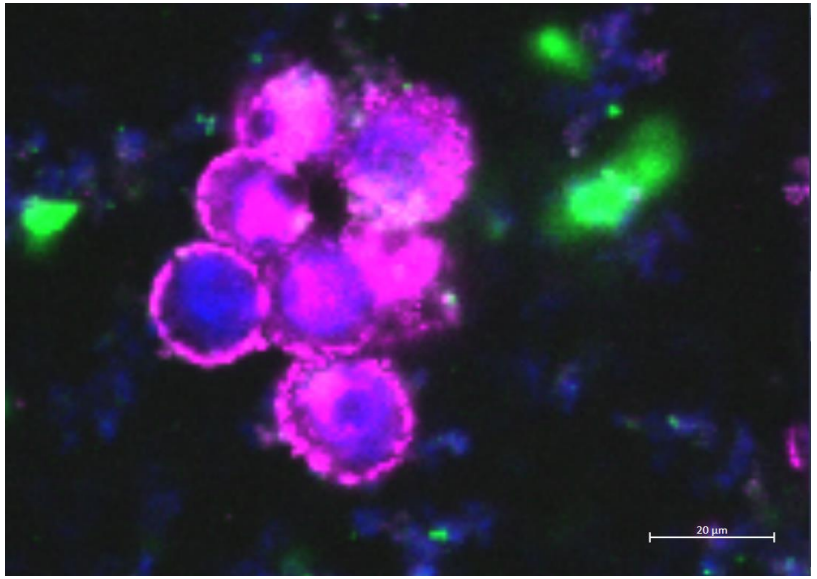
80 min



130 min



190 min



Key Observations – In vitro dynamic fluorescent cell imaging

- In HEK293 cells, LNC uptake is accompanied by prominent formation of endosome-like structures at approximately 6 hours, peaking at around 9 hours, with delivery of siRNA cargo occurring somewhat later (10 hours plus).
- This pattern is strongly suggestive of endocytotic uptake
- In SKBR3 cells, by contrast, LNC uptake and ASO delivery occurs much more rapidly, with almost immediate uptake and very early appearance of cargo in the cell membrane ruffles, followed relatively shortly thereafter by the appearance of cargo in the cytosol, with delivery complete by about 3 hours.
- The diffuse appearance of cytosolic labeled ASOs and the very rapid time course of uptake and delivery is more suggestive of a cell membrane fusion mechanism

Conclusions

As these data indicate, the LNC platform holds considerable promise for the intracellular delivery of complex therapeutics, including both single-stranded ASOs and double-stranded siRNA, particularly in inflammation and oncology.

In addition to prior clinical success with oral administration of Amphotericin-B in HIV patients with cryptococcal meningitis, LNCs can also be used to deliver small oligonucleotides to a variety of different cell types.

Uptake and delivery proceed differently in different cell lines:

- Ex vivo LNCs are avidly taken up by **innate immune cells,** particularly professional phagocytes, in blood; siRNA cargo delivery follows shortly thereafter.

  LNC siRNA delivery to immune cells is more efficient than uptake of naked small oligos.

- In somatic **HEK293 cells**, uptake and siRNA cargo delivery appears to primarily proceed via endocytotic mechanisms, and occur, sequentially, over a period of hours.
- In **SKBR3 tumor cells**, uptake is much more rapid, and ASO cargo delivery appears to be more related to cellular membrane fusion.

Further work is planned to clarify additional details of delivery mechanism(s) and endosomal escape in immune, somatic, and tumor cells.

Additional in vivo data on oral delivery of small oligonucleotides presented today

1709

Successful *in-vivo* oral delivery of biologically active and therapeutic anti-inflammatory mRNA-targeted oligonucleotides with a lipid nanocrystal delivery platform

Hui Liu, Vinod Ramgolam, Jeffrey Bender, Mariam Mikhael, Amra Tabakovic, Tzong-Jen Sheu, Partha Samadder, Thomas Hoover, James Ferguson

MATINAS BIOPHARMA

Abstract

Background:

There has been little progress in the oral delivery of nucleic acid therapeutics beyond the liver. Matinas BioPharma's lipid nanocrystal (LNC) platform has successfully delivered oral amphotericin-B in patients with cryptococcal meningitis; other ex-vivo work has shown avid LNC uptake by innate immune cells. Prior studies have shown the *in vitro* efficacy of two mRNA-targeted oligos – one knocking down *IL-17A*, the other knocking down *TNFα*. LNC formulations of both have shown greater cytokine knock-down than "naked" oligos *in vitro*. The present work evaluated the *in vivo* efficacy of oral LNC formulations of these oligos in two different inflammatory disease models.

Methods:

Psoriasis in BALB/C mice was induced with 31.25 mg of 5% Imiquimod (IMQ) applied daily for 6 days. There were 5 treatment groups (n=10 per group): untreated controls, IMQ alone, IMQ plus one of two different LNC-oligo formulations administered daily by oral gavage, and IMQ plus anti-IL17A antibodies. Skin erythema and scaling was scored daily. The study was terminated at day 7; cytokine mRNA levels in the psoriatic skin lesions were determined by qRT-PCR.

Colitis in C57BL/6 mice was induced with 3.5% DSS in drinking water for 5 days. There were 6 treatment groups: untreated controls, DSS alone, DSS plus one of two different LNC-oligo formulations, DSS plus LNC-formulated scrambled oligos, and DSS plus a TNFα neutralizing antibody. Daily disease activity scores were measured; animals were sacrificed at day 14 and serum TNFα and tissue (colon) *TNFα* mRNA (qRT-PCR) were measured.

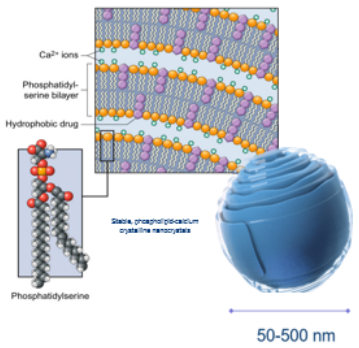
Results:

In the imiquimod psoriasis model (**Figure 1**) daily oral administration of an LNC formulation of the *IL-17A*-targeted oligos resulted in both knock-down of skin IL-17A mRNA and significant improvement in clinical parameters of redness and scaling.

Similarly, in the DSS colitis model (**Figure 2**), daily oral administration of an oral LNC formulation of the *TNFα*-targeted oligo resulted in reductions of colon *TNFα* mRNA and significant reductions in serum TNFα levels, as well as significant improvements in disease activity scores. Thus, we have shown successful oral delivery of two RNAi oligos targeting different inflammatory cytokines in two different disease models, with documented biological/molecular activity as well as therapeutic efficacy.

Conclusions:

The LNC delivery platform can successfully orally deliver biologically active (and potentially therapeutic) oligonucleotides targeting key cytokines in inflammatory disease models. While these initial results are promising, there is still some individual heterogeneity of response; future work will be focused on optimizing the LNC formulations to improve delivery efficiency, increase their potency, and extend the application of oral cytokine-targeting oligo therapeutics to other inflammatory disease models.



Delivery of small molecules and small oligonucleotides

- Successful oral delivery of therapeutics in infectious disease, inflammation, and oncology

Extra-hepatic targeting

- Selective delivery to targeted tissues facilitated by phosphatidylserine
- Validated Blood-Brain-Barrier penetration with MAT2203 in cryptococcal meningitis

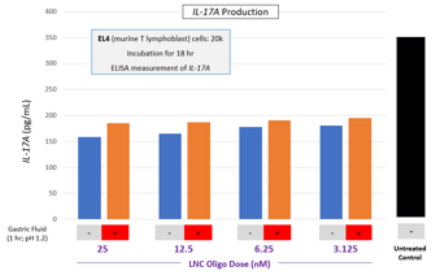
Oral delivery

- Unique structure protects cargo in GI tract
- Particle size obviates first-pass hepatic metabolism

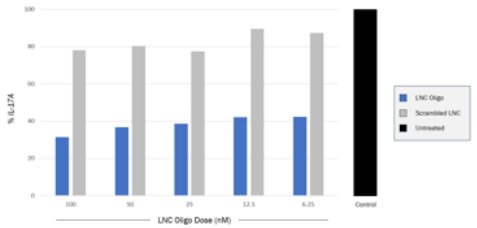
Safe & stable

- Deliver high-target tissue concentrations of drug with low plasma levels and greatly reduced uptake in non-target tissues
- No evidence of immunogenicity or cytotoxicity

LNC oligo formulations maintained full cytokine knockdown capabilities *in vitro* even after gastric fluid exposure

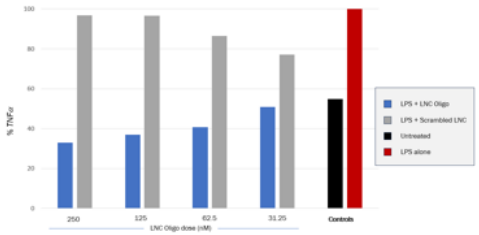


IL-17A knock-down in vitro



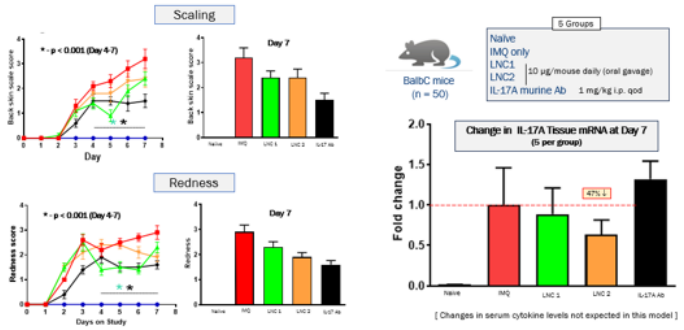
Substantial knock-down (up to 70%) of *IL-17A* with active LNCs
Comparable results in stimulated (IL-2) and unstimulated cells

TNFα* knock-down *in vitro



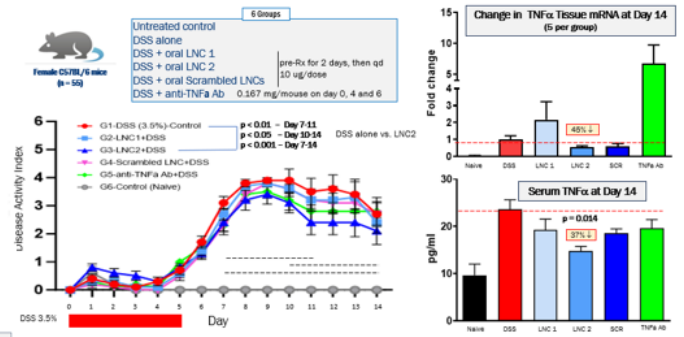
Substantial knock-down (up to 65%) of *TNFα* with active LNCs
No additional TNFα produced after LPS stimulation

Figure 1 Effect of Oral LNC *IL-17A* RNAi in a Murine Imiquimod (IMQ) Psoriasis Model



[Changes in serum cytokine levels not expected in this model]

Figure 2 Effect of Oral LNC-TNFα RNAi in a Murine DSS Acute Colitis Model



A lipid nanocrystal formulation was used to orally deliver small mRNA-targeted oligonucleotides in two different animal inflammatory disease models; each of the two oligos tested showed both biological activity and potential therapeutic effects.

Small therapeutic anti-inflammatory oligonucleotides can be orally delivered outside the liver.

Thank You

James J. Ferguson, MD

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