

Nanoformulation-Based Approaches to Deliver Biologicals to Dendritic Cells and Liver Non- Parenchymal Cells

Dissertation

zur Erlangung des Grades

„*Doktor rerum naturalium*“

(Dr. rer. nat.)

am Fachbereich Chemie

der Johannes-Gutenberg-Universität Mainz

vorgelegt von

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Geboren am [REDACTED]

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Mainz, April 2025

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Mainz, April 2025

(Unterschrift)

Acknowledgements

An dieser Stelle möchte ich allen danken, die mich die letzten (fast) 4 Jahre während meiner Zeit als Doktorandin begleitet und unterstützt haben.

Zuallererst möchte ich mich bei meinem Betreuer und Erstgutachter [REDACTED] dafür bedanken, dass er mich als Doktorandin aufgenommen hat und mir damit die Möglichkeit gegeben hat, neue und spannende methodische und theoretische Kompetenzen zu erlangen. Dabei bin ich besonders dankbar für die exzellente Betreuung, tolle Kommunikation und langjährige intensive Unterstützung während meiner Doktorarbeit. Außerdem möchte ich mich bei [REDACTED] bedanken, dass ich meine Arbeit in seiner Arbeitsgruppe anfertigen durfte, sowie für den großen wissenschaftlichen Input für meine Projekte. Auch an [REDACTED] möchte ich meinen herzlichen Dank aussprechen. Zum einen für die gute Zusammenarbeit an Projekten im SFB1066 und die Unterstützung seit meinem Studium an der JGU, außerdem auch für die Übernahme des Zweitgutachtens meiner Arbeit, wie auch bei meiner Masterarbeit.

Des Weiteren möchte ich meinen aktuellen und ehemaligen Arbeitskolleg*innen aus der AG Grabbe/Bros danken, für die tatkräftige Unterstützung und Motivation während meiner Zeit im Labor. Speziell möchte ich noch einmal [REDACTED] und [REDACTED] hervorheben, danke euch für die großartige Zusammenarbeit in jeglicher Hinsicht, sowie die ganzen unterhaltsamen und schönen Momente die wir zusammen verbringen durften. Auch die schwierigsten und anstrengenden Herausforderungen haben wir zusammen durchgestanden. Ich kann mich sehr glücklich schätzen, solche tollen Arbeitskollegen und Freunde wie euch zu haben.

Bedanken möchte ich mich auch bei [REDACTED], der mich bei jedem Hoch und Tief unterstützt hat, sowie meinen Freund*innen, vor allem für die mentale Unterstützung während meiner Zeit als Doktorandin. Dabei bedanke ich mich besonders bei [REDACTED], [REDACTED] und [REDACTED], die mich von Anfang bis zum Ende begleitet haben und immer für mich da waren. Genauso bedanken möchte ich mich bei [REDACTED], dafür, dass wir uns seit unserem Studium über alles austauschen konnten und uns gegenseitig bei unseren Doktorarbeiten unterstützt haben.

Zuletzt danke ich auch herzlich meinen Eltern und meinen Geschwistern, die mir immer mit einem offenen Ohr zur Seite standen, mein ganzes Leben lang für mich da waren und mich ermutigt haben, immer weiter zu machen und meinen Weg zu gehen.

Publications Included in This Thesis

This dissertation was conducted between July 2021 and April 2025 at the Department of Dermatology, University Medical Center of the Johannes Gutenberg University Mainz, Germany, in the lab of Prof. Dr. Stephan Grabbe and PD Dr. Matthias Bros.

The five publications listed below are the foundations for this dissertation, and their scientific relevance and key findings are outlined in the following sections.

- (1) Medina-Montano C, Cacicedo ML, Svensson M, Limeres MJ, **Zeyn Y**, Chaves- Giraldo JE, Rohrig N, Grabbe S, Gehring S, Bros M. Enrichment Methods for Murine Liver Non-Parenchymal Cells Differentially Affect Their Immunophenotype and Responsiveness towards Stimulation. *Int J Mol Sci*. 2022 Jun 11;23(12):6543. doi: 10.3390/ijms23126543.
- (2) Svensson M, Limeres MJ, **Zeyn Y**, Gambaro RC, Islan GA, Berti IR, Fraude-El Ghazi S, Pretsch L, Hilbert K, Schneider P, Kaps L, Bros M, Gehring S, Cacicedo ML. mRNA-LNP vaccine strategies: Effects of adjuvants on non-parenchymal liver cells and tolerance. *Mol Ther Methods Clin Dev*. 2025 Feb 4;33(1):101427. doi: 10.1016/j.omtm.2025.101427.
- (3) Haase F, Pöhmerer J, Yazdi M, Grau M, **Zeyn Y**, Wilk U, Burghardt T, Höhn M, Hieber C, Bros M, Wagner E, Berger S. Lipoamino bundle LNPs for efficient mRNA transfection of dendritic cells and macrophages show high spleen selectivity. *Eur J Pharm Biopharm*. 2024 Jan;194:95-109. doi: 10.1016/j.ejpb.2023.11.025. Epub 2023 Dec 6.
- (4) **Zeyn Y**, Hobernik D, Wilk U, Pöhmerer J, Hieber C, Medina-Montano C, Röhrig N, Strähle CF, Thoma-Kress AK, Wagner E, Bros M, Berger S. Transcriptional Targeting of Dendritic Cells Using an Optimized Human *Fascin1* Gene Promoter. *Int J Mol Sci*. 2023 Nov 29;24(23):16938. doi: 10.3390/ijms242316938.
- (5) Lantzberg B#, **Zeyn Y**#, Forster R#, Jian L, Schauenburg D, Hieber C, Nuhn L, Zhou T, Silva MJSA, Koynov K, Jiang HL, Kuan SL*, Bros M*, Opatz T*, Weil T*. Glycogen-inspired trimannosylated serum albumin nanocarriers for targeted delivery of toll-like receptor 7/8 agonists to immune cells and liver. *Journal of Controlled Release*. 2025. doi: 10.1016/j.jconrel.2025.113705.

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Abbreviations

APC	antigen presenting cell
BMDC	bone marrow-derived dendritic cell
CART	charge-altering releasable transporter
cDC	conventional dendritic cell
cGAMP	cyclic GMP-AMP
CCR	C-C chemokine receptor
CLEC9A	C-type lectin domain family 9 member
CLR	C-type lectin receptors
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
CXCL	C-X-C motif chemokine ligand
DAMP	damage-associated molecular patterns
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DC-STAMP	dendritic cell-specific transmembrane protein
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
FLT3L	Fms-related tyrosine kinase 3 ligand
FoxP3	forkhead-box-protein P3
Fscn1	Fascin1
hbPG	hyperbranched polyglycerol
HPMA	N-(2-hydroxypropyl)methacrylamide
HSA	human serum albumin
HSC	hepatic stellate cell
i.v.	intravenous
IL	interleukin
INF	interferon
IRF	interferon regulatory factor
KC	kupffer cell
KLF	Krüppel-like factor
LAF	lipo amino fatty acid

LAF-Stp	lipoamino xenopeptide carrier
LD	liver dissociation
LNP	lipid nanoparticle
LP	liver perfusion
LPEI	linear PEI
LPP	lipopolyplex
LSEC	liver sinusoidal endothelial cell
MDC	muramyl dipeptide
MHC	major histocompatibility complex
MMR	macrophage mannose receptor
moDC	monocyte-derived DC
MR	mannose receptor
mRNA	messenger RNA
MyD88	myeloid differentiation primary response 88
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	natural killer cell
NPC	non-parenchymal cells
ODN	oligodeoxynucleotide
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
pDC	plasmacytoid dendritic cell
PD-L1	programmed death-ligand 1
pDNA	plasmid DNA
PEG	polyethyleneglycol
PEI	polyethyleneimine
PolyI:C	polyinosinic-polycytidylic acid
PRR	pattern recognition receptor
R848	resiquimod
RNA	ribonucleic acid
Siglec-H	sialic acid binding Ig-like lectin H
SIRP α	signal regulatory protein α
SOD1	superoxide dismutase 1
ssRNA	single-stranded RNA

STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes
Stp	succinoyl tetraethylene pentamine
succPEI	succinylated branched PEI
TCR	T cell receptor
TGF- β	transforming growth factor β
Th	T helper
TLR	Toll-like receptor
TM	trimannose
TNF- α	tumor necrosis factor α
TNFR1	tumor necrosis factor receptor 1
Treg	regulatory T cell
XCR1	chemokine XC receptor 1

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Graphical Abstract

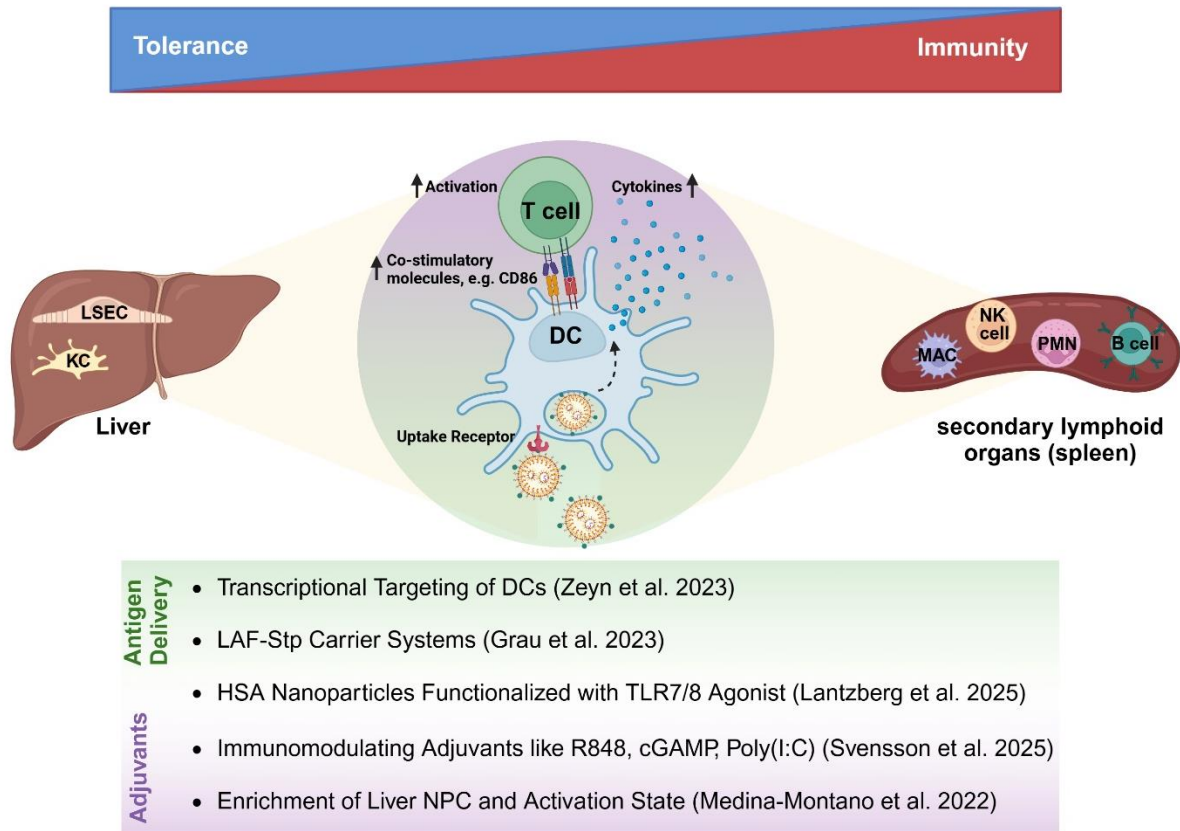


Figure 1. Graphical representation of main topics addressed in this dissertation, listed from antigen delivery to adjuvants. This work addresses strategies to overcome liver-induced immune tolerance mediated by non-parenchymal cells (NPCs), such as Kupffer cells (KCs), dendritic cells (DCs), and liver sinusoidal endothelial cells (LSECs). Vaccines targeting DCs in secondary lymphoid organs often accumulate unintentionally in the liver due to the expression of numerous distinct pattern recognition receptors (PRRs) on liver NPCs, which may consequently lead to antigen-specific tolerance rather than systemic anti-tumor immunity. To counteract the overall protolerogenic function of hepatic immune cells, several interconnected strategies were tested in this thesis. I) First, the protocol to isolate liver NPC was optimized, and responsiveness of thereby isolated cells towards adjuvants (e.g., R848, cGAMP, Poly(I:C)) in soluble form (Medina-Montano et al. 2022) II) and when loaded into mRNA-delivering lipid nanoparticles (LNPs) (Svensson et al. 2025) was studied. III) Trimannose-functionalized human serum albumin (TM-HSA) nanoparticles coupled with TLR7/8 ligands to target DC-SIGN and homologue receptors were generated in order to actively address hepatic immune cells as a prerequisite to reprogram these (Lantzberg et al. 2025). IV) To improve mRNA delivery, lipoamino xenopeptides (LAF-Stp) were formulated into LNPs (Grau et al. 2023). V) Lastly, an optimized DC-specific *Fascin1* promoter was created to drive selective transgene expression in pan-DCs, aiming to prevent unwanted transgene expression in non-DCs. All of these approaches are intended to activate DC in secondary lymphoid organs and to reprogram by default tolerance-promoting hepatic immune cells in order to achieve sustained adaptive immune responses directed against co-delivered antigens, as required, e.g., in tumor therapy.

Abstract

Liver non-parenchymal cells (NPCs), including Kupffer cells (KCs), dendritic cells (DCs), liver sinusoidal endothelial cells (LSECs), and hepatic stellate cells (HSCs), play a dual role in immunity. On the one hand, they exert tolerogenic activity under steady state conditions by default, while on the other hand, they are able to initiate adaptive immune responses. This may limit the efficacy of cancer immunotherapy, especially of vaccines targeting DCs in secondary lymphoid organs. Liver NPCs express numerous distinct pattern recognition receptors (PRRs), which are critical for pathogen detection, but also trap nanovaccines, that may be recognized by the immune systems as pathogens according to their size and non-self-surface properties. If a nanovaccine delivers antigen, this may risk antigen-specific tolerance. The active engagement of liver NPCs with antigen and adjuvant may dampen systemic anti-tumor T cell responses, highlighting the need for strategies that simultaneously activate DCs in secondary lymphoid organs and reprogram liver NPCs towards an immunostimulatory phenotype. The approaches taken in this thesis are visually summarized in the graphical abstract. First, methods for isolating liver NPCs, liver perfusion and liver dissociation, were established. These methods ensured reliability of the results regarding the activation states and responsiveness of liver NPCs to various well-defined molecular immunostimulatory adjuvants, such as Poly(I:C), R848, and cGAMP, which are frequently used in nanovaccine formulations. The effects of these adjuvants were analyzed *in vitro* as well as *in vivo*, simultaneously applied with mRNA lipid nanoparticles (LNPs), to analyze their effects on mRNA expression and immune cell activation. Furthermore, advanced nanocarrier systems were investigated to improve the delivery of antigens and adjuvants. This included the assessment of LNPs incorporating lipoamino xeno peptide carrier structures (LAF-Stp). The LAF-Stp units have a unique lipid architecture that is able to destabilize endosomal membranes and promote endosomal escape, thereby improving mRNA delivery and intracellular release. To refine immune cell targeting, trimannose- and TLR7/8 ligand-functionalized human serum albumin (TM-HSA) nanoparticles were designed, which target the DC-SIGN receptor and its homologues (e.g. L-SIGN), on DCs and other liver NPCs. These nanoparticles demonstrated strong immune activation due to TLR7/8 agonist engagement, effectively stimulating cytokine production and enhancing antigen presentation of immune cells. *In vivo* studies showed efficient liver targeting, rapid clearance from the bloodstream, and robust immune activation. Further, to achieve transcriptional targeting, an optimized *fascin1* gene-derived enhancer/promoter was used to drive DC-focused expression of DNA vaccines. Together, these efforts provide a foundation for the development for future immunotherapies that address the unique immunological environment of the liver while enhancing anti-tumor responses in secondary lymphoid organs.

Zusammenfassung

Nicht-parenchymale Zellen (NPCs) der Leber, darunter Kupffer-Zellen (KCs), dendritische Zellen (DCs), lebersinusoidale Endothelzellen (LSECs) und hepatische Stellat-Zellen (HSCs), spielen eine duale Rolle in der Immunantwort. Einerseits induzieren sie unter homöostatischen Bedingungen Toleranz, andererseits können sie adaptive Immunreaktionen initiieren. Dies könnte die Effektivität von Krebsimmuntherapien, z.B. DC-adressierenden Vakzinen, erheblich einschränken. Leber NPCs exprimieren zahlreiche *Pattern-Recognition*-Rezeptoren, die für die Erkennung von Pathogenen essenziell sind. Allerdings können diese Rezeptoren auch Nanovakzine abfangen, die vom Immunsystem aufgrund ihrer Größe und fremder Oberflächeneigenschaften als Krankheitserreger erkannt werden können. Trägt ein Nanovakzin ein Antigen, kann dies die antigenspezifische Toleranz induzieren. Die aktive Interaktion der Leber NPCs mit Antigen und Adjuvans kann die systemische Antitumor-T-Zell-Antwort dämpfen, weshalb Strategien erforderlich sind, die sowohl DCs in sekundären lymphatischen Organen aktivieren als auch Leber NPCs zu einem immunstimulierenden Phänotyp reprogrammieren. Die in dieser Arbeit verfolgten Ansätze sind im *Graphical Abstract* visuell zusammengefasst. Zu Beginn wurden Methoden zur Isolierung von Leber-NPCs entwickelt, darunter Leberperfusion und Leberdissoziation. Die erfolgreiche Isolierung von reaktiven und lebensfähigen Leber NPCs ermöglichte es detaillierte Analysen zur Charakterisierung des Aktivierungsstatus von NPCs nach Stimulation mit immunmodulatorischen Adjuvantien wie Poly(I:C), R848 und cGAMP durchzuführen, die häufig in Nanovakzin-Formulierungen eingesetzt werden. Die immunstimulierende Wirkung dieser Adjuvantien wurde auch *in vivo* evaluiert, indem sie gemeinsam mit mRNA-Lipidnanopartikeln (LNPs) appliziert wurden, um ihre Effekte auf die mRNA-Expression und die Aktivierung von Immunzellen zu analysieren. Darüber hinaus wurden weitere innovative Nanopartikelsysteme zum verbesserten Transport von Antigenen und Adjuvantien untersucht. Dazu gehörte die Entwicklung von LNPs, die Lipoamino-Xenopeptid-Trägerstrukturen (LAF-Stp) enthalten. Diese Einheiten weisen eine einzigartige Lipidarchitektur auf, die endosomale Membranen destabilisiert, den endosomalen Escape fördert und somit die intrazelluläre Freisetzung der mRNA optimiert. Zur weiteren Präzisierung des Immunzell-Targetings wurden mit Trimannose und TLR7/8-Ligand funktionalisierte Nanopartikel aus humanem Serumalbumin (TM-HSA) entwickelt, die den DC-SIGN-Rezeptor und seine Homologe (z. B. L-SIGN) auf DCs und anderen Leber NPCs adressieren. Diese Nanopartikel induzierten wegen des TLR7/8-Liganden eine starke Immunaktivierung was zu einer effektiven Zytokinproduktion und einer verbesserten Antigenpräsentation von Immunzellen führte. *In vivo*-Studien zeigten ein effizientes Lebertargeting, eine schnelle Clearance aus dem Blutkreislauf sowie eine ausgeprägte Immunaktivierung. Des Weiteren wurde für

transkriptionelles Targeting ein optimierter, vom *Fascin1*-Gen abgeleiteter Enhancer/Promoter verwendet, um eine DC-fokussierte Expression von DNA-Vakzinen zu erlangen. Die hier beschriebenen Forschungsansätze bilden eine vielversprechende Grundlage für zukünftige Immuntherapien, die sowohl das einzigartige immunologische Umfeld der Leber berücksichtigen als auch die Antitumor-Immunität in sekundären lymphatischen Organen verstärken können.

1. Composition and Functions of the Immune System

The immune system is a defense network that protects the organism from pathogenic threats while maintaining tolerance to self-components. The ability to distinguish between self and pathogen-derived antigens is central to immune function (Chi, Pepper et al. 2024). Specialized cells and molecules detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), resulting in targeted immune responses to eliminate threats (Li and Wu 2021). The immune system can be broadly divided into two main components that interact closely (Marshall, Warrington et al. 2018):

- Innate immunity provides rapid, nonspecific defense via phagocytes, complement proteins, and inflammatory cytokines.
- Adaptive immunity confers antigen-specific responses through T- and B-lymphocytes, generating long-lasting memory.

The immune system maintains a delicate balance between initiating protective responses against pathogens and retaining tolerance to self-antigens and harmless environmental agents. This balance is particularly apparent in organs like the liver and spleen, which play a key role in immune regulation (Jenne and Kubes 2013). Under homeostatic conditions, the liver's unique immunological milieu actively contributes to the induction and maintenance of tolerance (Horst, Neumann et al. 2016). This tolerogenic environment is created by a complex network of interactions between various immune cells in the liver. The liver's specialized microenvironment consists mainly of liver non-parenchymal cells (NPCs), including Kupffer cells (liver-resident macrophages, KCs, approximately 20% of liver NPC), dendritic cells (DCs, 25%) and liver sinusoidal endothelial cells (LSECs, 50%) which induce tolerance by default (Tiegs and Lohse 2010, Medina-Montano, Cacicedo et al. 2022). Additionally, a small subset of liver NPCs consists of innate immune cells, including natural killer (NK) cells and NKT cells (Racanelli and Rehermann 2006), as well as biliary epithelial cells and hepatic stellate cells (HSCs) (Pellicoro, Ramachandran et al. 2014). HSCs typically remain inactive under steady state conditions. However, when activated, HSCs play a key role in the development of liver diseases (Luo, Li et al. 2021). In response to liver injury, they get activated and promote liver fibrosis by producing extracellular matrix factors (Medina-Montano, Cacicedo et al. 2022). In addition, HSC are able to induce regulatory T cells (Tregs) through release of retinoic acid (Dunham, Thapa et al. 2013, Wu, Qian et al. 2023).

KCs phagocytose gut-derived pathogens and antigens from portal blood and contribute, together with LSECs, to immune regulation by producing immunomodulatory signals such as IL-10 (interleukin-10), TGF- β (transforming growth factor β), and retinoic acid (Fig. 2) (Cacicedo, Medina-Montano et al. 2020). These factors promote the development of Treg, establishing a tolerogenic milieu.

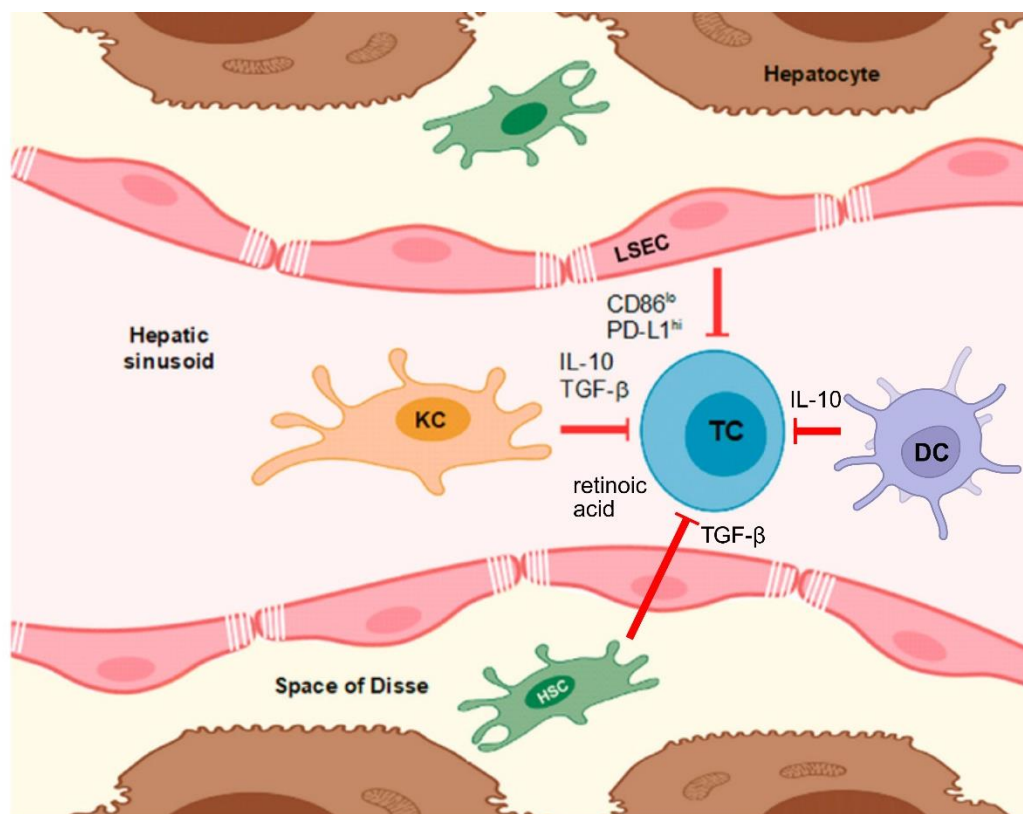


Figure 2. Liver NPCs induce T cell tolerance by presenting antigens without activation signals, expressing low CD86 but high PD-L1, and releasing anti-inflammatory cytokines like IL-10, TGF- β and retinoic acid (modified, Cacicedo, Medina-Montano et al. 2020).

LSECs, which line the hepatic sinusoids, are identified by the presence of fenestrae. These fenestrae structures are tiny, pore-like openings that allow efficient transport of substances like lipoproteins from hepatocytes directly into the sinusoidal bloodstream (Szafranska, Kruse et al. 2021). In addition to their structural role, LSECs play an important role in immune regulation. They express a variety of receptors, including endocytic scavenger receptors, C-type lectin receptors (CLR) (Bhandari, Larsen et al. 2021), and the Fc γ receptor IIb (CD32) (Ganesan, Kim et al. 2012), which are all involved in the uptake of pathogens and immune complexes and help maintain immune balance by promoting the activity of Tregs (Wohlleber and Knolle 2016, Medina-Montano, Cacicedo et al. 2022).

Liver DCs are also known to play a key role in promoting immune tolerance. They present antigens to T cells in a tolerogenic manner and promote T cell suppression and Treg differentiation (Dou, Ono et al. 2018). This tolerance is critical for preventing immune

reactions to harmless dietary antigens and gut microbes (Carambia and Herkel 2018). The high exposure of the liver to gut-derived antigens via the portal vein necessitates a balance between immune activation and suppression to avoid chronic inflammation (Wang, Tang et al. 2021). Understanding the liver's tolerogenic properties under homeostatic conditions is crucial for the development of new therapeutic approaches for liver diseases, cancer, transplantation, and autoimmune disorders.

In contrast, the spleen functions as a key lymphoid organ for systemic immunity. Its structure is divided into red pulp, which filters blood to remove pathogens and damaged cells, and white pulp, which organizes lymphocytes to initiate adaptive immune responses (Alexandre, Schienstock et al. 2022). There are several types of immune cells present in the spleen. DCs and macrophages detect and capture pathogens, process their antigens, and present them to T and B cells to initiate adaptive immune responses (Gaudino and Kumar 2019). These antigen presenting cell (APC) types also release cytokines, e.g. IL-6, TNF- α (tumor necrosis factor α) and IL-12, that regulate inflammation and immune cell activation (Xu, Cheng et al. 2022, Chen, Saeed et al. 2023). B cells in the spleen produce antibodies to neutralize pathogens and label them for subsequent recognition by the complement system and phagocytes, respectively, ultimately resulting in their destruction (Lewis, Williams et al. 2019). Some activated B cells rapidly generate IgM antibodies for immediate defense, while others undergo maturation processes to enhance antibody specificity and long-term immunity. Memory B cells remain in secondary lymphoid organs (e.g., spleen, lymph nodes) and bone marrow, but are also strategically distributed in non-lymphoid tissues (e.g., mucosal barriers) to enable rapid localized responses during reinfection (Allie and Randall 2020, Syeda, Hong et al. 2024). T cells play a crucial role in coordinating immune reactions. T helper cells (Th) activate B cells and other immune cells, while cytotoxic T cells eliminate virus-infected and malignant cells (Sun, Su et al. 2023). Tregs help prevent excessive immune activation, maintaining immune balance and preventing autoimmune and allergic reactions (Deo, Shrijana et al. 2024). NK cells provide a rapid response against infected and malignant cells (Chen, Zhu et al. 2024). Additionally, circulating monocytes in the bloodstream act as precursors that infiltrate tissues during infection or injury, where they differentiate into macrophages or DCs to enhance immune responses (Villar, Ouaknin et al. 2023). The spleen's unique organization ensures efficient pathogen detection, antibody production, and immune memory formation, which makes it essential for protection against infections.

All liver NPCs express a range of pattern recognition receptors (PRRs), including those found on DCs in secondary lymphoid organs, which are often targeted by DC-addressing nanovaccines (e.g., CD206, CD209) (Cacicedo, Medina-Montano et al. 2020). The amount

of diverse PRRs on liver NPCs may contribute to the unintended accumulation of i.v. (intravenous) injected nanovaccines in the liver. While these vaccines can trigger adaptive immune responses in lymph nodes and the spleen by targeting DCs, they may also promote antigen-specific tolerance through interaction with liver NPCs, which potentially restricts anti-tumor T cell responses (Svensson, Limeres et al. 2025). As DCs are found both in the liver and in secondary lymphatic organs and are able to induce immune responses by adequate use of adjuvants and nanoparticles, they are an important target for immunotherapy.

2. Dendritic Cells

DCs are specialized APCs that are essential in bridging the innate and adaptive immune systems. These cells, which originate from hematopoietic stem cells in the bone marrow, are essential for detecting pathogens, processing antigens, and subsequently activating T cells to initiate specific immune responses (Zanna, Yasmin et al. 2021). DCs exist in two functional states: mature and immature. A key distinction between them is that only mature dendritic cells can activate antigen-specific naïve T cells in secondary lymphoid organs (Zanna, Yasmin et al. 2021). Their maturation is triggered by disruptions in tissue homeostasis, which are detected through the recognition of PAMPs or DAMPs (Li and Wu 2021). Toll-like receptors (TLR), which are key PRRs, detect these specific molecular patterns from pathogens and damaged cells. Their activation triggers signaling cascades that initiate inflammatory responses and thereby link innate to adaptive immunity (Li and Wu 2021). PAMP or DAMP recognition initiates metabolic, cellular, and genetic changes that enable DCs to migrate from peripheral tissues to T cell-rich regions in secondary lymphoid organs. There, they present antigens and initiate immune responses. DCs consist of distinct subsets with specialized functions, including conventional DC1 (cDC1), conventional DC2 (cDC2), and plasmacytoid DC (pDC) (Fig 3). Furthermore, there are also tissue-specific and inflammation-dependent populations playing specialized roles in immune regulation and tolerance. For instance, Langerhans cells are resident in the epidermis, where they capture environmental antigens and migrate to lymph nodes to prime T cells (Wilcox, Taheri et al. 2024). Dermal DCs, including CD103⁺ and CD11b⁺ subsets, are found in the skin dermis, where they tailor immune responses to local pathogen exposure (Feng, Zhou et al. 2021). In addition, monocyte-derived DCs (moDCs) are a subset generated in response to inflammatory stimuli and are present in tissues and lymph nodes. Blood monocytes are recruited to inflamed skin via CCR2 (C-C chemokine receptor type 2), where they differentiate into moDCs, inducing localized Th1 responses at the site of infection (Liu, Zhang et al. 2021).

In general, cDC1 are a DC subpopulation specialized for cross-presentation and express distinct receptors like CLEC9A (C-type lectin domain family 9 member) and XCR1 (chemokine XC receptor 1) (Macri, Jenika et al. 2023). They are defined by the transcription factor interferon regulatory factor 8 (IRF8), which is essential for their development and function (Lança, Ungerback et al. 2022). In tissues like liver and spleen, cDC1 play critical roles in activating CD8⁺ cytotoxic T lymphocytes (CTL). In this process, exogenous antigens, such as those derived from tumors or viruses, are presented on major histocompatibility complex (MHC) class I molecules to activate CTLs. cDC1s prime T cells

in lymph nodes by increasing the expression of costimulatory molecules (e.g., CD80/86, CD40) and releasing cytokines like IL-12, which promotes Th1 differentiation of CD4⁺ T cells (Bödder, Zahan et al. 2021). The resulting Th1 response boosts CTL activity by stimulating IFN- γ (interferon- γ) production and thereby supporting cytotoxic T cell expansion (Jorgovanovic, Song et al. 2020). In addition, cDC1s produce chemokines such as CXCL9 (C-X-C motif chemokine ligand 9) and CXCL10 to attract CTLs to sites of infection or tumors (Abdul-Rahman, Ghosh et al. 2024).

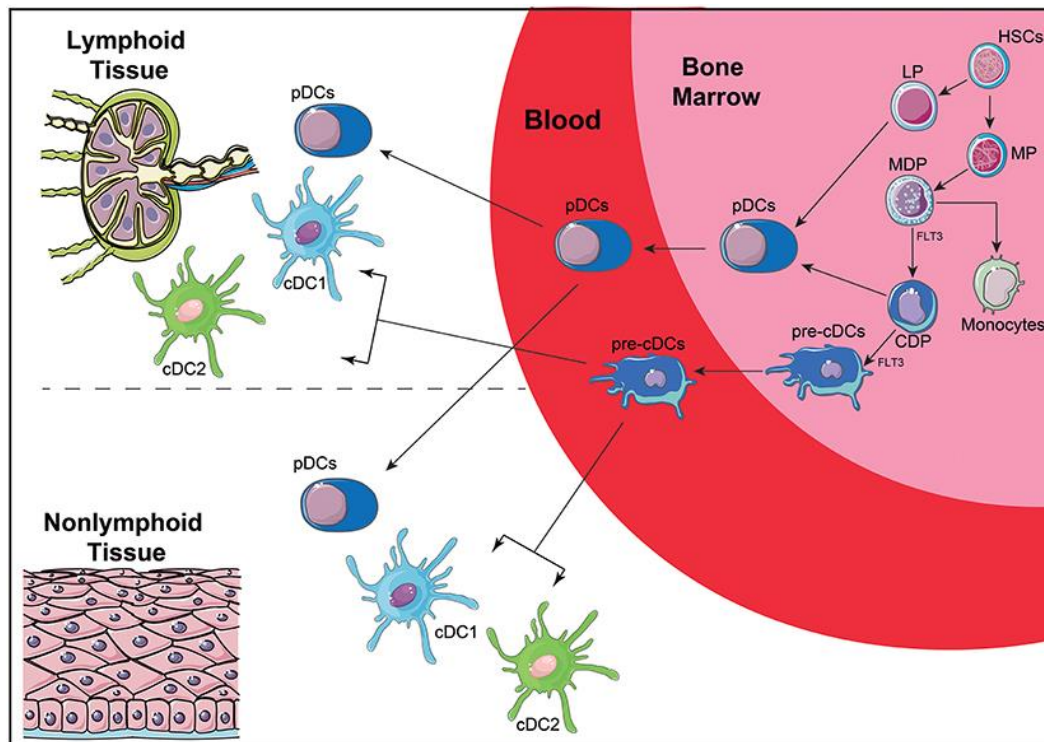


Figure 3. Dendritic cell maturation and differentiation into pDC, cDC1 and cDC2. DCs originate from hematopoietic stem cells (HSCs), which give rise to myeloid and lymphoid progenitors (MP and LP). Myeloid progenitors differentiate into MDP (macrophage-DC precursors), which further differentiate into CDP (common DC precursors) and monocytes. CDPs give rise to pre-cDC or pDC. Lymphoid progenitors (LP) can also differentiate into pDCs. Pre-cDCs then differentiate into the two main types of conventional DCs: cDC1 and cDC2. Both pDCs and cDCs can migrate from the blood to lymphoid and non-lymphoid tissues (Patente, Pinho et al. 2019).

cDC2, known for their primary role in activating CD4⁺ cells, express CD11b and SIRP α (signal regulatory protein α) (Szulc-Dabrowska, Biernacka et al. 2024). Their development is driven by transcription factors such as IRF4 and KLF4 (Krüppel-like factor 4) (Minutti, Piot et al. 2024). cDC2 are adept at activating CD4⁺ T cells by presenting antigens on MHC class II molecules, thereby inducing Th2 and Th17 responses, which are critical for fighting e.g. parasites (Amon, Hatscher et al. 2020). In addition, cDC2s play regulatory roles, particularly in tissues like the intestine and liver, where they promote the differentiation of

Tregs to maintain tolerance and prevent autoimmunity (Hasegawa and Matsumoto 2018). These cells also express diverse PRRs to detect extracellular pathogens.

pDCs are unique in their capacity for rapid and massive production of type I IFN (IFN- α/β) in response to viral infections and express markers like Siglec-H (sialic acid binding Ig-like lectin H) and B220 (Ye, Gaugler et al. 2020). Unlike conventional DCs, pDCs have limited antigen-presenting capacity but are highly effective in detecting viral nucleic acids for rapid antiviral defense. In addition to their antiviral role, pDCs are able to induce Tregs via PD-L1 (programmed death-ligand 1) expression, balancing antiviral and tolerogenic responses (Yun, Igarashi et al. 2021). Hyperactivation of pDC has been linked to autoimmune diseases like lupus, resulting from aberrant type I IFN production (Psarras, Alase et al. 2020).

In general, pathogen recognition by DCs relies on PRRs such as TLRs and MHC molecules. For instance, MHCI presents antigens to CD8⁺ T cells, leading to their activation if the T cell receptor (TCR) recognizes the antigen and receives the necessary costimulatory signals. This activation triggers T cell proliferation and the release of cytotoxic granules (e.g., perforin, granzymes) to eliminate infected or cancerous cells (Raskov, Orhan et al. 2021). This interaction also triggers cytokine production by activated CD8⁺ T cells (e.g., IFN- γ) to amplify immune responses against intracellular pathogens (Ngo, Garrec et al. 2024).

3. Dendritic Cells as Target for Immunotherapy

3.1 Targeting DC with Nanovaccines

DCs are promising targets for nanovaccines due to their central role in orchestrating adaptive immunity and tolerance and their localization in both the liver and spleen (Clemente, Denis et al. 2023). Tolerance-promoting nanovaccines targeting DCs are being investigated as promising therapeutic approaches for a wide range of autoimmune diseases (Kim, Xie et al. 2023, Arve-Butler and Moorman 2024). These nanovaccines aim to restore or to induce immune tolerance to self-antigens by reprogramming DCs to activate Tregs and suppress pro-inflammatory immune responses (Arve-Butler and Moorman 2024). For example, a dexamethasone-loaded nanovaccine targeting DCs via CD80/CD86 blockers (e.g., abatacept) suppressed autoreactive T cell activation in experimental autoimmune encephalomyelitis (EAE), preventing demyelination and clinical symptoms (Park, Le et al. 2023). Another strategy employs rapamycin-loaded nanoparticles to generate immunosuppressive DCs and Foxp3⁺ (forkhead-box-protein P3) Tregs, effectively preventing disease progression in EAE (Maldonado, LaMothe et al. 2015). DEC-205, a DC-specific endocytic receptor, has also been used for tolerance induction (Li, Yang et al. 2022). DEC-205-targeted delivery of self-antigens like proteoglycan peptide 70-84 inhibited germinal center formation and autoantibody production in rheumatoid arthritis models (Spiering, Margry et al. 2015).

For cancer immunotherapy, DC-targeted nanovaccines aim to enhance antigen cross-presentation and T cell priming. DCs are highly specialized APCs capable of capturing, processing, and presenting tumor antigens to T cells, thereby inducing a targeted immune response against cancer cells (Fig 4.) (Del Prete, Salvi et al. 2023). Several receptors on DCs can be targeted, including PRRs such as TLRs and CLRs, to enhance DC maturation and antigen presentation (Hossain and Wall 2019). Additionally, endocytic receptors like CD209 (DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) can be utilized for antigen delivery to DCs, while costimulatory molecules such as CD86 can be targeted by adjuvants to enhance DC activation and T cell priming (Sehgal, Dhodapkar et al. 2014). For instance, glucosylated nanovaccines use the expression of glucose transporter 1 on DCs to deliver tumor antigens, promoting DC maturation and cross-presentation to CD8⁺ T cells, as demonstrated in melanoma and MC38 tumor models (Liu, Cui et al. 2024). Similarly, CLEC9A-targeted nanovaccines leverage the cDC1-specific receptor CLEC9A to deliver tumor antigens and STING (stimulator of interferon genes) agonists, amplifying cytotoxic T cell responses while minimizing off-target effects

(Hussain, Zhang et al. 2025). Such approaches synergize with checkpoint inhibitors like anti-PD-1, achieving robust antitumor immunity (Nguyen, Le et al. 2024). Trimannose, a carbohydrate structure recognized by receptors like DC-SIGN on DCs, has been successfully employed as a targeting moiety in drug delivery systems. In a study by Wagener et al., liposomes carrying a trimannose moiety on their surface demonstrated preferential binding to DCs both *in vitro* and *in vivo*, with significant accumulation in the spleen and liver after i.v. injection (Wagener, Bros et al. 2020). This targeted approach highlights the potential of trimannosylated liposomes to improve nanovaccine delivery and optimize immune responses against tumors.

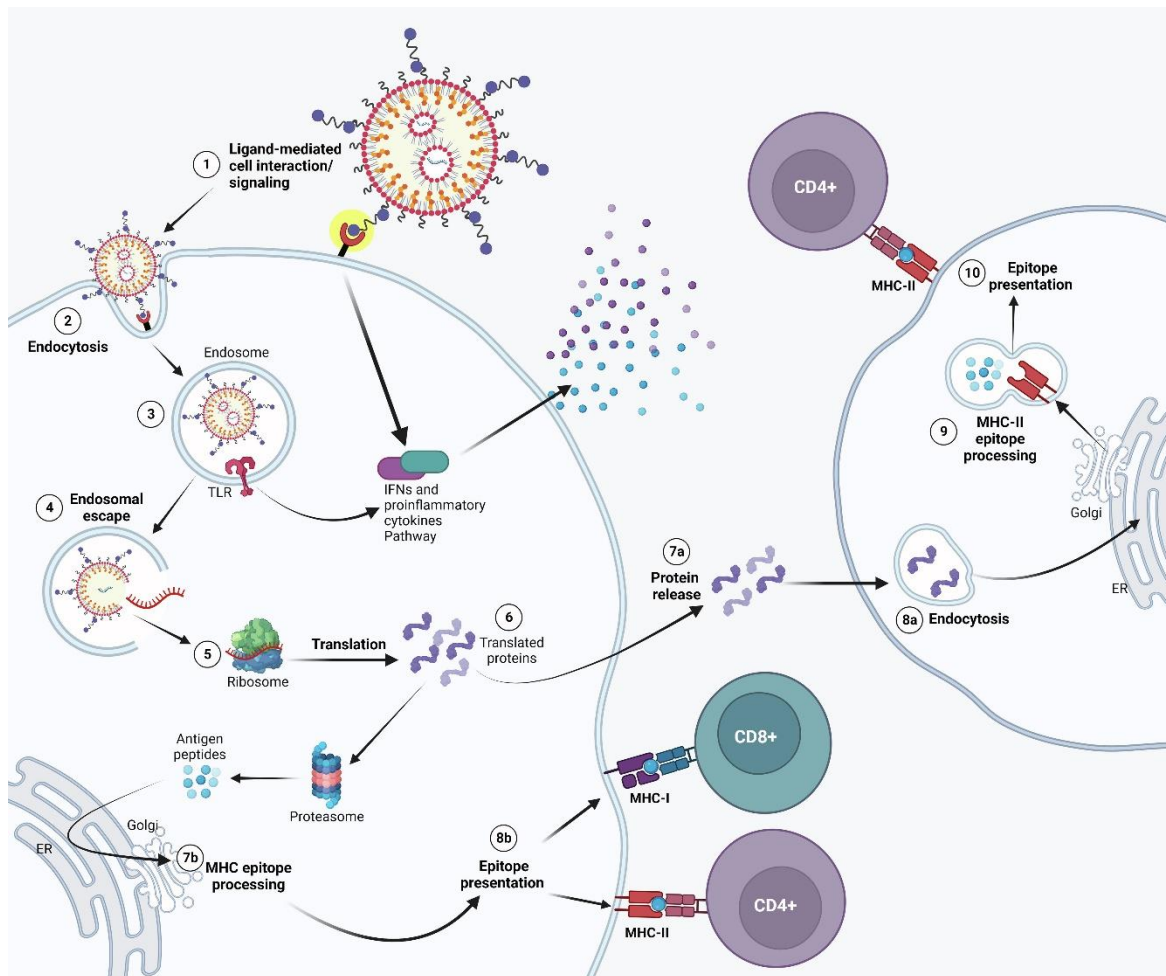


Figure 4. Possible mechanism of action of DC-targeted LNP encapsulating mRNA. mRNA-LNPs with specific ligands bind to surface receptors on APCs and trigger receptor-mediated endocytosis. Within endosomes, the mRNA interacts with TLRs, which activates signaling pathways that induce type I IFNs and proinflammatory cytokines. Following endosomal escape, the mRNA is translated into proteins, which are either secreted and taken up by neighboring APCs or processed intracellularly via the proteasome for MHC class I presentation to CD8⁺ T cells or MHC class II presentation to CD4⁺ T cells (Clemente, Denis et al. 2023) (see Abbreviations).

The potential of these dual applications, either activating DCs for cancer or inducing tolerance for autoimmunity, underscore the versatility of nanovaccines. By targeting

receptors that are preferentially expressed by DCs (e.g., CLEC9A, DEC-205, DC-SIGN) and organ-specific biodistribution (e.g., liver, spleen), nanovaccines achieve precise immune modulation, balancing therapeutic efficacy with minimized systemic toxicity.

3.2 Nanovaccine Delivery Systems

Nanovaccine delivery systems include a diverse range of platforms, e.g., lipid nanoparticles (LNPs), liposomes, polyplexes and protein nanoparticles, each offering unique advantages for vaccine delivery and immunomodulation (Fig. 5).

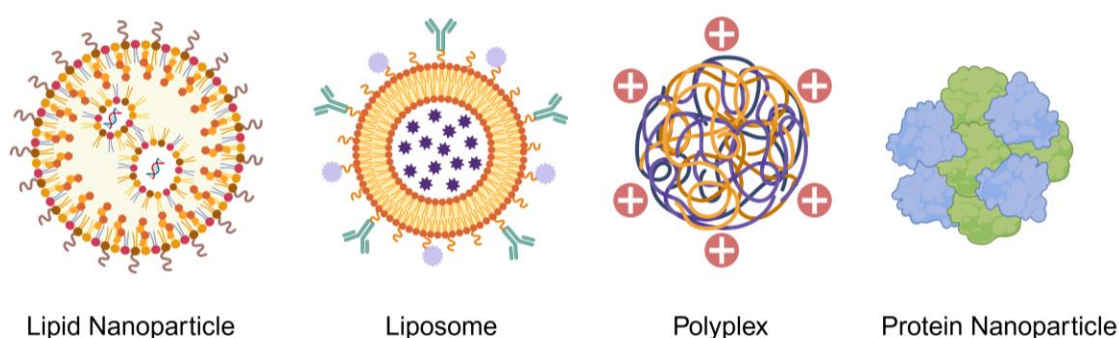


Figure 5. Overview of several nanoparticle systems. LNPs are composed of ionizable lipids, cholesterol, helper lipids, and PEG, to enable efficient drug delivery through endosomal escape and membrane fusion (Albertsen, Kulkarni et al. 2022). Liposomes are spherical vesicles with phospholipid bilayers and are used for drug delivery due to their biocompatibility and ability to encapsulate hydrophilic/hydrophobic agents (Nsairat, Khater et al. 2022). Polyplexes are formed by electrostatic interactions between cationic polymers and nucleic acids and protect and transport genetic material (Ita 2020). Protein nanoparticles use natural proteins to enhance drug stability and targeting (Hong, Choi et al. 2020) (see Abbreviations). Figure created with BioRender.

LNPs became significantly prominent, particularly in messenger ribonucleic acid (mRNA) vaccine delivery, as demonstrated by their success in COVID-19 vaccines (Kowalzik, Schreiner et al. 2021). LNPs protect mRNA from degradation and facilitate cellular uptake, making them a preferred choice for nucleic acid-based vaccines (Cacicedo, Limeres et al. 2022). Traditional LNPs consist of four primary components: ionizable lipids, structural lipids (cholesterol), phospholipids, and PEGylated (polyethyleneglycol) lipids. Ionizable lipids are particularly critical for complexing negatively charged mRNA and facilitating endosomal escape upon cellular uptake (Mendonca, Kont et al. 2023). The main advantages of mRNA vaccines include rapid development and production, as they can be designed and manufactured quickly once the genetic sequence of a pathogen is known (Al Fayez, Nassar et al. 2023). They offer flexibility and adaptability, allowing for easy modifications to target new variants of a given antigen. mRNA vaccines have a favorable

safety profile as they do not integrate into the host genome and are quickly degraded in the body (Liao and Liu 2025). LNPs have been successfully employed in vaccines such as those developed for COVID-19 (Albertsen, Kulkarni et al. 2022). Co-application of adjuvants and mRNA-LNP formulations has been shown to potentiate liver NPC activation, enhancing the overall immune response to the vaccine (Svensson, Limeres et al. 2025).

Polyplexes also play an important role in cancer immunotherapy. They are particularly effective for delivering nucleic acids while protecting them from enzymatic degradation (Mendes, Conniot et al. 2022). Due to their positive charge, polyplexes have the ability to condense genetic material into compact particles which facilitates cellular uptake and endosomal escape (Zakeri, Kouhbanani et al. 2018). These features make them suitable for applications like gene silencing or antigen expression in cancer therapy (Vetter and Wagner 2022). Polyplexes have also shown promising results in delivering plasmid DNA (plasmid deoxyribonucleic acid) to DCs, underlining their potential as a tool in vaccine development (**Zeyn**, Hobernik et al. 2023)

Liposomes, with their phospholipid bilayer structure, can encapsulate both hydrophilic and hydrophobic vaccine components. Their surface can be easily modified with targeting ligands to enhance delivery to specific cell types, such as DCs (Wagener, Bros et al. 2020).

Protein nanoparticles, particularly those based on endogenous proteins, represent a promising platform for targeted vaccine delivery to DCs. These nanoparticles offer several advantages, including biocompatibility, biodegradability, and the ability to be easily functionalized with targeting moieties and adjuvants (Hong, Choi et al. 2020). For example, protein nanocapsules can be engineered to encapsulate TLR7/8 and STING agonists within their core, while the proteins forming the capsule serve as the antigen (Schunke, Hueppe et al. 2024). Human serum albumin (HSA) can be used as a platform for conjugating TLR7/8 agonist together with a trimannose targeting unit, effectively targeting and activating DCs while using the protein's inert nature to escape from degradation (**Lantzberg, Zeyn et al. 2025**)*. This multifunctional approach enables the delivery of immunostimulatory signals to DCs, potentially enhancing their activation and subsequent T cell responses.

The ability of nanocarriers platforms to co-deliver antigens and adjuvants within the same nanoparticle has shown promising effects in stimulating potent immune responses, making them useful to develop customized immunotherapies against infectious diseases, autoimmune disorders, and cancer (Zhuang, Holay et al. 2019).

3.3 Enrichment Methods for Liver NPC

Liver NPCs play a key immunological role and often accumulate nanoparticles designed to target other cell types (Tsoi, MacParland et al. 2016). This highlights the need to study their interactions with nanoparticles and the resulting functional outcome. While their natural tolerance-inducing properties can help to suppress unwanted immune responses, like in allergies (Kusumoputro, Au et al. 2024), overcoming this tolerance is vital for effective anti-tumor nanovaccination, requiring the use of appropriate adjuvants, as explored in the study by Medina-Montano and coworkers (Medina-Montano, Cacicedo et al. 2022).

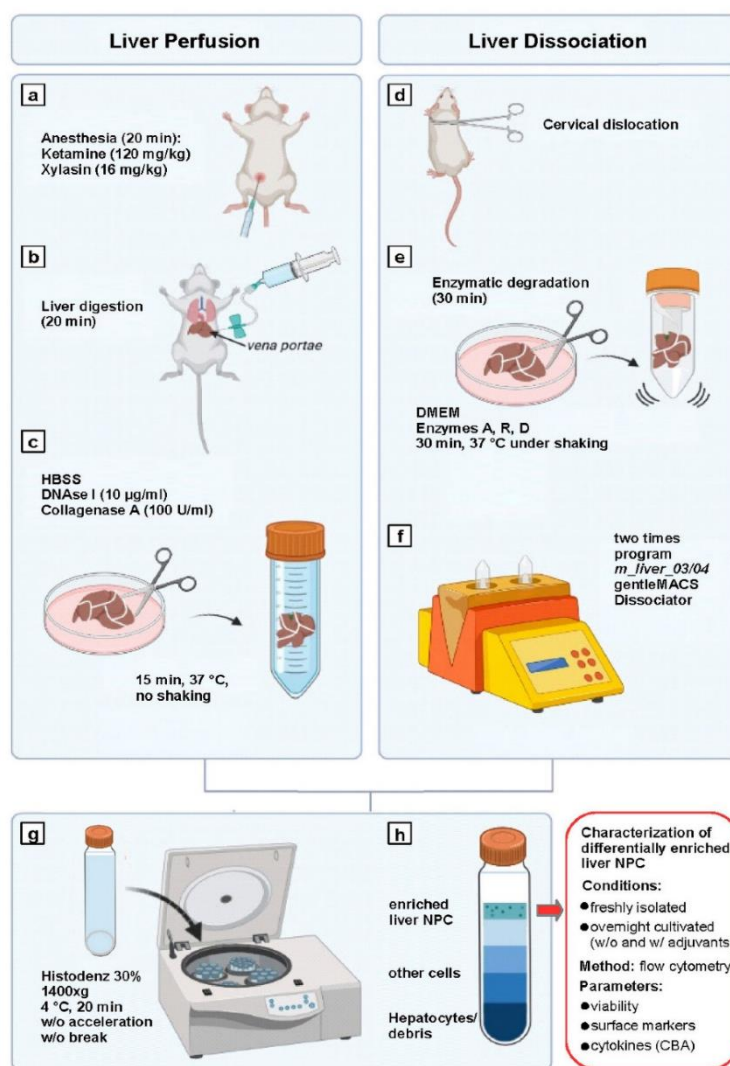


Figure 6. Schematic representation of liver NPC isolation through liver perfusion and liver dissociation. Two methods for enriching liver non-parenchymal cells (NPCs) were optimized: liver perfusion (LP) involving *in situ* enzymatic digestion via portal vein cannulation, and liver dissociation (LD) using an enzyme cocktail as well as mechanical disruption. Both approaches yield single-cell suspensions, which were subjected to Histodenz gradient centrifugation to enrich NPCs. The isolated cells were then analyzed for viability, surface markers, and cytokine release, both freshly and after incubation with or without adjuvants (Medina-Montano, Cacicedo et al. 2022).

The optimization of liver NPC isolation techniques has been important for developing effective and reproducible *ex vivo* assays. Two primary methods, liver perfusion (LP) and liver dissociation (LD), are commonly employed for this purpose, which were compared in a study by Medina-Montano and coworkers (**Medina-Montano, Cacicedo et al. 2022**). Both techniques aim to isolate NPCs, including KC, DC and LSEC, which are essential for understanding liver immunology and nanoparticle interactions.

While LP and LD are similar in comprising enzymatic digestion and density gradient centrifugation, they differ in their specific approaches (Fig. 6). LP starts with *in situ* perfusion of the liver via the portal vein, whereas LD utilizes mechanical dissociation followed by enzymatic digestion. In the efforts to refine these isolation techniques, Medina-Montano et al. encountered challenges in accurately assessing cell viability. To address this, various live/dead staining reagents were tested, where Sytox and 7-AAD were found to be reliable for viability analysis. This optimization step was essential for ensuring the quality and reliability of the isolated NPC populations. Liver NPCs isolated by LD also showed higher basal expression of costimulatory receptors CD80 and CD86 compared to LP, indicating a pre-activated state. Upon stimulation with TLR ligands, LP-derived liver NPCs demonstrated greater responsiveness, with increased expression of activation markers and cytokines, while LD-derived NPCs were more refractory to further activation. In summary, LP, although more time-consuming and labor-intensive, yields less activated NPCs, making it preferable for certain functional studies. Conversely, LD allows for parallel processing of multiple samples, making it suitable for high-throughput experiments.

For further research, this refinement has several important implications. It allows the study of specific nanoparticle uptake and adjuvant activity by different NPC subsets, including DCs, which is essential for understanding the biodistribution and efficacy of nanovaccine candidates.

3.4 Adjuvants for Nanovaccination

3.4.1 Toll-Like Receptor Agonists

Adjuvants play a crucial role in activating DCs and liver NPCs to induce effective immune responses. A significant approach to activate DCs and NPCs is the use of TLR agonists. TLR7/8 are closely related PRRs that play a significant role in the innate immune system. These receptors are located in endosomes of immune cells and are specialized in recognizing viral single-stranded RNA (ssRNA) molecules (Sun, Li et al. 2022). TLR7/8

share structural similarities, but have distinct characteristics in terms of expression patterns and signaling cascades. In humans, TLR7 is predominantly expressed in pDCs and B cells (Wen, Zhang et al. 2023), while TLR8 is found in myeloid dendritic cells, monocytes, macrophages and neutrophils (Martinez-Espinoza and Guerrero-Plata 2022). This differential expression contributes to their specialized roles in immune responses.

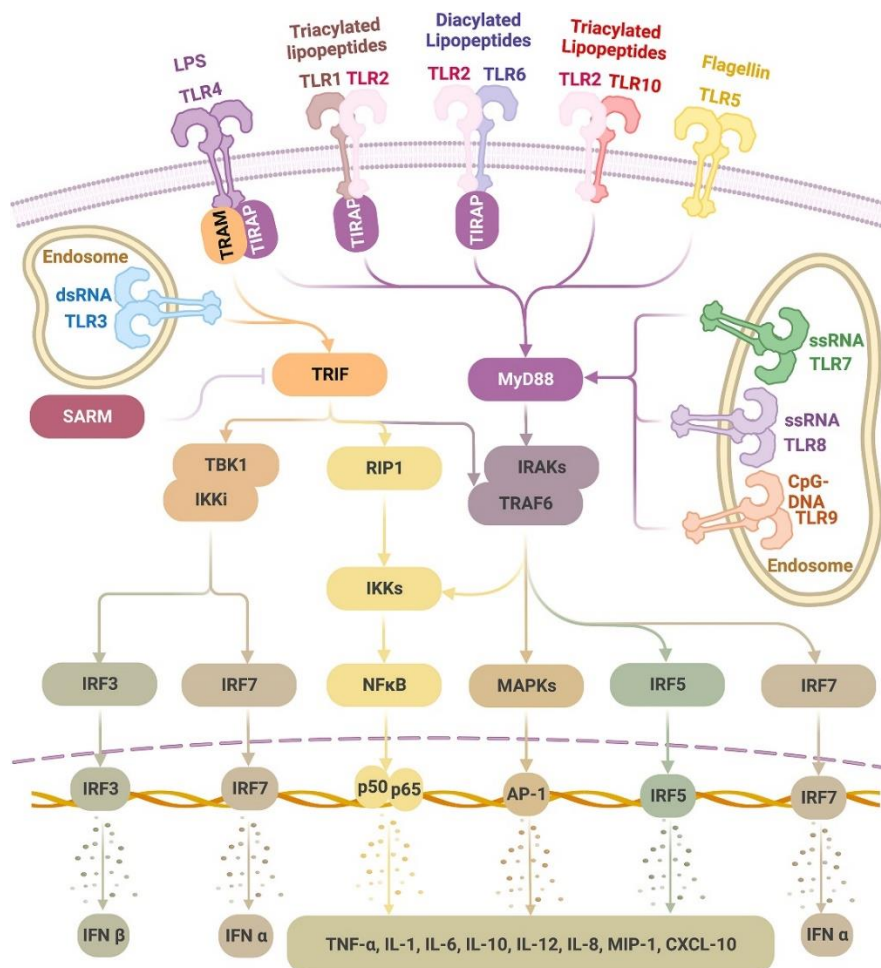


Figure 7. Overview of TLR families, their ligands, and signaling pathways. Stimulation of TLRs leads to a signaling cascade resulting in activation of transcription factors (e.g. NFkB) and therefore to release of proinflammatory cytokines and type-I IFNs (Yang, Li et al. 2022).

Upon activation by ssRNA, both TLR7 and TLR8 initiate MyD88 (myeloid differentiation primary response 88)-dependent signaling cascades (Fig. 7). However, the downstream effects of these cascades differ slightly between the two receptors. TLR7 activation strongly induces the IRF pathway, leading to robust production of type I IFNs, particularly IFN- α . In contrast, TLR8 activation predominantly stimulates the NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway, resulting in the secretion of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-12 (Bender, Tzvetkov et al. 2020). In cancer models, the TLR7/8 agonist R848 (resiquimod) has demonstrated efficacy in various cancer types, including breast, pancreatic, and colorectal cancer (Bhaliya, Anwer et al. 2025). In lung

cancer, systemic administration of R848 led to enhanced activation of DCs and NK cells (Zhou, Xu et al. 2022).

TLR9 agonists, particularly CpG oligodeoxynucleotides (ODNs), are another important class of adjuvants. CpG ODNs are synthetic ssDNA molecules containing unmethylated CpG motifs (Hartmann 2023). They are classified into three main classes (Li, Hua et al. 2020). Class A ODNs strongly stimulate IFN- α production by pDC but are weak stimulators of TLR9-dependent NF- κ B signaling. Class B ODNs effectively activate B cells but weakly induce IFN- α secretion. In contrast, class C ODNs combine properties of both previous classes, activating both IFN- α production and evoking B cell stimulation.

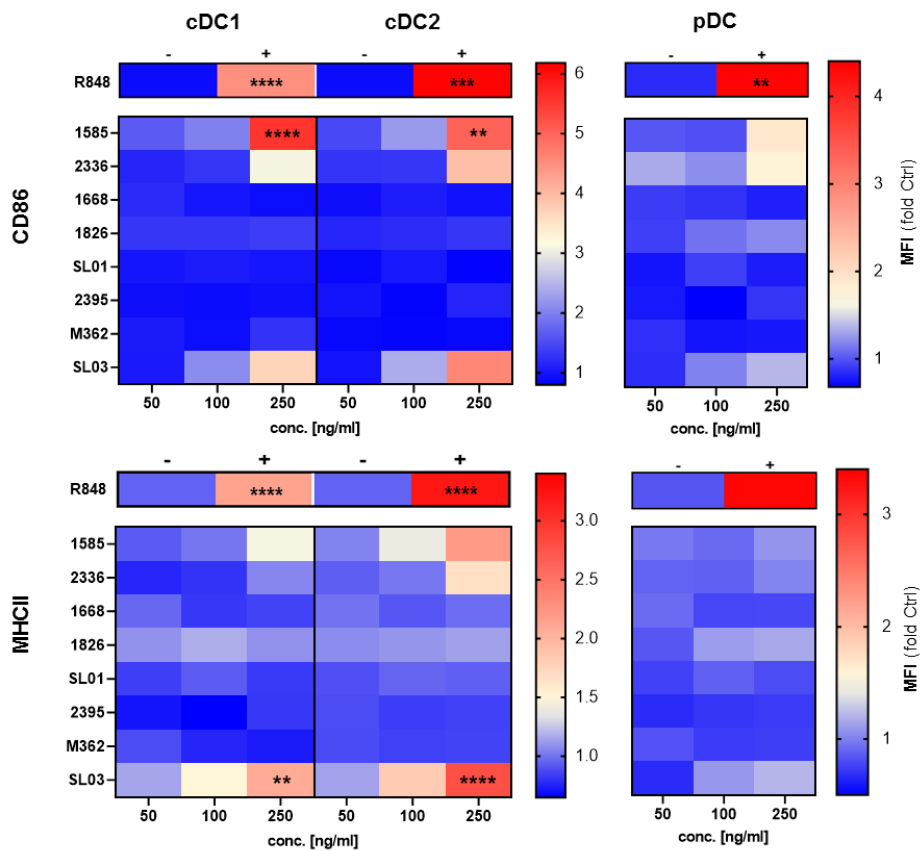


Figure 8. CpG ODNs enhance MHCII and CD86 expression of FLT3L BMDC. FLT3L differentiated BMDC were incubated with different concentrations of CpG ODN (50, 100 or 250 ng/ml) or the TLR7/8 ligand R848 (1 μ g/ml). On the next day, expression of MHCII and CD86 was assessed by flow cytometric analysis. Graphs denote fold Ctrl of fluorescence intensities (MFI) (mean $\pm$ SEM of 4 experiments) of marker expression. Statistical differences versus *Ctrl are indicated (one way ANOVA, Tukey test, **p<0.01, ***p<0.001, ****<0.0001 (original data; unpublished).

CpG ODNs have demonstrated significant potential as vaccine adjuvants and immunotherapeutic agents in preclinical studies and clinical trials (Desai, Gotru et al. 2025). They enhance antigen presentation by APCs and promote Th1-directed immunity, e.g., vaccine-specific Th1 associated antibody responses, which is particularly beneficial in combating infections and cancer (Zhao, Cai et al. 2023, Wang, Liu et al. 2024).

In a yet unpublished study, the optimal activation of DCs by CpG ODNs was investigated. FLT3L (Fms-related tyrosine kinase 3 ligand)-differentiated bone marrow-derived DC (BMDC) cultures mimic steady-state DC populations found *in vivo*, including conventional cDC1, cDC2, and pDCs, which differ in their primary functions but play essential roles in antigen presentation and immune modulation.

Several CpG ODNs from the above mentioned three classes were tested to identify the most effective adjuvant for DC activation (Fig. 8). Among the CpG ODNs tested, ODN1585 (class A) demonstrated the strongest dose-dependent induction of CD86 expression across all DC populations. A similar trend was observed for ODN2336 (class A) for CD86 expression. SL03 (class C) emerged as a particularly potent activator, strongly enhancing both MHCII and CD86 expression in cDC1 and cDC2 subsets. Due to its superior performance also in activating liver NPCs and splenic DCs (data not shown), SL03 was selected for further experiments.

To simulate vaccine-like intracellular delivery of SL03, this CpG ODN was complexed with DOTAP, a commonly used transfection reagent (Simberg, Weisman et al. 2004). This approach significantly enhanced DC activation compared to soluble SL03. Transfected BMDCs showed strong upregulation of activation markers and pro-inflammatory cytokine release (data not shown). These findings suggest that intracellular delivery of SL03 may potentiate its adjuvant effects by facilitating direct interaction with intracellular TLR9 (Comberlato, Koga et al. 2022).

To evaluate the consequences of SL03 transfection on T cell activation, co-culture experiments using murine OVA (ovalbumin) responsive CD8⁺ or CD4⁺ T cells were performed. These cells were co-cultured with BMDCs, which had been treated with complexed SL03, to subsequently assess proliferation and cytokine production (Fig. 9).

Intracellular delivery of SL03 into BMDCs demonstrated a significantly stronger T cell activation than the soluble form of SL03 on both CD8⁺ and CD4⁺ T cell responses. For CD8⁺ T cells, prior complexation of SL03 resulted in strong proliferation and elevated secretion of Th1-/CTL-related cytokines such as IFN γ and TNF α , indicating that intracellular delivery of SL03 amplifies antigen presentation and promotes a strong cytotoxic immune response (Fig 9A). The enhanced TNF α production further highlights the ability of transfected BMDCs to enhance pro-inflammatory signaling, which is important for effective immune activation.

CD4⁺ T cells showed a distinct response to DCs that had intracellular delivery of SL03, with trends indicating increased proliferation and cytokine production (Fig 9B). Interestingly, transfected BMDCs induced an altered cytokine pattern in CD4⁺ T cells, suggesting that

intracellular delivery of SL03 may modulate Th-cell differentiation pathways. While IFN γ levels were elevated under certain conditions, TNF α production in CD4 $^{+}$ T cells mirrored the trends observed by CD8 $^{+}$ T cells, indicating a partial shift towards a Th1-like inflammatory profile.

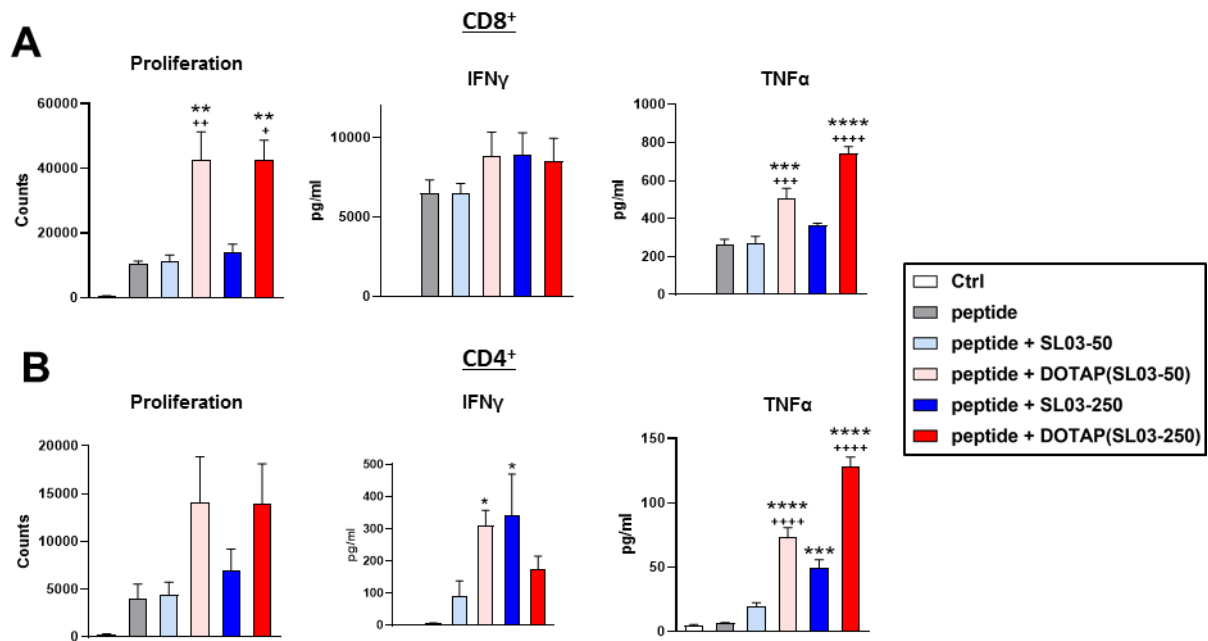


Figure 9. Transfection of CpG ODN SL03 to FLT3L differentiated BMDC enhances CD8 $^{+}$ and CD4 $^{+}$ T cell proliferation and cytokine release. FLT3L differentiated BMDC were incubated with soluble or DOTAP-complexed SL03 (50 or 250 ng/ml). On the next day, BMDCs were co-cultured with CFSE stained (A) CD8 $^{+}$ and SIINFEKL peptide or (B) CD4 $^{+}$ T cells and GREY peptide, respectively. After 3-5 days, T cells were harvested and subjected to flow cytometry. In addition, supernatants were collected and analyzed for cytokine levels. Proliferation graphs denote counts, cytokine graphs denote cytokine concentration (pg/ml) (mean $\pm$ SEM of 3-5 experiments). Statistical differences versus *Ctrl or *soluble of same concentration are indicated (one way ANOVA, Tukey test). *, + p<0.05; **, ++ p<0.01; ***, +++ p<0.001; ****, ****+<0.0001 (original data; unpublished).

In conclusion, these data highlight the potential of intracellular delivery of SL03 as a promising adjuvant strategy for nanovaccine applications. The ability to modulate cytokine profiles and to optimize antigen presentation through intracellular delivery offers promising opportunities for designing customized immunotherapies targeting specific immune pathways. Further studies are needed to explore how these findings translate into *in vivo* settings and how they can be used to improve vaccine efficacy against cancer.

3.4.2 Other Adjuvants

While TLR agonists have shown promising results in immunomodulation, other adjuvants present considerable alternatives. For example, STING agonists such as cyclic GMP-AMP

(cGAMP) represent another promising class of adjuvants. Activation of STING by agonists like 3'3'-cGAMP leads to phosphorylation of STING, IRF3, and STAT1 (signal transducer and activator of transcription 1), which in turn induces the production of type I IFNs (Zhu, An et al. 2019). STING agonists have proven to be potent activators of the innate immune system and have yielded promising results in cancer immunotherapy (Wang, Yu et al. 2024).

Furthermore, polyinosinic-polycytidylic acid (PolyI:C) is an adjuvant that is able to induce the production of cytokines such as CXCL10 and IFN- β independently of STING. It activates TLR3 and can induce strong type I IFN responses (Araya, Men et al. 2024).

In addition to these approaches, there are other strategies for activating DCs and NPCs, like the use of cytokines, chemokines, or specific antibodies targeting certain surface receptors on these cells. The combination of different adjuvants could result in synergistic effects and lead to even more effective immune activation.

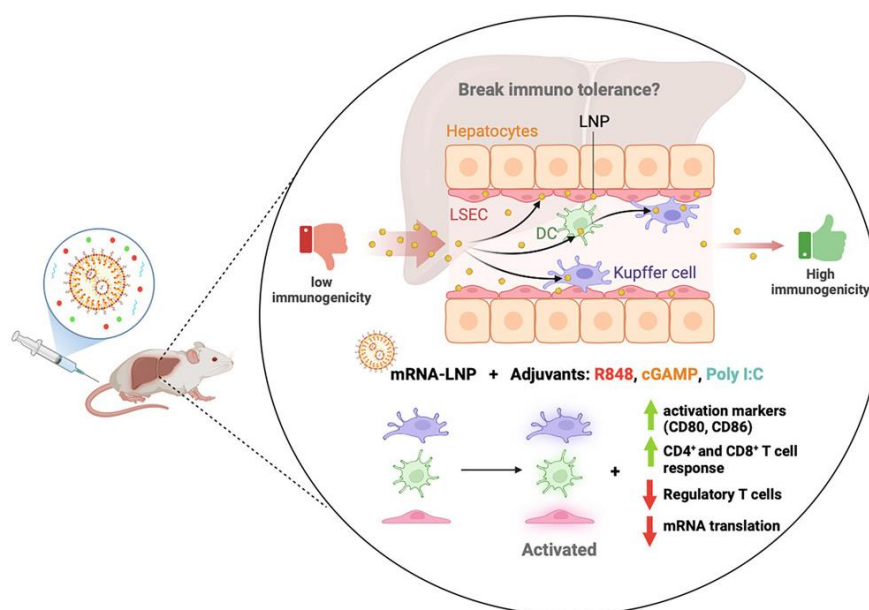


Figure 10. Schematic overview of the study by Svensson et al. evaluating different adjuvant combinations to overcome liver NPC tolerance. Commonly used adjuvants and combinations thereof were tested *in vitro*, as well as *in vivo* applied together with mRNA-LNPs to achieve enhanced activation of liver NPCs and T cell responses (Svensson, Limeres et al. 2025).

The study by **Svensson et al.** investigated the strategic use of adjuvants to enhance the immunogenicity of mRNA-LNP vaccines in the liver (**Svensson, Limeres et al. 2025**). Three well-characterized adjuvants were selected: R848, cGAMP, and PolyI:C. All of them are known to activate distinct PRRs and to promote immune stimulation. The goal was to exploit the diverse immunostimulatory properties of the adjuvants to overcome the liver's inherent tolerance and promote robust immune responses after vaccination (Fig. 10). For

instance, stimulation of LSECs with TLR1/2 ligand can enhance T cell-mediated immunity (Liu, Jiang et al. 2013).

In vitro, **Svensson et al.** observed that R848 was most effective at upregulating the expression of co-stimulatory molecules CD80 and CD86 on all three liver NPC populations monitored. Combinations of adjuvants often resulted in additive effects on liver NPC activation. Furthermore, use of R848, either alone or in combination with cGAMP, resulted in the strongest production of various cytokines and chemokines. Additionally, the T cell / liver NPC co-culture experiments revealed that R848 in combination with cGAMP induced the strongest T cell proliferation.

In vivo, the impact of these adjuvants on the response to mRNA-LNP vaccines in mice was assessed. mRNA-LNPs without adjuvants failed to upregulate the expression of CD80 and CD86 on NPCs after i.v. injection. However, additional i.v. application of R848, either alone or in combination with cGAMP, enhanced the expression of co-stimulatory molecules on NPCs. Experiments using luciferase-encoding mRNA revealed that LNPs accumulated mostly in the liver, but the presence of adjuvants negatively affected translation efficiency, especially the use of R848. To further investigate the effects of these adjuvants in combination with mRNA LNPs, a repetitive administration was performed. When applied without additional adjuvants, this resulted in similar co-stimulatory molecule upregulation on liver NPCs compared to mRNA-LNP + adjuvants. Repeated administration of R848 or the R848 + cGAMP combination alongside mRNA-LNPs resulted in improved cellular immune responses compared to mRNA-LNP alone, evidenced by enhanced numbers of cytokine-releasing CD4⁺ and CD8⁺ T cells. The results highlight the importance to carefully select adjuvants to overcome the tolerogenic microenvironment of the liver and induce effective immune responses following mRNA-LNP vaccination.

3.5 Targeting Immune Cells with Newly Developed Nanocarrier Systems

mRNA therapeutics have emerged as a transformative platform for vaccines (Al Fayez, Nassar et al. 2023), protein replacement therapies (Papukashvili, Rcheulishvili et al. 2022), and immunotherapy (Liu, Shi et al. 2023). However, the success of mRNA-based treatments is highly dependent on the delivery system used to transport the mRNA molecules into target cells (Malla, Srilatha et al. 2024). As conventional LNPs have become the gold standard for non-viral mRNA delivery and have demonstrated remarkable efficacy, particularly in the context of COVID-19 vaccines, researchers are now exploring innovative

particle designs to overcome limitations such as tissue specificity (Dilliard, Cheng et al. 2021), immune modulation (Kiaie, Majidi Zolbanin et al. 2022), and intracellular barriers (Zhao, Wang et al. 2024).

In the previous chapter, the potential of external adjuvants such as R848 and cGAMP to enhance immune responses while potentially affecting mRNA translation efficiency was investigated. These findings underline the delicate balance between immunostimulation and maintaining optimal mRNA functionality. Recent advancements in LNP technology have focused on intrinsic modifications of the nanoparticle itself, such as novel lipid compositions and targeting strategies, to improve delivery efficiency and therapeutic outcomes without relying only on external adjuvants (Wang, Ding et al. 2024).

Recent innovations in nanoparticle design have introduced new classes of ionizable structures with enhanced properties. One notable example are CARTs (charge-altering releasable transporters), which are amphiphilic diblock copolymers that form complexes with mRNA through electrostatic and hydrophobic interactions. CARTs contain a polycarbonate block incorporating cationic hydroxyethyl glycine repeat units, which upon intracellular rearrangement degrade to neutral small molecules, facilitating mRNA release into the cytoplasm (Berger, Lächelt et al. 2024). Modified versions of CARTs have demonstrated organ-specific targeting, such as K-CART that conferred lung-selective delivery and GSer-CART that mediated lung- and spleen-specific delivery (Blake, Haabeth et al. 2023, Li, Amaya et al. 2024).

Another promising innovation are lipopolyplexes (LPPs). These constitute a hybrid system that combines liposomal and polymeric components to enhance the intracellular delivery of nucleic acids such as mRNA or DNA (Pei, Deng et al. 2022). Unlike traditional polyplexes, which are composed of polymers complexed with nucleic acids, LPPs incorporate lipids into their structure, creating composite particles that take advantages of both systems. This combination results in improved transfection efficiency and reduced toxicity compared to more simple delivery systems (Rezaee, Oskuee et al. 2016, Persano, Guevara et al. 2017).

Lipoamino xeno peptide carriers (LAF-Stp), as described by Thalmayr et al., combine lipo amino fatty acids (LAFs) with polar cationizable succinoyl tetraethylene pentamine (Stp) units (Fig. 11) (Thalmayr, Grau et al. 2023). Thalmayr et al. synthesized a library of LAF-Stp carriers using solid-phase peptide synthesis, creating molecules with defined 2D sequences and 3D topologies, so called bundles or U-shapes. This design equips the LPPs with a unique “molecular chameleon” behavior. This effect describes the transition from hydrophobic at physiological pH to hydrophilic in the acidic environment of endosomes,

potentially enhancing endosomal escape. The study by Thalmayr and coworkers found that certain LAF-Stp carriers, particularly those with bundle topologies, exhibited superior pDNA and mRNA encapsulation and delivery compared to particles formulated with standard ionizable lipids.

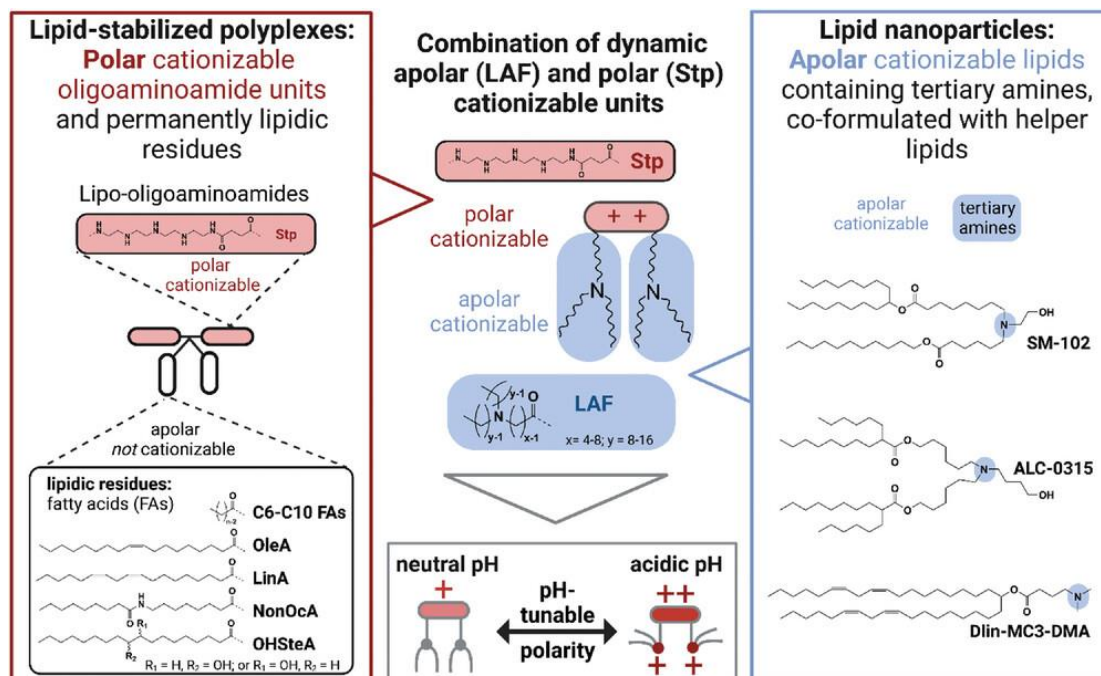


Figure 11. Design concept of LAF–Stp carriers. On the left are visualized lipo-oligoaminoamides as polar, cationizable units along with lipidic residues. On the right are apolar cationizable lipids that contain tertiary amines. In the center, the newly designed LAF carriers that incorporate both apolar (LAF) and polar (Stp) components (Thalmayr, Grau et al. 2023).

In a following study, Haase et al. formulated mRNA-LNPs containing LAF-Stp structures instead of standard ionizable lipids (**Haase, Pohmerer et al. 2024**). The LNPs containing these carriers demonstrated faster and more effective transfection compared to standard LNP formulations. *In vitro*, they showed high transfection efficacy for various cell types, including tumor cell lines, macrophages, and DCs. Remarkably, these LNPs yielded high transfection levels even in the presence of serum and at very low mRNA doses. *In vivo* experiments revealed that mRNA-LNPs incorporating LAF-Stp structures exhibited a high spleen/liver expression ratio after i.v. administration, suggesting a preferential targeting of DCs and macrophages in the spleen. This contrasts with conventional LNPs, which tend to accumulate in the liver due to apolipoprotein E-mediated uptake (Kimura and Harashima 2023). Preferential targeting of immune cells, especially DCs, in the spleen could be advantageous for vaccine and immunotherapy applications (Wang, Zhang et al. 2024). The research of Haase et al. demonstrates that LNPs containing LAF-Stp units significantly enhance endosomal escape, leading to improved intracellular cargo release and higher mRNA expression levels both *in vitro* and *in vivo*. LAF-Stp carriers represent a promising

alternative to conventional ionizable lipids in LNPs. Their ability to promote efficient mRNA encapsulation and mRNA delivery to immune cells in the spleen makes them interesting candidates for the development of targeted mRNA therapeutics and vaccines. In conclusion, the LAF-Stp LNPs are a novel approach to mRNA delivery that may have significant advantages over conventional LNPs, specifically for immune cell targeting and enhancing therapeutic efficacy.

3.6 Protein Nanoparticles Carrying Targeting Structures

DCs play an essential role in the coordination of immune responses, making them excellent targets for immunotherapy and vaccine development. Their exceptional ability to process and present antigens to naive T cells allows them to effectively initiate and drive adaptive immune responses (Mac Keon, Ruiz et al. 2015). To exploit this potential, several targeting strategies have been developed to deliver antigens or immunomodulatory molecules directly to DCs, which may enhance specificity and therapeutic efficiency (Baldin, Savvateeva et al. 2020). These approaches include antibody-based targeting (Bourque and Hawiger 2022), carbohydrate ligands like mannose or trimannose (Krumb, Frey et al. 2020), and other molecular structures designed to interact with DC-specific receptors (Fig. 12) (Hossain and Wall 2019).

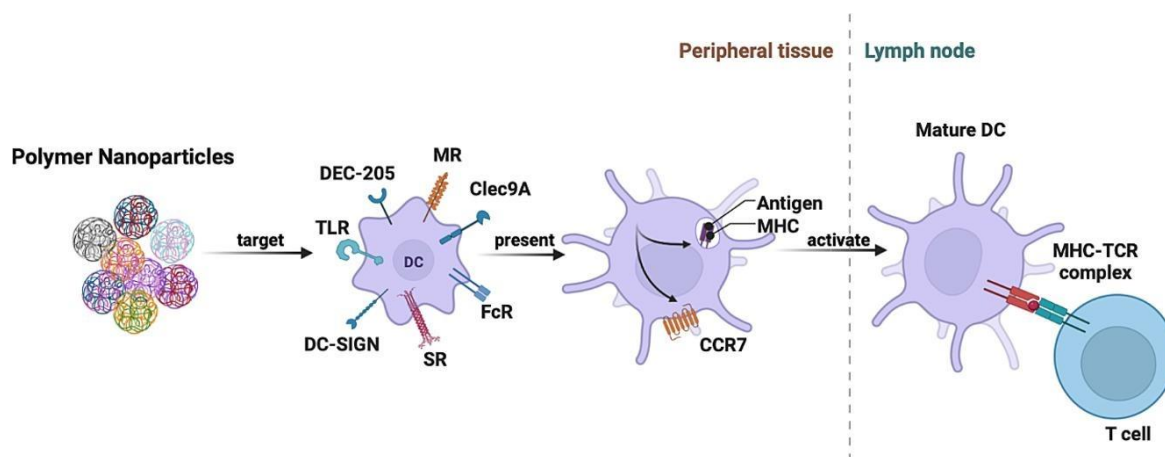


Figure 12. Schematic overview of targeting structures on the DC surface and DC maturation and activation after transfection. Nanoparticles target DCs via receptors such as DEC-205, TLR, DC-SIGN, MR, CLEC9A, FcR (Fc receptor), and SR (scavenger receptor). The DCs process and present delivered antigens via MHC molecules and migrate to the lymph node via CCR7. In the lymph node, mature DCs activate T cells via the MHC-TCR complex (Hu, Zhang et al. 2023) (see Abbreviations).

Antibody-based cell targeting is one of the most established methods. Antibodies designed to bind DC-specific receptors such as DEC-205 have shown remarkable success in

delivering antigens to DCs (Silva, Almeida et al. 2021). DEC-205, also known as CD205, is a type I transmembrane protein and a member of the macrophage mannose receptor (MMR) family (Shrimpton, Butler et al. 2009, Gully, Venugopal et al. 2021). It is highly expressed by DCs and plays an important role in antigen uptake and presentation (Silva, Almeida et al. 2021). Several studies demonstrated that coupling tumor antigens to anti-DEC-205 antibodies led to efficient uptake by DCs, enabling robust antigen presentation and activation of T cells (Birkholz, Schwenkert et al. 2010, Dhodapkar, Sznol et al. 2014, Sutherland, Ju et al. 2021). This approach has also been validated in animal models, where targeted delivery resulted in significant tumor-specific immune responses (Mahnke, Qian et al. 2005).

Other molecular structures are also being explored for DC targeting. For example, synthetic peptides or small molecules which are designed to bind specific surface markers on DCs can be conjugated with antigens or immune stimulators. An example includes CLEC9A-targeting peptides, which specifically bind to CD8⁺ and CD103⁺ DC subsets. When conjugated with tumor antigens, these peptides improved antigen cross-presentation by targeted DCs, which in turn stimulated CD8⁺ T cells, reducing metastasis in preclinical models (Yan, Wu et al. 2016).

Carbohydrate ligands like mannose and trimannose represent another promising strategy. Sugars offer high targeting specificity, minimal toxicity, and excellent biocompatibility (Paurevic, Gajdosik et al. 2024). They have emerged as effective tools for targeting DCs due to their ability to bind specific CLRs expressed on DC surfaces. These receptors, including DC-SIGN and mannose receptor (MR, CD206), mediate the uptake of glycosylated molecules (Kuipers, Nolte-'t Hoen et al. 2020). Both mannose-based and trimannose-modified carriers have demonstrated significant potential in improving the specificity and efficacy of DC-targeted therapies (Li, Luo et al. 2023).

DC-SIGN is a tetrameric CLR expressed predominantly on DCs and recognizes high-mannose glycans and fucosylated structures (van Liempt, Bank et al. 2006). Its carbohydrate recognition domain allows binding to multivalent glycan ligands, making it an ideal target for vaccine development. For instance, synthetic oligomannose dendrimers have been developed to mimic high-mannose structures (Li, Hogervorst et al. 2019). These structures enhanced binding to DC-SIGN, promoted antigen presentation and were particularly effective in inducing robust CD8⁺ T cell responses, as shown by Li et al., who combined them with TLR7 agonists for synergistic immune activation (Li, Hogervorst et al. 2019). Another approach by Kramer et al. introduced HPMA [N-(2-hydroxypropyl)methacrylamide]-based block copolymers that self-assemble into core-

crosslinked micelles, which were functionalized with mannose or trimannose for DC targeting. These micelles, loaded with the lipophilic adjuvant L18-MDP (muramyl dipeptide) and conjugated to peptide antigens, demonstrated mannose/trimannose-dependent binding to DC-SIGN and MRs, selectively activated DCs, and, in line, induced antigen-specific CD8⁺ T cell responses (Kramer, Langhanki et al. 2019). In a further study by Wagener et al., trimannosylated liposomes have been evaluated for their ability to target DCs *in vitro* and *in vivo* (Wagener, Bros et al. 2020). Trimannose, known to bind DC receptors like DC-SIGN, was coupled to hyperbranched polyglycerol (hbPG) lipids using copper catalyzed cycloaddition and was further radiolabeled for tracking. Wagner and coworkers showed that trimannosylated liposomes enhanced nanoparticle binding to DCs *in vitro*, and increased accumulation in spleen and liver *in vivo*, compared to non-mannosylated liposomes. This confirms their targeting ability and demonstrates the potential of trimannose-functionalized particles as effective drug delivery systems for DC-targeted immunotherapies.

Human serum albumin (HSA), a widely studied protein, has proven to be an excellent platform for nanoparticle-based drug delivery due to its biocompatibility, non-immunogenicity, and functional versatility (Qu, Song et al. 2024). HSA nanoparticles can be designed with surface modifications like targeting peptides to enhance selective uptake by immune cells or tumor tissues (Sun, Fu et al. 2025). HSA serves as a multifunctional platform for nanoparticle-based drug delivery systems in both research and clinical applications. Its advantages include biodegradability and the ability to bind diverse drugs, which enhances their pharmacokinetics and targeted delivery (Asrorov, Mukhamedov et al. 2024). Clinically, HSA nanoparticles have been employed in Nab-Paclitaxel (Abraxane). Abraxane is a formulation used to treat breast cancer and other cancers like non-small cell lung cancer (Cucinotto, Fiorillo et al. 2013). In addition, Abraxane uses the natural affinity of HSA for tumor-associated albumin-binding proteins to enhance drug delivery to tumors while reducing systemic toxicity (Stukan, Zuk et al. 2025).

In research, HSA nanoparticles have been explored for diverse applications. For example, gemcitabine-loaded HSA nanoparticles have shown to be promising for targeting pancreatic cancer cells by inhibiting proliferation and reducing resistance mechanisms (Stukan, Zuk et al. 2025). Similarly, HSA nanoparticles have been used as non-viral vectors for gene therapy to deliver copper-zinc superoxide dismutase (SOD1) genes to treat ocular diseases (Mo, Barnett et al. 2007). These HSA particles demonstrated high transfection efficiency and stability against enzymatic degradation.

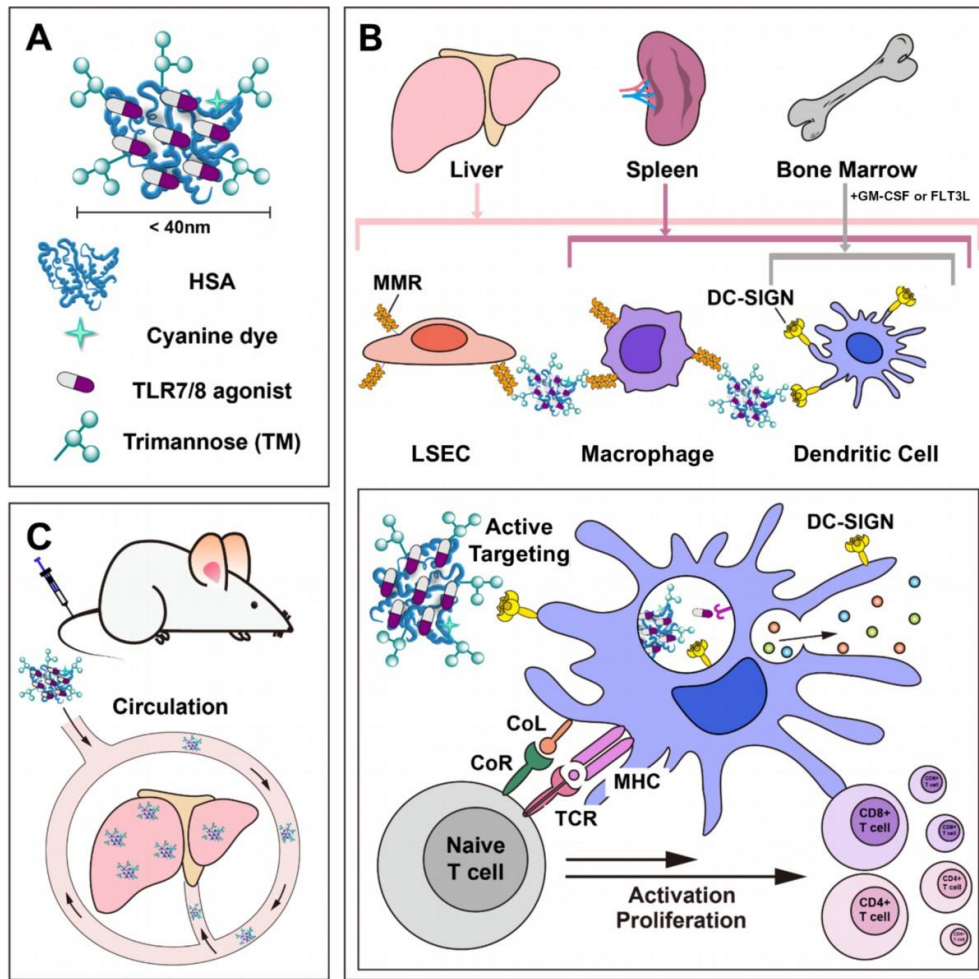


Figure 13. Design and application concept of trimannosylated HSA nanoparticles for targeted delivery to DCs and liver NPCs. A) Structure of the trimannosylated HSA nanocarrier conjugated with a TLR7/8 agonist. B) Target cell populations analyzed in *ex vivo* studies for nanocarrier uptake. These populations include liver NPCs (LSECs, KCs, and DCs), splenic macrophages and DCs, and bone marrow-derived DCs (top) and DC-specific effects of HSA nanocarrier (bottom). C) *In vivo* biodistribution of the trimannosylated HSA nanocarrier in healthy mice (Lantzberg, Zeyn et al. 2025) (see Abbreviations).

HSA has also been employed in neurological research due to its ability to cross the blood-brain barrier (Shastri, Raj et al. 2024). For instance, polyethyleneimine (PEI)-coated HSA nanoparticles have been employed to deliver genetic material into neuronal cells without inducing toxicity or immune responses (Langiu, Dadparvar et al. 2014). Additionally, albumin fragments crosslinked with genipin have been optimized for paclitaxel delivery. This modification achieves higher drug-loading efficiency and slower release compared to conventional HSA nanoparticles (Ge, You et al. 2018).

In general, HSA-based nanocarriers are widely studied for their potential in cancer therapy (Li, Zhang et al. 2023), gene delivery (Look, Wilhelm et al. 2015), and immunotherapy (Wang, Zhou et al. 2021). While formulations like Abraxane have achieved clinical success, ongoing research continues to expand the applicability of HSA nanoparticles for treating

various diseases. For example, recombinant HSA mutants carrying mannose units can be selectively delivered to the liver via mannose receptors on KCs, showing potential for treating hepatic diseases (Hirata, Maruyama et al. 2010).

In a recent study, Lantzberg, **Zeyn** et al. developed functionalized HSA nanoparticles to target DCs via DC-SIGN receptors (Lantzberg, **Zeyn** et al. 2025). The innovative development included the conjugation of trimannose (TM) to HSA, which resulted in a glycogen-like nanocarrier structure with a size below 40 nm (Fig. 13A). These TM-HSA particles exhibited rapid and highly selective uptake into immune cells. In addition, TM-HSA was functionalized with TLR7/8 agonists by using redox-sensitive linkers to enable targeted immune activation. TLR7/8 agonists are known for their ability to induce robust innate and adaptive immune responses but often suffer from poor solubility and systemic toxicity (Varshney, Qiu et al. 2021). A selective uptake of TLR7/8-functionalized TM-HSA particles into DC-SIGN expressing cells has been observed by Lantzberg, **Zeyn** and coworkers (Fig. 13B). Furthermore, in comparison to soluble TLR7/8 agonist, a higher cytokine release by BMDCs as well as similar activation and induction of CD8⁺ and CD4⁺ T cell proliferation was achieved. *In vivo*, fast clearance of TM-HSA from the blood stream and its accumulation in the liver was observed (Fig 13C). These findings suggest that TM-HSA conjugates have the potential to function as adaptable nanocarriers for targeted treatment of liver diseases, thereby maximizing therapeutic outcomes (Lantzberg, **Zeyn** et al. 2025). The approach holds promise for treatment of a wide range of diseases related to immune dysfunction, including infections, autoimmune disorders, liver fibrosis and cancer. Altogether, TM-HSA carriers represent a significant step forward in nanomedicine, offering improved immune modulation and minimal side-effects for future therapies.

3.7 Transcriptional Targeting of DCs

Polyplexes form through the electrostatic interaction of nucleic acids with cationic polymers and are widely studied as non-viral vectors for gene delivery. These systems offer advantages like reduced immunogenicity and enhanced safety compared to viral vectors (Khan 2024). While polyplexes have shown potential for nucleic acid delivery, the optimization of their selectivity and minimizing off-target effects remains a major area of research (Winkeljann, Keul et al. 2023). A commonly used polyplex agent is PEI. High molecular weight PEI showed superior transfection efficiency but also increased cytotoxicity, while low molecular weight PEI was less toxic but also less effective (Hall, Lächelt et al. 2017). The positive charge of polyplexes can lead to toxicity within biological

systems (Casper, Schenk et al. 2023). To overcome these issues, researchers have developed various strategies. These include PEGylation to reduce toxicity and to improve stability (Grun, Suberi et al. 2021), designing biodegradable derivatives (Porello, Bono et al. 2024), incorporating hydrophobic modifications to enhance polyplex stability (Wang, Ye et al. 2019), or introducing negative charges into PEI derivatives (Zintchenko, Philipp et al. 2008). Other approaches focus on promoting endosomal escape through mechanisms like incorporating endosomolytic peptides (Grau and Wagner 2024).

Transcriptional targeting enhances gene therapy specificity by using tissue- or cell-specific promoters that ensure expression of a therapeutic gene or antigen only in target cells (Montaño-Samaniego, Bravo-Estupiñan et al. 2020). This approach improves treatment efficacy while reducing off-target effects. For example, DC-specific antigen expression could be achieved using a human CD83 promoter. This promoter, active in mature DCs, was integrated into adenoviral vectors to drive the expression of therapeutic antigens selectively in DCs to induce potent, antigen-specific immune responses (Knippertz, Deinzer et al. 2016).

Transcriptional targeting in the context of DNA-based vaccines was explored by **Zeyn** et al. In their study, human *fascin1* (*Fscn1*) gene promoter was investigated to achieve DC-focused transcriptional targeting (**Zeyn**, Hobernik et al. 2023). *Fscn1* is an actin-bundling protein and is almost exclusively expressed in activated DCs (**Zeyn**, Harms et al. 2022). *Fscn1* expression is central for the ability of DCs to form dendritic protrusions, which contribute to their migratory activity. Inhibiting *Fscn1* in stimulated DCs diminishes their interaction with antigen-specific T cells and consequently reduces their T cell stimulatory capacity, suggesting that *Fscn1* plays a vital role in DC-mediated immune responses (**Zeyn**, Harms et al. 2022). Additionally, *Fscn1* expression is precisely regulated at the transcriptional level (Bros, Ross et al. 2003, Mohr, Gross et al. 2015). *Fscn1* can also be expressed by tumor cells *de novo* (Grothey, Hashizume et al. 2000). The upregulation of *Fscn1* in malignant cells has been associated with enhanced tumor cell motility, contributing to increased invasiveness and metastatic potential (Li, Chan et al. 2022). Transcriptional targeting in cancer gene therapy is conferred for example by the tyrosinase promoter to achieve melanoma-specific gene expression. Tyrosinase is a key enzyme in melanin synthesis that is highly expressed in melanoma cells (Honda, Matsuda et al. 2024). The tyrosinase promoter was used to control the expression of the E1A gene in an oncolytic adenovirus. This resulted in more than 200-fold selective viral replication and cytolysis in melanoma cells compared to normal cells (Nettelbeck, Rivera et al. 2002).

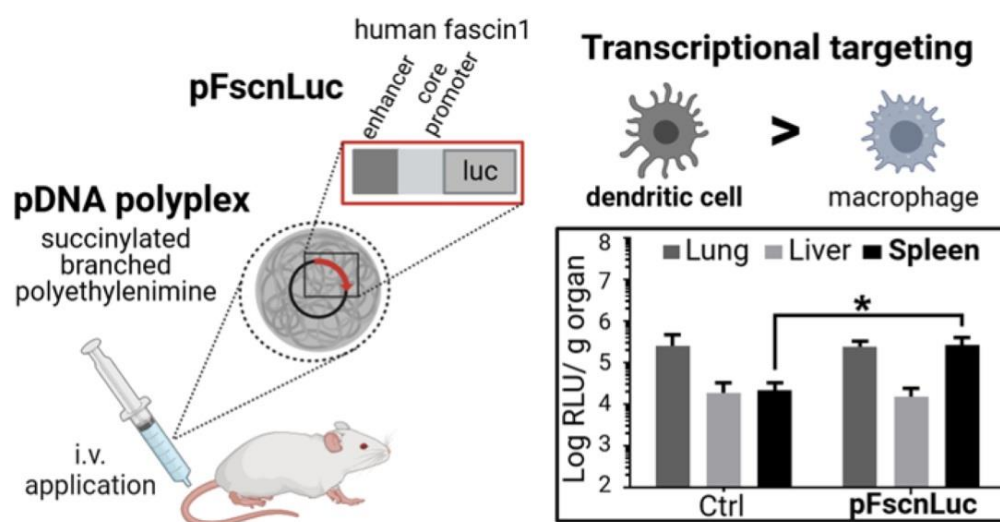


Figure 14. Graphical representation of the study by Zeyn et al. investigating an optimized human *Fscn1* promoter *in vitro* and *in vivo*. Human *fascin1* promoter/enhancer construct was complexed with succinylated branched polyethylenimine to achieve DC-focused transcriptional targeting. Intravenous application of complexed pFscnLuc led to enhanced luciferase expression in the spleen compared to a control vector. (Zeyn, Hobernik et al. 2023).

The suitability of an optimized human *Fscn1* promoter to drive DC-focused transgene expression in DNA vaccines was investigated by **Zeyn** and coworkers, and its performance was compared to the commonly used CMV (cytomegalovirus) promoter (**Zeyn**, Hobernik et al. 2023). *In vitro* experiments revealed that the optimized *Fscn1* promoter demonstrated significantly higher activity in DC2.4 cells, as reflected by elevated Luciferase reporter expression compared to the CMV promoter, while showing minimal activity in other cell types like macrophages (RAW264.7) or B cells (A20). The DC-focused activity was attributed to the hybrid design of the promoter, which combines the DC-specific enhancer with the core promoter of the *Fscn1* gene to limit off-target expression. Interestingly, the *Fscn1* promoter also showed activity in tumor cells. Certain cancer types, including neuroblastoma (N2a) and hepatoma (Hepa1-6) cells, had detectable *Fscn1* expression, which consequently enabled the promoter to achieve expression levels comparable to the CMV promoter in these cancer cells.

Furthermore, the study by **Zeyn** et al. evaluated different PEI carriers for DNA delivery. Linear PEI (LPEI) (Wightman, Kircheis et al. 2001) at higher N/P ratios (N/P 9) improved transfection in DC2.4 cells accompanied by increased cytotoxicity, whereas succinylated branched PEI (succPEI) (Zintchenko, Philipp et al. 2008) showed slightly lower efficiency but maintained viability of the tested cell lines. These findings highlight the need for carrier optimization to maximize DNA vaccine safety and efficacy.

In vivo experiments further validated the targeting specificity of the *Fscn1* promoter. Following i.v. injection of polyplexes containing the *Fscn1*-driven luciferase construct

(pFscnLuc), its expression was predominantly observed in the spleen. pFscnLuc complexed with succPEI exhibited significantly stronger luciferase expression in the spleen than pCMVLuc, a luciferase encoding vector, which is driven by the strong and ubiquitously active CMV promoter. While pCMVLuc demonstrated high, ubiquitous expression, pFscnLuc showed a more DC-focused expression, especially in spleen and lymph nodes. The ratio of transfection of DC compared to macrophages was higher for pFscnLuc, confirming its potential for DC-focused transcriptional targeting.

Overall, the *Fscn1* promoter appears to be a promising tool for DC-focused DNA vaccines by offering targeted transfection of DCs and direct tumor targeting. This dual functionality suggests that vaccines containing the *Fscn1* promoter could simultaneously activate DCs for antigen presentation and deliver therapeutic genes (e.g., TNF- α) directly to tumors, what may enhance antitumor efficacy (**Zeyn**, Hobernik et al. 2023). In this context, TNF- α may function as an adjuvant within APCs, while also exerting direct cytotoxic effects on tumor cells through receptor-mediated signaling, particularly via TNFR1 (tumor necrosis factor receptor 1), which can induce apoptotic cell death (Jiang, Yu et al. 2017).

4. Outlook

Targeting and activating DCs and hepatic APCs to overcome liver-induced immune tolerance was the common aim of the studies presented in this thesis. This issue was addressed through the evaluation and optimization of adjuvants, antigen, and suitable delivery systems.

The results of this work (Fig. 15) provide a basis for further investigations. The transfection efficiency and immunostimulatory properties of CpG ODNs could be examined *in vivo*, co-formulated with antigen mRNA to determine its potential for enhancing antigen-specific immune responses. Additionally, the effects of the discussed adjuvants require further *in vivo* characterization. Specifically, co-encapsulating adjuvants alongside mRNA or antigen peptide by nanocarriers may yield stronger effects than their parallel application. Beyond, the efficacy of LAF-Stp carrier systems could be studied in both *ex vivo* and *in vivo* models to evaluate their potential for enhancing T cell responses. In addition, its performance in disease models should be investigated to assess its potential for immunotherapeutic applications. A further optimization of LAF-Stp carrier structure and formulation could lead to even more potent and selective mRNA delivery systems. The transcriptional targeting of DCs using the *Fscn1* promoter provided a way to drive antigen expression rather selectively within DCs. Its dual potential to be also active in *Fscn1* expressing tumor cells may be exploited in further *in vivo* studies. For instance, in a tumor model, mice could be treated with the *Fscn1* enhancer/promoter construct encoding a tumor antigen to evaluate the antigen expression specificity. Finally, another promising strategy for future investigation involves the integration of antigen peptides into the TM-HSA. This approach could be explored in *in vivo* tumor models for its therapeutic potential, particularly in the context of liver cancer. This could provide valuable insights into their ability to induce anti-tumor immune responses and improve treatment outcomes in hepatic malignancies. In a further approach, trimannose-mediated targeting could also be combined with transcriptional targeting to achieve more precise and efficient delivery and expression in DCs. For example, nanoparticles carrying trimannose-moiety on their surface and encapsulating a *Fscn1* promoter driving TNF- α gene expression could specifically target and activate DCs.

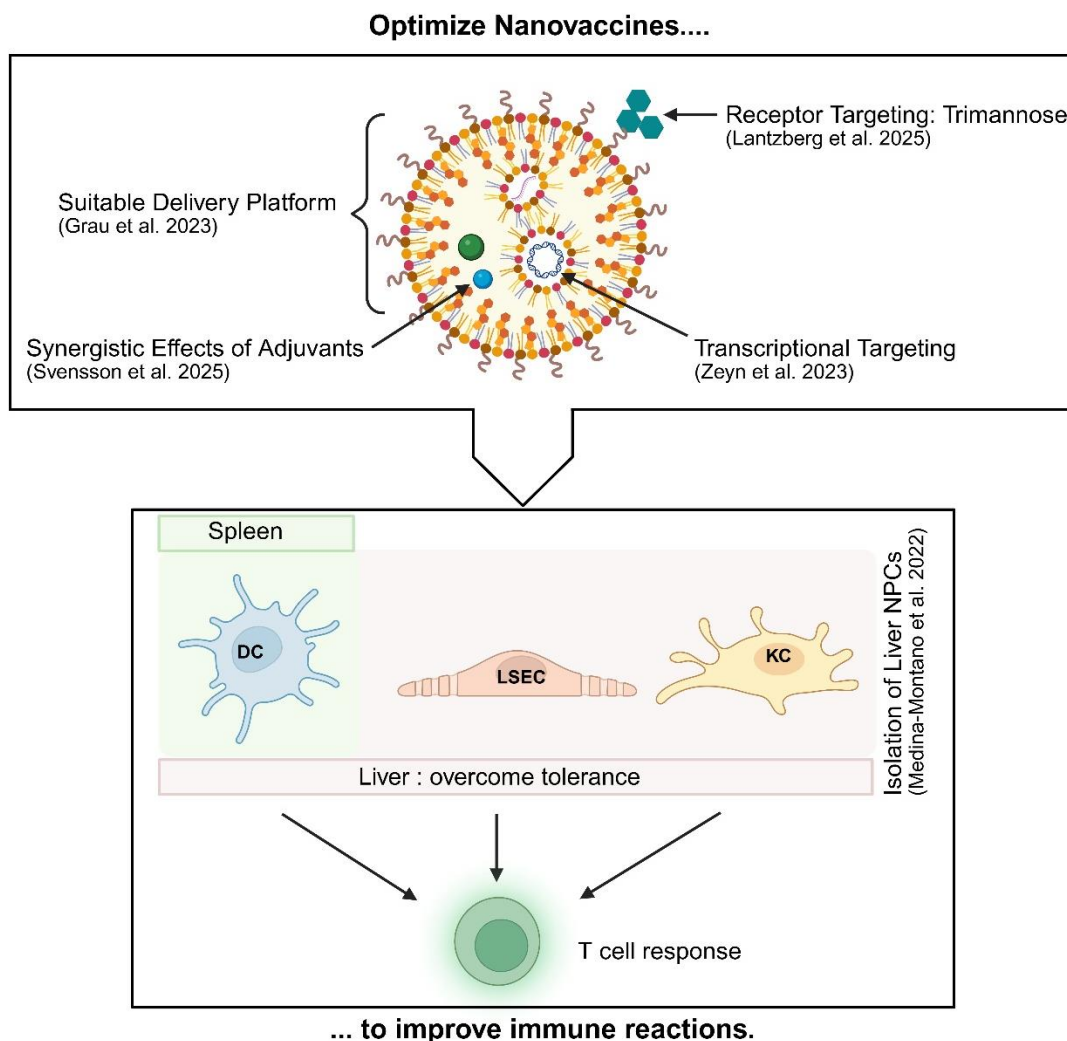


Figure 15. Graphical summary and correlations of the studies investigated in this thesis. The isolation methods for liver NPCs were optimized to ensure reproducibility and reliability in downstream assays (Medina-Montano et al. 2022). Furthermore, the effects of combinations of commonly used adjuvants for nanovaccination were systematically evaluated and refined (Svensson et al. 2025). Selective targeting of liver NPCs was optimized by incorporating a DC-SIGN-targeting moiety like trimannose (Lantzberg et al. 2025). Transcriptional targeting using a DC-focused promoter/enhancer was evaluated to achieve reporter gene expression in DCs (Zeyn et al. 2023). In addition, nanovaccine delivery platforms were further optimized by integrating lipoamino xenopeptide units into LNPs, resulting in improved transfection efficiency (Grau et al. 2023). Collectively, these approaches aim to enable precise activation and antigen expression in DCs and liver NPCs, thereby promoting T cell responses for enhanced immunological efficacy. These strategies may be combined into one approach to synergistically improve selective uptake, cellular activation, and translational efficiency in liver NPCs (see Abbreviations). Figure Created with BioRender.

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6. Appendix

6.1. Publication 1

“Enrichment Methods for Murine Liver Non-Parenchymal Cells Differentially Affect Their Immunophenotype and Responsiveness towards Stimulation”

Published in: *International Journal of Molecular Sciences*. **2022** Jun 11;23(12):6543

DOI: 10.3390/ijms23126543

This study evaluates liver perfusion (LP) and liver dissociation (LD) methods for isolating murine liver non-parenchymal cells (NPCs), including Kupffer cells, dendritic cells, and liver sinusoidal endothelial cells. Both methods yielded similar cell numbers and compositions, but LD-derived NPCs exhibited a higher basal activation state after overnight cultivation, making them less responsive to stimulation with TLR ligands. In contrast, LP-derived NPCs were less pre-activated and more responsive to immunostimulatory signals, making LP the preferred method for *in vitro* functional studies. LD, however, is better suited for high-throughput experiments and *ex vivo* analysis of freshly isolated samples due to its efficiency in processing multiple samples simultaneously.

Contribution:

As co-author of this paper, I carried out several *in vitro* experiments, their analysis and visualization.

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Medina-Montano C, Cacicedo ML, Svensson M, Limeres MJ, **Zeyn Y**, Chaves- Giraldo JE, Rohrig N, Grabbe S, Gehring S, Bros M. Enrichment Methods for Murine Liver Non-Parenchymal Cells Differentially Affect Their Immunophenotype and Responsiveness towards Stimulation. *Int J Mol Sci*. 2022 Jun 11;23(12):6543. doi: 10.3390/ijms23126543. PMID: 35742987; PMCID: PMC9223567.



Communication

Enrichment Methods for Murine Liver Non-Parenchymal Cells Differentially Affect Their Immunophenotype and Responsiveness towards Stimulation

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Citation: Medina-Montano, C.; Cacicedo, M.L.; Svensson, M.; Limeres, M.J.; Zeyn, Y.; Chaves-Giraldo, J.E.; Röhrig, N.; Grabbe, S.; Gehring, S.; Bros, M. Enrichment Methods for Murine Liver Non-Parenchymal Cells Differentially Affect Their Immunophenotype and Responsiveness towards Stimulation. *Int. J. Mol. Sci.* **2022**, *23*, 6543. <https://doi.org/10.3390/ijms23126543>

Academic Editor: Jin-Kyu Park

Received: 20 May 2022

Accepted: 9 June 2022

Published: 11 June 2022

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Abstract: Hepatocytes comprise the majority of the liver and largely exert metabolic functions, whereas non-parenchymal cells (NPCs)—comprising Kupffer cells, dendritic cells and liver sinusoidal endothelial cells—control the immunological state within this organ. Here, we compared the suitability of two isolation methods for murine liver NPCs. Liver perfusion (LP) with collagenase/DNase I applied via the portal vein leads to efficient liver digestion, whereas the modified liver dissociation (LD) method combines mechanical dissociation of the retrieved organ with enzymatic degradation of the extracellular matrix. In cases of both LP and LD, NPCs were enriched by subsequent gradient density centrifugation. Our results indicate that LP and LD are largely comparable with regards to the yield, purity, and composition of liver NPCs. However, LD-enriched liver NPCs displayed a higher degree of activation after overnight cultivation, and accordingly were less responsive towards stimulation with toll-like receptor ligands that are frequently used as adjuvants, e.g., in nano-vaccines. We conclude that LP is more suitable for obtaining liver NPCs for subsequent in vitro studies, whereas LD as the less laborious method, is more convenient for parallel isolation of larger numbers of samples for ex vivo analysis.

Keywords: liver perfusion; liver dissociation; liver sinusoidal endothelial cells; Kupffer cells; dendritic cells; adjuvant; toll-like receptor ligand; liposome

1. Introduction

The liver is a central metabolic organ, but also plays an important immunological role, serving to maintain tolerance under homeostatic conditions [1–3]. The liver is comprised of hepatocytes, which make up approximately two thirds of its total cell population and exert largely metabolic functions, comprising the synthesis of numerous types of plasma proteins—for example, albumin [4], fatty acids [5], carbohydrates [6], and bile acid metabolism [7]—but are also pivotal for detoxification [8]. The heterogeneous group of non-parenchymal cells (NPCs) plays an important role in immune regulation [9]. Liver NPCs comprise liver sinusoidal endothelial cells (LSECs, approximately 50% of NPCs), Kupffer cells (KCs, 20%) that largely constitute the resident macrophage population, and dendritic cells (DCs, 25%). The minor fraction of liver NPCs is mainly composed of innate immune cells comprising natural killer (NK) cells and NKT cells [10], biliary epithelial cells [7],

and hepatic stellate cells (HSC) [11]. The latter are located in the perisinusoidal region between the sinoids and hepatocytes, and under steady state conditions remain in a quiescent state, but play an important role in the induction of liver diseases [12]. HSC are activated in response to liver damage and in this state constitute the main producers of extracellular matrix factors—thereby promoting liver fibrosis [13]. Furthermore, chronic inflammation of a fibrotic liver enhances the likeliness of hepatocellular carcinoma (HCC) development [14]. HCC in turn reprograms HSC to differentiate towards cancer-associated fibroblasts, which besides other cell types such as tumor-associated macrophages, shape the immune-evasive tumor microenvironment [15]. With regard to the major liver NPC fractions, KCs were recognized early on as promoting liver diseases [16] such as nonalcoholic fatty liver disease [17] and alcoholic hepatitis [18]. In general, KCs have been attributed protolerogenic functions [19,20]. Similar to KCs, liver DCs have been reported to exert protolerogenic functions under steady state conditions [21], but have also been reported to display a stimulated, proimmunogenic state at later stages of NAFLD and to contribute to liver fibrosis [22].

LSECs form the lining of hepatic sinusoids and are characterized by numerous pore-like structures, termed fenestrae, which allow the rapid transport of, e.g., lipoproteins from hepatocytes into the sinusoidal blood stream [23]. Furthermore, LSECs release vasodilatory mediators upon shear stress, and impaired generation of these factors has been implicated in non-alcoholic fatty liver disease [24]. Moreover, LSECs express numerous types of endocytic scavenger and C-type lectin receptors [25] as well as the Fcγ receptor IIb (CD32) [26], which enable the uptake of different types of pathogens [27,28] and immune complexes [26] and promote immune homeostasis by inducing regulatory T cells [29].

Altogether, liver NPCs on the one hand have the common property of maintaining antigen-specific T cell tolerance under homeostatic conditions [30,31], but on the other hand, may contribute to NAFLD and other liver diseases in cases of dysfunction [24,32,33]. Liver NPCs have also come into the focus of nanodrug-associated research as a large fraction of systemically applied nano-formulations accumulates in the liver [34–36]. Nanodrugs have the potential to co-deliver various agents in a tissue- and even cell type-specific manner, thereby reducing toxicity and other unwanted side effects associated with non-specific uptake of conventional therapeutics [37]. Therefore, both with regard to nanodrugs designed for the treatment of liver diseases [38] as well as nano-formulations developed to address other organs and cell types, it is highly important to address potential uptake by the liver.

In this regard, most studies have demonstrated endocytic uptake of nanodrugs by KCs [39–42]. Moreover, we and others have reported that DCs and LSECs may also significantly contribute in this regard [36]. The overall T cell tolerance-promoting function of liver NPCs may even be detrimental in cases of nano-vaccines developed to address antigen-presenting cells such as DCs in secondary lymphoid organs aimed at inducing tumor antigen-specific immune responses [9]. Therefore, it is of general interest to study in detail the interaction of liver NPCs with biologicals in vitro to test and eventually optimize their cell-targeting properties for subsequent in vivo studies. Along this line, ex vivo analysis of liver NPCs at the end of in vivo experiments enables the evaluation of in vivo cell type-targeting behavior and payload-dependent functional effects.

So far, liver perfusion (LP) is the standard method for NPC enrichment but requires laborious in situ pretreatment of the organ with collagenase and DNase I [43–46]. Hence, this approach limits the number of samples that can be processed. This issue may be especially important when liver NPCs need to be isolated from a number of animals after in vivo treatment for ex vivo analysis. Therefore, we compared the suitability of LP with ex vivo liver dissociation (LD) using a commercially available kit that allows for the parallel processing of numerous samples. We showed that both liver NPC enrichment procedures yielded similar results in terms of overall cell numbers, composition, and viability when assessed directly after isolation. However, after overnight cultivation, NPCs enriched by LD displayed a higher state of activation and were less responsive towards stimulation with different toll-like receptor (TLR) ligands. Altogether, our results show that both methods are suitable for liver NPC isolation. LD proved less laborious than LP but might be more

suitable in cases of direct ex vivo analysis of freshly isolated samples, whereas LP is the method of choice when liver NPCs are to be subjected to functional studies in vitro.

2. Results

2.1. LP and LD Yield Comparable Numbers of Enriched Viable NPCs

LP-mediated NPC enrichment starts with in situ perfusion of the liver via the portal vein with a collagenase/DNase I digestion mixture (Figure 1, left panel), whereas in cases of LD, only extra-corporal enzymatic tissue digestion of liver tissue is performed (Figure 1, right panel). Hence, LP is more demanding and laborious compared to LD. We modified the LD protocol by performing subsequent density centrifugation to enrich NPCs in the interphase (Figure 1, lower panel). This step is part of the LP method by default.

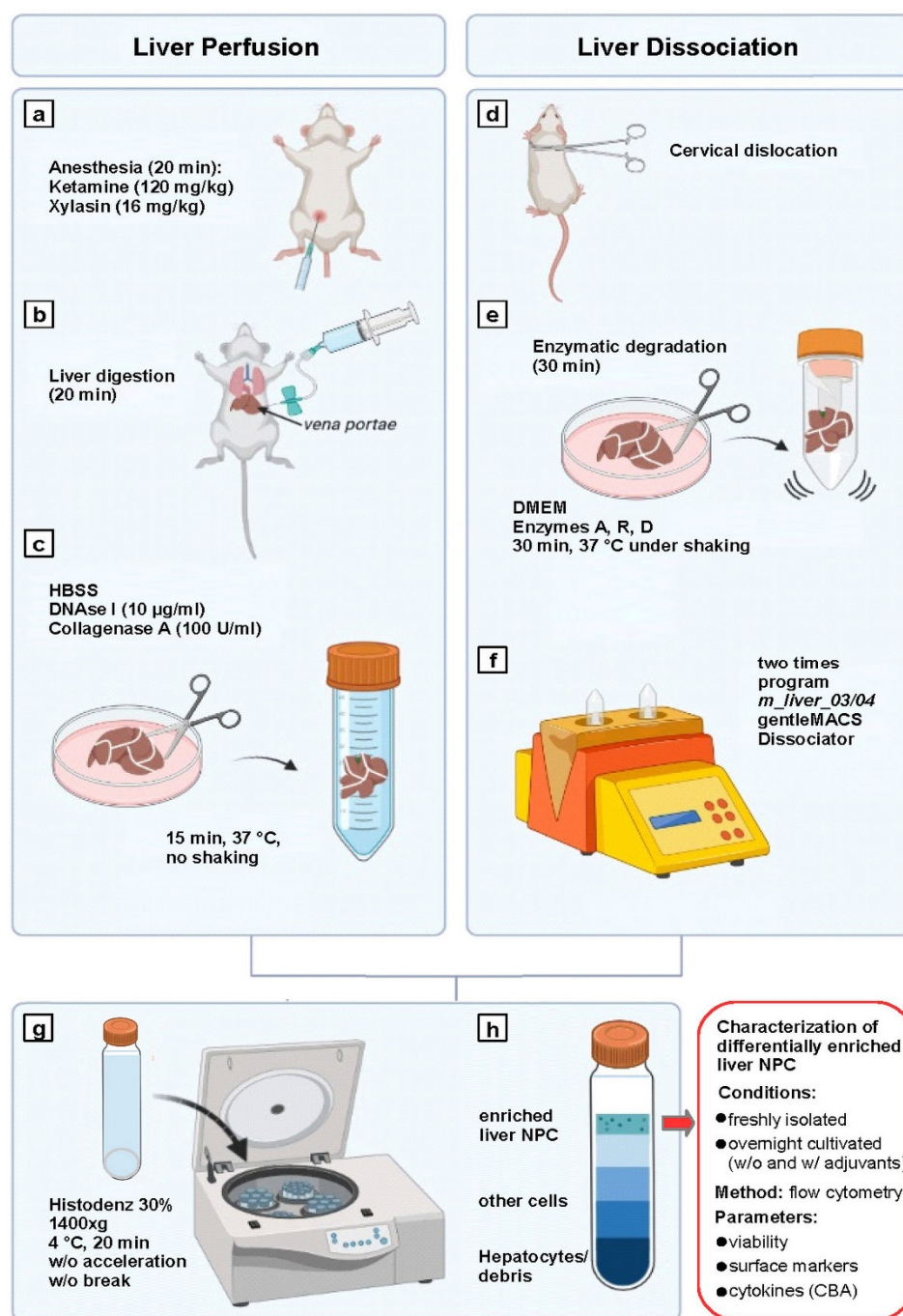


Figure 1. Schematic overview of liver non-parenchymal cell (NPC) enrichment by liver perfusion (LP)

and liver dissociation (LD) and subsequent experimental readout. Left panel: LP mice were (a) anesthetized with ketamine/Xylazine, (b) the vena portae was cannulated and collagenase A/DNase I-containing PBS was infused for in situ tissue digestion. (c) The liver was retrieved, cut into pieces and further digested with collagenase A/DNase I. Liver tissue pieces were mashed to obtain a single cell suspension (not shown). Right panel: LD mice were (d) killed by cervical dislocation, and (e) the retrieved liver was cut into pieces and digested in an enzyme cocktail of proprietary composition (Miltenyi Biotec), (f) followed by mechanical dissociation using a gentleMACS (Miltenyi Biotec). Lower left panel: Liver cell suspensions generated by either method were (g) subjected to Histodenz density gradient centrifugation, (h) resulting in enrichment of liver NPCs in the interphase that was subjected to subsequent experiments. Lower right panel: differentially enriched liver NPCs were characterized in a freshly isolated state and after overnight incubation w/o and in the presence of adjuvants with regard to viability, surface marker expression, and cytokine contents by flow cytometry. (created with BioRender.com; accessed on 1 June 2022).

Both liver NPC enrichment methods resulted in similar outcomes in terms of total yield (Figure 2A), the frequency of enriched $CD45^+$ liver NPCs (50–60%; Figure 2B) and the composition of the NPC population with regard to $F4/80^+$ macrophages (MACs), $CD11c^+$ DCs, and $CD32b^+$ LSECs (Figure 2C). However, it is worth mentioning that after density centrifugation, cell suspensions obtained by LD contained more debris than observed when performing LP (not shown).

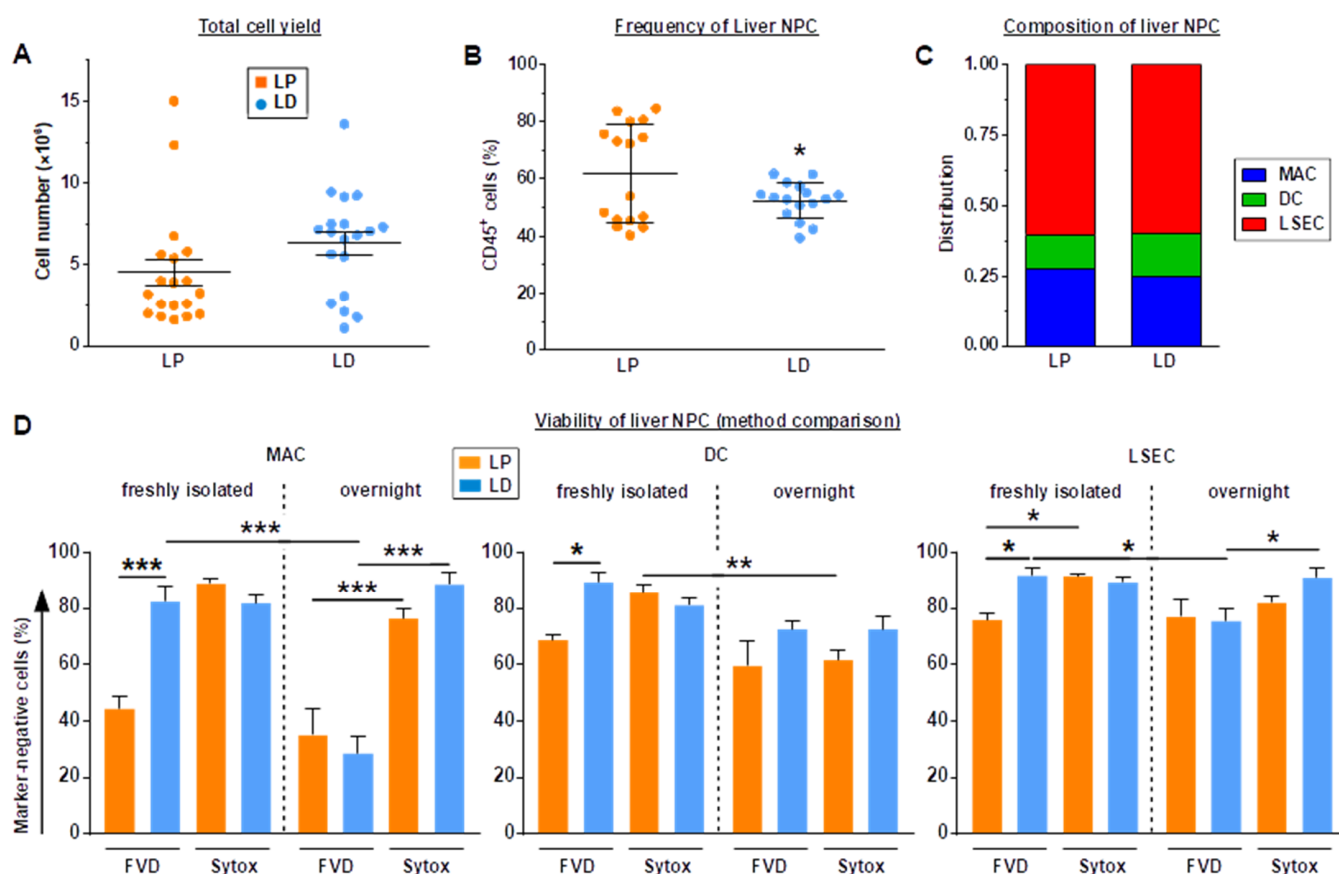


Figure 2. LP and LD yield comparable numbers and composition of liver NPCs, but LD-enriched cells appear dead when using FVD. (A) Total cell yield obtained after density centrifugation (mean $\pm$ SEM, each $n = 19$). (B) Frequencies of $CD45^+$ cells within the enriched NPC suspensions were assessed by flow cytometry (mean $\pm$ SEM, each $n = 19$). (C) Composition of liver NPCs ($CD11c^+$ DCs, $F4/80^+$ MACs, $CD32b^+$ LSECs) within the $CD45^+$ cell population (mean, each $n = 19$; the gating strategy is shown in Figure S1). Statistically significant differences versus (B) *LP (t -test) and (D) * between groups (two-way ANOVA/Tukey test) are indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Next, we investigated the viability of enriched liver NPC populations. To this end, we employed FVD, which binds intracellular primary amines of dead cells [47]. In contrast to other live/dead markers, FVD-treated samples can be fixated, which, in contrast to DNA-intercalating agents (see below), allows flow cytometric analysis at later time points.

Unexpectedly, LP-enriched MACs displayed rather strong interactions with the FVD, which suggested that their viability was about 42% only as compared to about 80% in the case of LD-enriched MACs (Figure 2D, left panel). After overnight incubation, MACs interacted with the FVD to a high extent, irrespective of the liver NPC enrichment method—suggesting viability rates below 40%. On the contrary, incubation of liver NPCs both directly after isolation as well as after overnight cultivation with Sytox, which intercalates the genomic DNA of dead cells [48], in general delineated >75% of MACs as viable. Freshly isolated DCs (Figure 2D, middle panel) and LSECs (Figure 2D, right panel) displayed similar FVD/Sytox binding patterns, showing moderately lower viability when enriched by LP and incubated with FVD, whereas no difference in viability was apparent in the case of Sytox incubation. These method-dependent differences were less obvious after overnight incubation, suggesting largely comparable viability rates determined by either viability dye. Similar results were obtained when using FVD conjugated with a distinct fluorochrome, and 7-AAD instead of Sytox (not shown).

Taken together, these observations suggest that LP-mediated enrichment of liver NPCs resulted in stronger interactions with the FVD than after LD enrichment (MACs > DCs, LSECs).

The unexpected finding of enhanced engagement of the FVD by MACs prompted us to perform a comparative viability analysis of spleen cells after isolation as well as after overnight incubation using FVD and Sytox in parallel (Figure S2). None of the freshly isolated splenic immune cell types showed stronger engagement of the FVD than of Sytox. However, after overnight culture B cells, MACs and PMNs displayed a lower viability rate when using FVD for assessment as compared to Sytox. Comparable findings were made when using FVD with a distinct fluorochrome and 7-AAD instead of Sytox (not shown).

Due to the cardinal advantage of FVD over Sytox/7-AAD to fixate samples after treatment, enabling flow cytometric measurements at later time points, we also tested whether Annexin V—which detects phosphatidyl serin on the surface of early/late apoptotic cells [49] and allows for sample fixation as well—could be used instead of FVD. However, a considerable proportion of LD-enriched liver NPCs engaged Annexin V to a similar extent as that observed for FVD (Figure S3).

Altogether, these observations suggest that DNA-intercalating agents (Sytox, 7-AAD) are more reliable for the viability analysis of liver NPCs.

2.2. Liver NPCs Enriched by LD Express Costimulatory Receptors at Higher Levels and Are Partially Refractory towards Stimulation

Our findings on the differential binding of liver NPCs to FVD viability dyes suggested that both liver NPC enrichment procedures may also affect their overall state of activation. Overnight-cultivated liver NPC populations displayed no method-associated differences in expression of the antigen-presenting receptor MHCII (Figure 3, Ctrl groups). In contrast, MACs, DCs and LSECs expressed the costimulatory receptors CD80 and CD86 to a higher extent when enriched by LD as compared to LP.

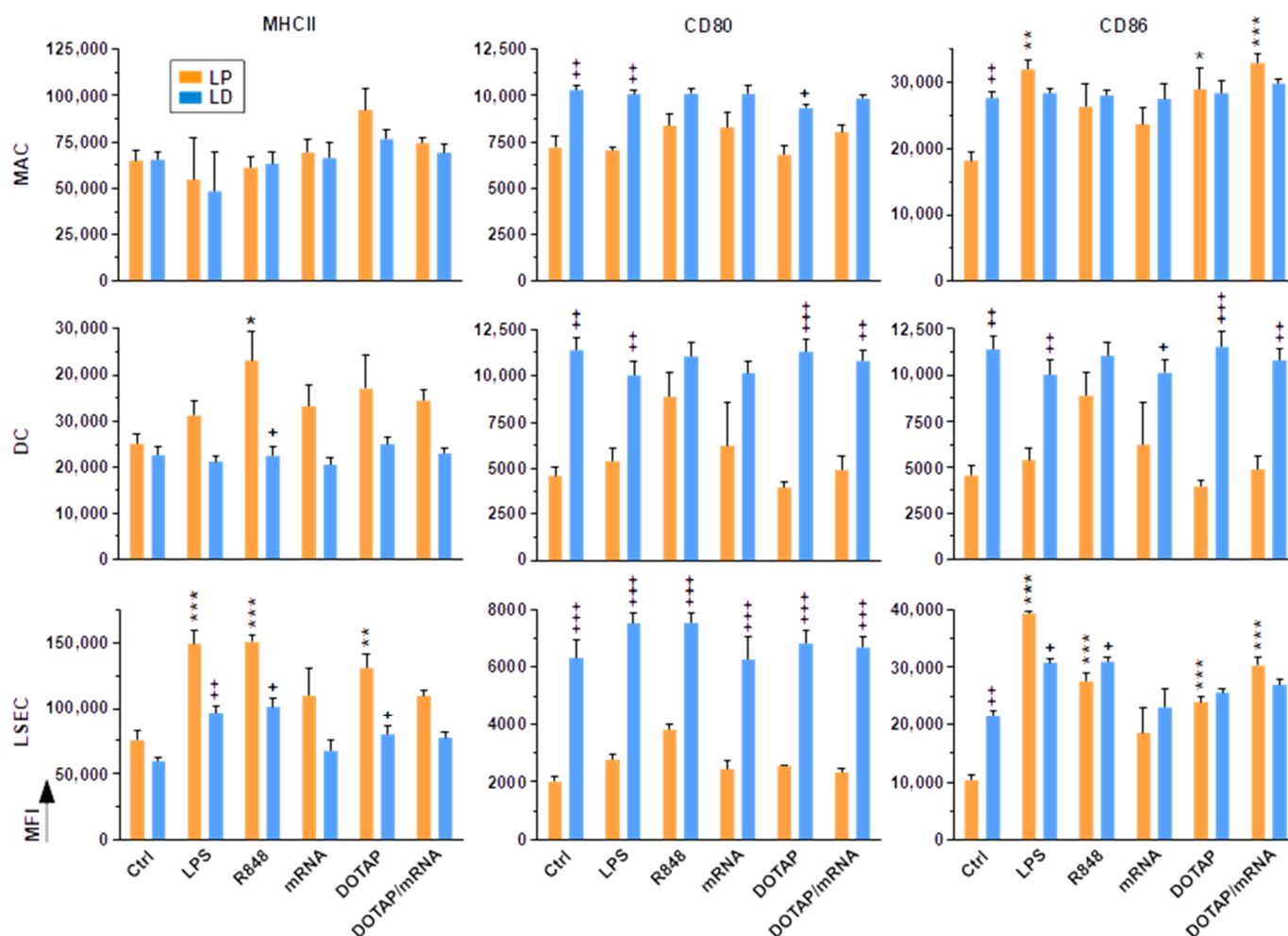


Figure 3. LD-enriched liver NPCs are pre-activated, as deduced from elevated activation marker expression. Liver NPCs enriched by LP and LD were seeded into wells of 96 well plates ($10^5/100 \mu\text{L}$) and stimulated overnight with the agents indicated or left untreated. The man fluorescence intensity (MFI) of surface markers was assessed by flow cytometry (mean $\pm$ SEM, $n = 4$). Statistically significant differences versus * according Ctrl and versus +LP are indicated (one way ANOVA, Tukey test). *, + $p < 0.05$, **, ++ $p < 0.01$, ***, +++ $p < 0.001$.

To assess the suitability of LP- and LD-derived liver NPCs for in vitro testing of adjuvants, e.g., as a component of nano-vaccines, we applied several TLR ligands—including the TLR4 ligand LPS, the TLR7/8 agonist R848, native mRNA (TLR3 agonist), the well-established liposomal carrier DOTAP, and DOTAP/mRNA complexes. MHCII expression of MACs enriched by either of the methods displayed was not altered by either stimulus. Similar observations were made for the co-stimulator CD80. In contrast, CD86 expression was upregulated in the case of LP-enriched MACs in response to either stimulus, whereas LD-derived MACs remained unresponsive in this regard. LP-enriched DCs upregulated MHCII and showed similar tendencies regarding both co-stimulators in response to stimulation, whereas DCs pre-activated due to enrichment by LD were refractory.

LP-enriched LSECs showed increased MHCII expression in response to LPS, R848 and DOTAP, but LD-enriched LSECs displayed only very moderately elevated levels. Similar to MACs and DCs, LD-derived LSECs in general scarcely upregulated either co-stimulator in response to stimulation. In contrast, LP-derived LSECs showed somewhat increased CD80 expression when stimulated with R848 and significant upregulation of CD86 upon application of most stimuli.

In line with the partially activated state of LD-enriched liver NPC populations after overnight cultivation, the respective cell culture supernatants contained higher concentrations of TNF- α , IL-6, and IL-10 than in the case of LP-derived NPCs (Figure 4). Type I (IFN- α and IFN- β) and type II (IFN- γ) interferons were scarcely detectable under basal conditions. Most cytokines, except for IFN- α , were upregulated to the highest extent by stimulation with R848 and to a similar extent, in an enrichment method-independent manner. Only IL-10 showed markedly higher absolute levels in the case of LD-enriched NPCs than those observed for LP-derived NPCs. Stimulation with LPS also increased cytokine concentrations, except for IFN- α , albeit to a lower extent than that induced by R848. Again, for some cytokines (TNF- α , IL-6, IL-10), levels were somewhat higher in the case of LD-enriched NPCs. Only in the case of IFN- γ , LP-derived NPCs produced more cytokine than observed for LD-enriched NPCs. In RNAi and DOTAP RNAi applied separately, applied separately, exerted no effect on the production of either cytokine, whereas for IFN- γ , which was markedly upregulated in the NPCs of enriched with DOTAP RNAi. In RNAi, DOTAP RNAi, specifically increased specifically increased type-I IFN, interestingly, to a degree, interestingly, to a higher extent LD-derived liver NPCs LD-derived liver NPCs.

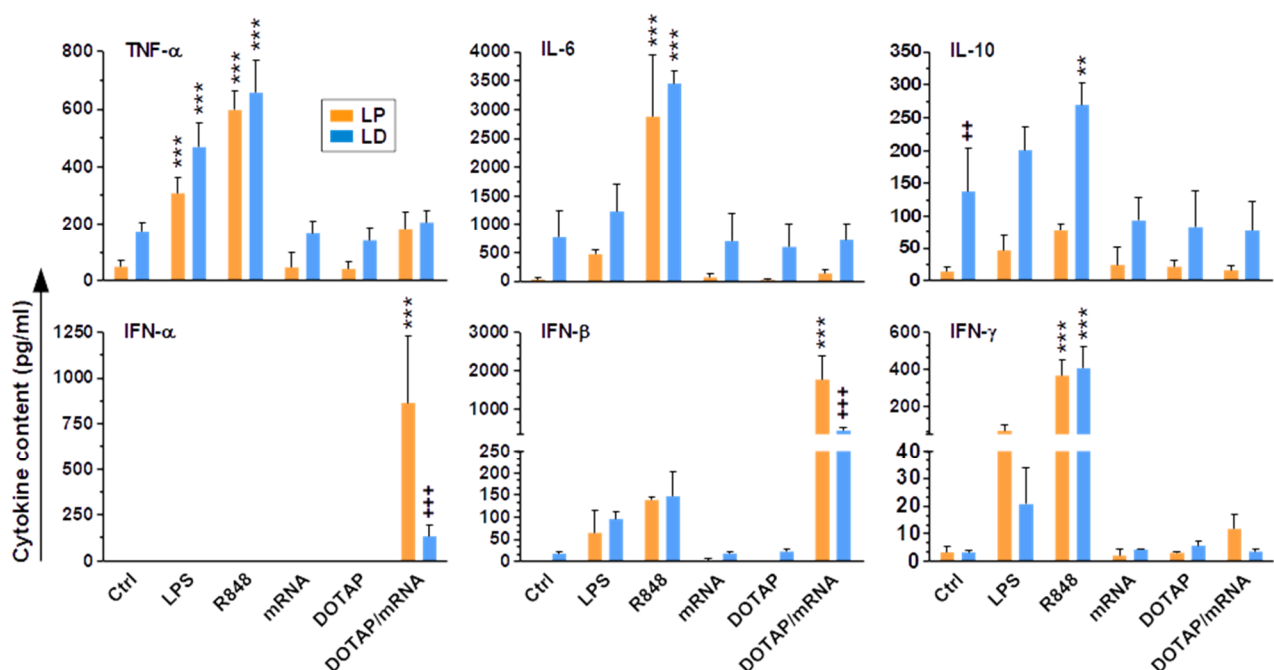


Figure 4. Liver NPCs generate cytokines in an adjuvant-specific manner and depending on the enrichment method used. Supernatants of differentially stimulated liver NPCs (see Figure 3) were assayed for cytokine contents in culture supernatants (mean $\pm$ SEM, $n = 3$). Statistically significant differences versus * according Ctrl and versus +LP are indicated (one way ANOVA, Tukey test). **, ++ $p < 0.01$, ***, +++ $p < 0.001$.

Altogether, these results suggest that LD results in the partial activation of enriched liver NPCs, which in the case of surface activation markers prevented stimulation-dependent upregulation. In agreement, LD-enriched liver NPCs showed higher basal levels of some cytokines, but in contrast to inducing non-responsiveness on the level of surface markers, were capable of upregulating cytokine production in response to stimulation.

3. Discussion

The immunological role of liver NPCs, and the frequent observation that nanodrugs intended to address other cell types are frequently enriched in this organ, underline the importance of studying the interactions of liver NPC populations with nanodrugs and the functional consequences thereof. Furthermore, liver NPCs have also been recognized as

interesting target population for nanodrug-mediated approaches aimed at exploiting their intrinsic protolerogenic potential to inhibit unwanted immune reactions, as in the case of allergies [50]. Moreover, in the case of anti-tumor nano-vaccination approaches, it may be an important issue to overcome their tolerance-promoting properties by applying suitable adjuvants, as assessed in this study [9].

Therefore, the suitability of liver NPC isolation methods is an important issue. In this regard, LP has been well established over the last decades, and numerous modifications have been tested—as summarized in Table 1. However, LP requires time-consuming in situ treatment of the liver, which may limit the number of samples that can be processed, e.g., for ex vivo analysis after in vivo treatments—as for example in the case of biodistribution studies of nano-carriers [36]. Therefore, we compared LP with LD as an alternative approach in this study.

Table 1. Protocols for liver NPC enrichment.

Liver Digestion	Access to the Liver	Species	NPC Yield ($\times 10^6$)	Gradient Centrifugation	Viability (%) ¹	Reference
Perfusion (aeration apparatus)	Vp	r	-	-	-	[51]
0.05% Collagenase/0.10% hyaluronidase	Vp	r	-	-	-	[52]
Pronase (<i>Streptomyces griseus</i>)	Vp	r	2–15	yes	-	[53]
Pronase (<i>Streptomyces griseus</i>)	-	r	14/gr	-	-	[54]
Collagenase	-	r	0.5–0.6/gr	Percoll	-	[55]
Pronase/DNase/Collagenase	Vp	r	26 (LSEC), 13 (KC)	Stratan	90	[56]
Collagenase	Vp	m	9	Metrizamide	-	[57]
Collagenase/DNase	Vp	m	-	Histodenz	-	[43]
Collagenase/DNase	Vp	m	-	Histodenz	-	[44]
Collagenase IV/Pronase	Vp	m	-	Percoll	97	[9]
Collagenase IV (<i>C. histolyticum</i>)	ex vivo	h	KC: 1.8, LSEC: 4.3/gr	yes	-	[58]
EGTA/collagenase P	ex vivo	h	1.9 (KC), 0.27 (LSEC)/gr		>90	[59]
According to [53]	Vp	m	-	yes	-	[60]
Collagenase II	ex vivo	m	5/gr tissue	-	-	[61]
Collagenase I	Vp	r	KC: 3–5, LSEC: 18–20	Percoll	-	[62]
EGTA/collagenase P (two-step)	ex vivo	h	-	Yes	-	[63]
Collagenase/DNase	Vp	m	3.33	Histodenz	-	[36]

h: human, KC: Kupffer cell; LSEC: liver sinusoid endothelial cell; m: murin, Vp: vena portae. ¹ numbers given when viability was assessed in the study.

We show that both enrichment methods yielded overall comparable results in terms of yield of CD45⁺ liver NPCs [9], displaying similar compositions of NPC populations with regard to DCs, MACs, and LSECs. Surprisingly, however, we observed that LD-enriched liver NPCs, and MACs especially, showed unspecific interactions with the FVD, and thereby would be considered dead. Similar findings were also made for the other liver NPCs populations. Furthermore, both LP- and LD-enriched MACs displayed low viability after overnight cultivation when using FVD. In contrast, DNA-intercalating agents (Sytox, 7-AAD) identified only <25% of MACs as dead, even after overnight incubation. Besides this, splenic MACs and PMN were also characterized by stronger interaction with FVD than DNA-intercalating dyes after overnight cultivation. Therefore, these findings suggest that endocytically active cell types in a viable state are prone to internalize FVD, which

favors the use of DNA-binding agents for delineating viability. It is unclear as of yet which receptors may play a role in this regard.

However, LD enrichment also resulted in partial activation of the assessed liver NPC types, which may compromise the outcome of in vitro studies aimed, e.g., at assessing the stimulatory activity of agents—as exemplified in this study for several TLR ligands. In this regard, the responsiveness of LD-dependently pre-activated liver NPCs on the level of surface markers was strongly impaired. LD-enriched NPCs generated easily detectable amounts of TNF- α , IL-6, and IL-10—even in the absence of stimulatory agents—but the difference towards LP-derived NPCs was most apparent in the case of IL-10. This potent anti-inflammatory cytokine has been implicated as an effector molecule upregulated in response to different types of stress [64] and has been shown to contribute in an essential manner to the overall pro-tolerogenic role of liver NPCs [65]. KCs have been reported to express IL-10 receptor at high levels, resulting in sustained activation of the transcription factor signal transducer and activator of transcription (STAT)3 [66]. STAT3 in turn is a major driver of IL-10 expression, thereby establishing a positive feedback loop for this cytokine [67]. However, LD-derived liver NPCs were still largely responsive towards stimulation on the cytokine level. Here, we observed that R848, and to lesser extent LPS, was a potent stimulator of TNF- α , IL-6, IL-10, and IFN- γ production—as previously shown by us and others [68]. In addition, we also demonstrated that complexed mRNA yielded considerable induction of type I IFN, as has also been described for antigen-presenting cells in secondary lymphoid organs upon transfection with mRNA-delivering liposomes [69–73].

Our results show that LP-derived liver NPCs can be used to study, e.g., the stimulatory effects of adjuvant-loaded nanodrugs in terms of surface activation marker expression and cytokine production. With regards to the latter, it will also be interesting to assess cytokine production on the single cell level by intracellular cytokine detection [74]. In the case of fluorescence-labeled nano-carriers, it will also be possible to study their liver NPC type-specific engagement—especially in the case of nanodrugs decorated with, e.g., C-type lectin receptor-targeting moieties such as carbohydrates [75]. Finally, in the case of nano-vaccines that co-deliver an antigen, subsequent cocultures of accordingly pretreated liver NPCs with antigen-specific T cells will show whether adjuvant-mediated NPC activation is able to overcome their intrinsic protolerogenic properties and result in efficient activation of antigen-specific T cells, e.g., to induce profound anti-tumor T cell responses [76].

Altogether, we conclude that LP is more reliable than LD for obtaining liver NPCs for subsequent in vitro studies. However, LP represents a laborious technique and well-trained operators are needed (Table 2). Therefore, LD is the method of choice in the case liver NPCs that need to be derived from a larger number of in vivo-treated animals for subsequent ex vivo analysis. In this case, the potential of LD to process a larger number of samples in parallel enables the completion of NPC isolation within a shorter period of time as compared to the rather continuous mode of handling in the case of LP. Additionally, our finding that LD partially activates NPCs in the course of subsequent overnight culture is not of primary concern when samples are to be analyzed directly after NPC isolation.

Table 2. Advantages and disadvantages of using LP or LD for NPC enrichment.

Aspect		LP	LD
Total yield		+++	+++
Purity (CD45 ⁺)		62%	52%
NPC viability ¹	MAC	89% ² , 76% ³	82%, 89%
	DC	86%, 62%	81%, 72%
	LSEC	92%, 82%	90%, 91%
NPC activation state		o/+	+++
NPC responsiveness towards stimulation		++	o/+
Preparation time		4–5 h	2–3 h
Costs		+	+++

¹ given for Sytox; ² freshly isolated; ³ after overnight culture. O, unaltered; +, low; ++, intermediate, +++, high.

4. Materials and Methods

4.1. Animals

C57BL/6 mice were bred and housed in the Translational Animal Research Center (TARC) of the Johannes Gutenberg-University Mainz under specific pathogen-free conditions on a standard diet, according to the guidelines of the regional animal care committee. The guide for the care and use of laboratory animals [77] as well as the 3R principles in laboratory animal experiments [78] were followed. Mice were sacrificed at an age of between 12–16 weeks for organ retrieval according to § 4(3) TierSchG.

4.2. Spleen Cells

Spleens were mashed using a sterile syringe plunger on a PBS pre-soaked 40 µm cell strainer (EASYstrainer™; Sarstedt, Nümbrecht, Germany). Mashed spleen tissue was washed through the strainer using 10 mL of PBS/2 mM EDTA buffer, and cells were pelleted by centrifugation (1200 rpm, 10 min, 4 °C).

4.3. Enrichment of Liver NPCs

Mice NPCs were enriched by liver perfusion (LP) and liver dissociation (LD). For LP, mice were anesthetized with a Ketamine/Xylazine mixture (Ketamine 120 mg/Kg; Xylasin 16 mg/kg) and the abdominal cavity was opened (see Figure 1, left panel). The vena portae was cannulated and flushed with 20 mL of HBSS (Hank's Balanced Salt Solution; ThermoFisher, Waltham, MA, USA) containing 100 U/mL of collagenase A (Sigma-Aldrich, St. Louis, MO, USA) and 10 µg/mL of DNase I (Sigma-Aldrich, St. Louis, MO, USA). Then, the liver was retrieved, cut into pieces, and incubated for 15 min at 37 °C in a 50 mL tube with PBS containing 100 U/mL of collagenase A and 10 µg/mL of DNase I. Afterwards, the liver tissue was mashed through a 70 µm nylon cell strainer and medium (DMEM (Dulbecco's Modified Eagle Medium)/F-12 GlutaMAX™, ThermoFisher Scientific) was applied. The cell suspension was subjected to density centrifugation using 30% Histodenz-HBSS (both from Sigma-Aldrich, St. Louis, MO, USA) gradient centrifugation as described [45] (see Figure 1, lower panel).

For LD-mediated NPC enrichment, mice were killed by cervical dislocation and the liver was retrieved (see Figure 1, right panel). An enzyme-dependent dissociation mixture of proprietary composition (Liver dissociation kit; Miltenyi Biotec, Bergisch-Gladbach, Germany) was preincubated for 15 min in C tubes (Miltenyi Biotec). Then, liver tissues were cut in little pieces and transferred into prepared C tubes. The latter were placed into a gentleMACS Dissociator and the tissue was minced (two times program m_liver_03). Afterwards, the cell suspension was incubated for 30 min at 37 °C under continuous shaking, followed by another round of gentleMACS-mediated disintegration (two times program m_liver_04). The samples were cleared using a Cell Strainer (100 µm; Sarstedt) and the liver NPCs were enriched by density centrifugation (see above).

4.4. Stimulation of Liver NPCs

Liver NPCs were seeded into wells of 96-well plates (10^5 /100 µL) and were treated overnight with LPS, (100 ng/m; Kenilworth, NJ, USA), R848 (1 µg/m, InvivoGen, San Diego, CA, USA), ovalbumine-encoding mRNA (1 µg/mL; Tri-Link, San Diego, CA, USA), DOTAP (6.4 µL/mL; Roth, Weiden, Germany), and DOTAP/mRNA complexes, which were formed by mixing both components at the mentioned amounts as recommended by the manufacturer (Roth).

4.5. Flow Cytometry

Single cell suspensions were washed (2% FCS in PBS), and Fc receptors were blocked with aCD16/CD32 (clone 2.4G2 clone; Biolegend, San Diego, CA, USA) for 10 min. Afterwards, cells were incubated with receptor-specific antibodies (Table S1) obtained from ThermoFisher (Waltham, MA) or BD Biosciences (Franklin Lakes, NJ, USA) for 20 min at 4 °C. Afterwards, cell viability was assessed by using fixable viability dye (FVD)-450

and -780, 7AAD, Sytox AADvanced (all from ThermoFisher) and Annexin V-AF647 (Biolegend), as recommended by the manufacturer. Samples were subjected to flow cytometry using a Attune NxT Flow Cytometer (Thermo Scientific) and an LSRII (BD Biosciences), and analyzed with Attune NxT ((ThermoScientific and FlowJo software, respectively (BD Biosciences)). The gating strategies for liver NPCs (see Figure S1) and splenic leukocytes (see Figure S2A) are depicted in the supplementals.

4.6. Cytokines

Supernatants of cell cultures were retrieved and stored at -20°C for subsequent analysis. $\text{TNF-}\alpha$, $\text{IFN-}\gamma$, IL-6, IL-1 β , and IL-10 were quantified using a Cytometric Bead Assay (CBA; BD Biosciences) as recommended by the manufacturer. Results were analyzed using FCAP Array Analysis Software v.1.0.1 (BD Biosciences). $\text{IFN-}\alpha$ and $\text{IFN-}\beta$ were measured using Legendplex reagents as recommended and data were analyzed using Qognit Legendplex Analysis Software (both from Biolegend).

4.7. Data Analysis

Data were analyzed using GraphPad PRISM v5 and v9 software (GraphPad Software Inc., San Diego, CA, USA). Data are given as mean $\pm$ SEM for the indicated number of independent experiments. Statistical differences were determined by unpaired Student's *t*-tests when comparing two groups, one way ANOVA followed by post-hoc Tukey's test when comparing multiple groups monitored at a given time point, and two-way ANOVA/Tukey tests when comparing multiple groups assessed at different time points—assuming significant differences at $p < 0.05$ in either case.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms23126543/s1>.

Author Contributions: Conceptualization, C.M.-M., M.L.C. and M.B.; methodology, C.M.-M. and M.L.C.; software, C.M.-M., M.L.C., M.S., M.J.L., Y.Z., J.E.C.-G. and N.R.; validation, C.M.-M., M.L.C., M.S., M.J.L., Y.Z., J.E.C.-G., N.R. and M.B.; formal analysis, C.M.-M., M.L.C., M.S., M.J.L., Y.Z., J.E.C.-G. and N.R.; investigation, C.M.-M., M.L.C., M.S., M.J.L., Y.Z., J.E.C.-G. and N.R.; resources, C.M.-M., M.L.C., M.S., M.J.L., Y.Z., J.E.C.-G. and N.R.; data curation, C.M.-M., M.L.C., Y.Z. and M.B.; writing—original draft preparation, C.M.-M., M.L.C. and M.B.; writing—review and editing, C.M.-M., M.L.C., S.G. (Stephan Grabbe), S.G. (Stephan Gehring) and M.B.; visualization, C.M.-M., M.L.C., Y.Z. and M.B.; supervision, M.L.C., S.G. (Stephan Gehring) and M.B.; project administration, S.G. (Stephan Grabbe), S.G. (Stephan Gehring) and M.B.; funding acquisition, S.G. (Stephan Grabbe), S.G. (Stephan Gehring) and M.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Deutsche Forschungsgemeinschaft (SFB1066), grant numbers B4 (S.G. (Stephan Grabbe)) and B15 (S.G. (Stephan Gehring), M.B. (Matthias Bros)).

Institutional Review Board Statement: Mice were sacrificed for organ retrieval according to § 4(3) TierSchG.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank the Research Center for Immunotherapy (FZI; University Medical Center, Mainz, Germany) for their continuous support.

Conflicts of Interest: The authors declare no conflict of interest.

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6.2. Publication 2

“mRNA-LNP vaccine strategies: Effects of adjuvants on non-parenchymal liver cells and tolerance”

Published in: *Molecular Therapy Methods & Clinical Development*. **2025** Feb 4;33(1):101427

DOI: 10.1016/j.omtm.2025.101427

The induction of a tolerogenic immune environment by liver non-parenchymal cells (NPCs) is a significant challenge for therapeutic applications. In this study, mRNA lipid nanoparticles (LNPs) were combined with adjuvants, including R848, cGAMP and PolyI:C, to overcome this immunological barrier. The adjuvants boosted immune responses, either alone or in combination, by upregulating co-stimulatory molecule expression and inducing cytokine production, with R848 showing the strongest activation across NPC subsets *in vitro*. Combining R848 and cGAMP further increased cytokine secretion and T-cell proliferation. However, R848 interfered *in vivo* with mRNA translation efficiency in some cases. When mRNA-LNPs were administered repeatedly together with adjuvants, immune responses varied significantly. These results highlight the importance of achieving a balance between immunostimulatory effects and mRNA translation efficiency when designing effective liver-targeted vaccines for cancer treatment.

Contribution:

As co-author of this paper, I contributed to all *in vivo* experiments.

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Svensson M, Limeres MJ, **Zeyn Y**, Gambaro RC, Islan GA, Berti IR, Fraude-El Ghazi S, Pretsch L, Hilbert K, Schneider P, Kaps L, Bros M, Gehring S, Cacicedo ML. mRNA-LNP vaccine strategies: Effects of adjuvants on non-parenchymal liver cells and tolerance. *Mol Ther Methods Clin Dev*. 2025 Feb 4;33(1):101427. doi: 10.1016/j.omtm.2025.10142

mRNA-LNP vaccine strategies: Effects of adjuvants on non-parenchymal liver cells and tolerance

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The liver, which plays pivotal roles in metabolism and immunity, often confers tolerance, suppressing immune responses to pathogens. Adjuvanted, lipid nanoparticle-encapsulated mRNA vaccines (mRNA-LNPs) offer a promising approach to overcome immune tolerance. In this study, the immunostimulatory activity of well-documented adjuvants, *i.e.*, 2'3'-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), resiquimod (R848), and polyinosinic:polycytidylic acid (Poly I:C), on non-parenchymal liver cells was determined. When co-applied with mRNA-loaded LNPs, these adjuvants enhanced immune responses at variable extents. Moreover, the efficiency of mRNA translation in the presence of cGAMP was comparable with the non-adjuvanted control. Repetitive co-application of adjuvants with mRNA-LNPs showed improvement in cellular responses when R848 or R848/cGAMP treatments were used. These findings emphasize the need to delineate the delicate balance between immunomodulatory properties and the efficiency of mRNA translation when selecting adjuvants for mRNA-LNP vaccines and offer insights on how to enhance immunity to infectious diseases and cancers that affect the liver.

INTRODUCTION

The liver filters circulating blood that enters via the hepatic artery and portal vein.¹ Consequently, the liver constantly encounters large quantities of innocuous antigens such as gut-associated bacteria, bacterial products, harmless food-derived antigens, and cellular debris. Immune tolerance to these antigens is fundamental to maintain homeostasis. Tolerance is controlled in large part by various populations of conventional and unconventional antigen presenting non-parenchymal cells (NPCs) consisting of Kupffer cells (KCs), dendritic cells (DCs), and liver sinusoidal endothelial cells (LSECs).^{1–3} Notably, the same cell populations play pivotal roles in recognizing antigens as non-self, *i.e.*, danger signals, and eliciting potent *ad hoc* immune responses.

KCs secrete anti-inflammatory, immunosuppressive cytokines (e.g., interleukin [IL]-10, transforming growth factor [TGF]- β) in response

to the continued exposure to bacterial endotoxin.² Similarly, hepatic DCs, which differ fundamentally from the DCs that reside outside the liver, secrete large amounts of IL-10.⁴ The presence of high IL-10 concentrations in the microenvironment contribute to the low-level, cell-surface expression of major histocompatibility complex (MHC I and MHC II) and co-stimulatory molecules, which is characteristic of KCs, DCs, and LSECs residing in the liver under steady-state tolerogenic conditions.⁴

This tolerogenic microenvironment is characterized by T cell dysfunction that occurs due to clonal deletion, anergy, senescence, deviation, exhaustion, and the expansion of the Foxp3⁺ regulatory T (T_{reg}) cell population.⁵ The generation of T_{reg} cells depends on factors secreted by NPCs under steady-state conditions.⁶ For example, TGF- β , secreted mainly by KCs and all-trans retinoic acid produced by LSECs, are essential for the production and maintenance of T_{reg} cells.⁷ Additionally, IL-10 is required to stabilize Foxp3 and to maintain the suppressive T_{reg} cell phenotype.⁸ Furthermore, T_{reg} cells inhibit hepatic DC maturation by physical interaction, thereby preventing up-regulated expression of CD80 and CD86 culminating in a tolerogenic DC state.^{5,8,9}

Immune tolerance may prevent the liver from recognizing foreign antigens associated with infection or malignancy, resulting in chronic pathologies.^{9,10} Novel liver-directed vaccine approaches are urgently needed to overcome the consequences of tolerance and reverse ineffective innate and adaptive immune responses in this organ. The molecular mechanisms involved in regulating tolerance and strategies to boost immunity in the liver remain under investigation.^{11–14} In this

Received 15 August 2024; accepted 31 January 2025;
<https://doi.org/10.1016/j.omtm.2025.101427>.

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regard, adjuvanted immunotherapies offer a means to stimulate strong immune responses against infectious diseases and malignancies.^{9,11,15,16} However, the cellular mechanisms activated in the liver by different adjuvants remain to be elucidated.

Adjuvants, such as 2′3′-cyclic guanosine monophosphate-adenosine monophosphate (cGAMP), resiquimod (R848), and polyinosinic:polycytidylic acid (Poly I:C), elicit innate immune responses by stimulating pattern recognition receptors (PRRs) expressed intracellularly and extracellularly by antigen-presenting cells (APCs).^{17,18} Receptor recognition promotes the cell surface expression of major histocompatibility and co-stimulatory (e.g., CD80 and CD86) molecules, and the secretion of proinflammatory cytokines (e.g., IL-6, IL-12, and tumor necrosis factor [TNF]- α). As such, stimulating PRRs with adjuvants represents a rational approach to reversing tolerance and thereby to enhance hepatic immunity.^{2,19}

mRNA-based vaccines and therapeutics that emerged during the COVID-19 pandemic promise a novel approach to combat infection, cancer and metabolic diseases.^{20,21} In addition to delivering the message encoding the vaccine antigen, mRNA act as a self-adjuvant stimulating toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I), and melanoma differentiation-associated gene 5 (MDA-5), resulting in the release of various cytokines.²² Such a strong immunological response to mRNA inhibits the translation and synthesis of the encoded protein. However, incorporation of modified nucleosides into the message sequence partially renders the molecule immunosilent.²³ The lipids that comprise the nanoparticles, which form mRNA delivery vesicles, are also capable of stimulating innate immunity although the mechanisms are not yet clear. In general, nucleoside-modified lipid nanoparticle-encapsulated mRNA vaccine (mRNA-LNPs) induce robust humoral and cell-mediated immune responses.²⁴ Within the liver, however, this may not be enough to circumvent tolerance. Supplementation of mRNA-LNP vaccines with adjuvants offers an approach to overcome this problem.

An ideal adjuvant for mRNA-based vaccines would boost immunity while maintaining optimal translation efficiency.²⁵ The current study was undertaken to compare the effects of well-known adjuvants, i.e., R848, cGAMP, and Poly I:C, on freshly isolated liver NPC subpopulations. Further, adjuvants in combination with mRNA-LNPs were tested to determine the ability of novel vaccine strategies to overcome the protolerogenic immunophenotype of liver NPCs. The results presented herein demonstrate the capacity of adjuvants to up-regulate co-stimulatory molecule expression and to induce pro-inflammatory cytokine secretion by NPCs without formulation in the LNP structure. Importantly, the data generated highlight the importance of the delicate balance between the immunomodulatory properties of adjuvants and their effects on mRNA translation efficiency and the consequent immune response generated upon its incorporation into an mRNA-LNP vaccine.

RESULTS

Effects of adjuvants on liver NPCs

The abilities of R848, Poly I:C, and cGAMP to activate different liver NPC populations in culture were tested. Activation was evaluated by assessment of the expression of the co-stimulatory markers CD80 and CD86. None of the adjuvants tested up-regulated CD86 expression by CD45⁺F4/80⁺ KCs; only treatment with R848 upregulated CD80 (Figures 1 and S1). However, R848 treatment up-regulated the expression of both CD86 and CD80 by CD45⁺CD11c⁺ DCs. Poly I:C and cGAMP promoted slight albeit not statistically significant increases in CD86 expression. Both R848 and cGAMP stimulated marked up-regulation in CD86 expression by CD45⁺CD32b⁺ LSECs. Only R848, however, induced a significant increase in CD80 in LSECs at every tested dose. Conversely, cGAMP induced activation at the CD80 level only when the dose was significantly increased (Figure S1).

Combined adjuvant effects

NPC populations were treated with a combination of Poly I:C, R848, and/or cGAMP *in vitro* in an effort to stimulate different cell receptors and induce strong innate immune responses. Additive effects were frequently observed when cell populations were treated with a combination of adjuvants compared with treatment with either adjuvant alone. KCs treated with cGAMP combined with either R848 or Poly I:C, for example, exhibited a detectable, albeit statistically insignificant increase in CD86 expression (Figure 2A). In contrast, only treatment with R848 added alone enhanced CD80 expression by KCs.

Non-parenchymal liver DCs exhibited similar trends regarding the expression of CD80 and CD86 in response to adjuvant treatment. Cells treated with a combination of cGAMP and either R848 or Poly I:C exhibited a marked increase in CD86 expression relative to cells treated with cGAMP alone. CD80 expression, in contrast, was only up-regulated by treatment with R848 alone. In the case of LSECs, treatment with single adjuvants induced modest increases in CD80 and CD86 level, whereas adjuvant combinations induced substantially increased levels.

The response to adjuvants added alone or in combination was studied further by quantifying cytokine and chemokine production by cells stimulated in culture. Although varied in extent, R848 added alone to NPC cultures increased the production of most cytokines and chemokines tested (Figure 2B). In comparison, cGAMP added alone induced comparatively less production of most cytokines (aside from interferon [IFN]- α and IFN- β) though production was greater than that determined for untreated, control cultures. Similarly, IP-10, RANTES, and MCP-1 concentrations were greater in cGAMP-treated NPC cultures, relative to the untreated control group. Likewise, cytokine/chemokine concentrations were often elevated in NPC cultures treated with Poly I:C alone though generally less than those determined in cultures treated with either R848 or cGAMP.

NPCs cultured with R848 combined with Poly I:C or cGAMP exhibited modest increases in cytokine production compared with cells

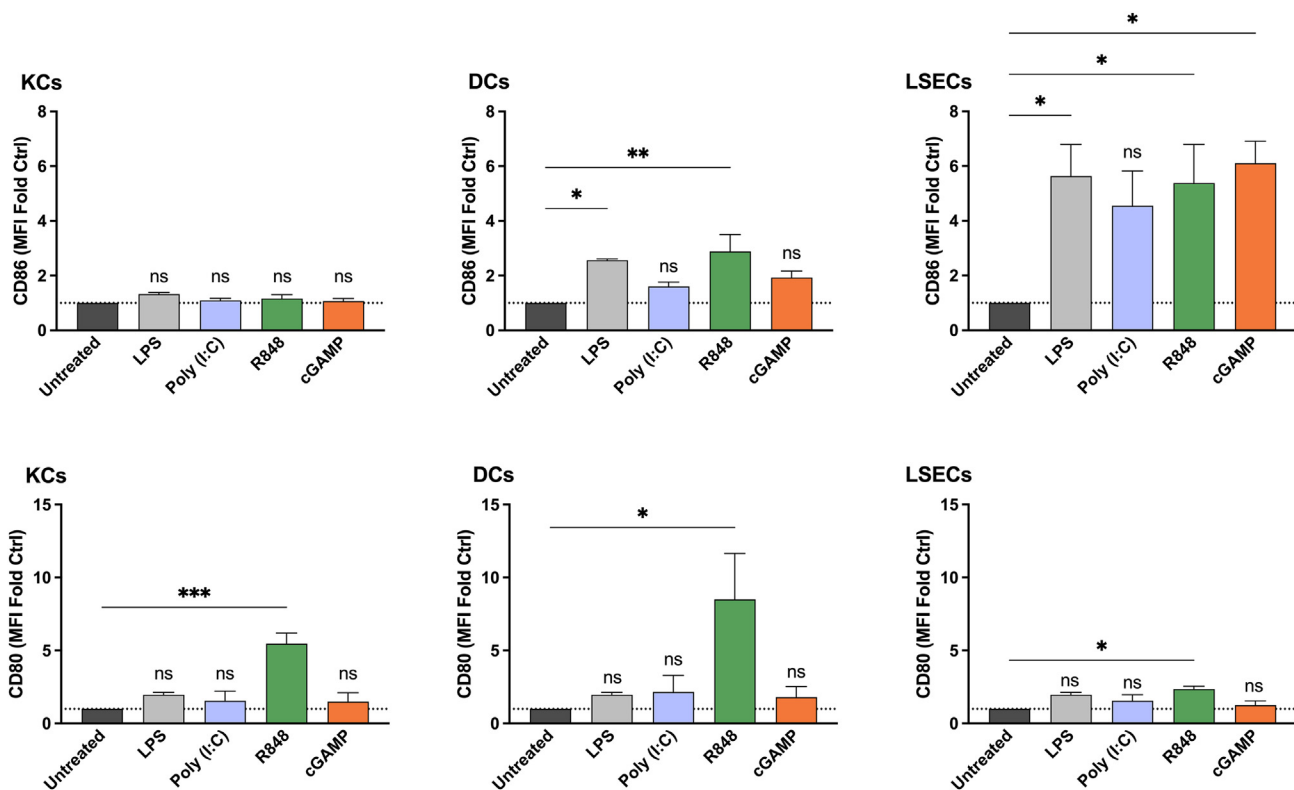


Figure 1. Adjuvants stimulate the expression of CD86 and CD80 by NPC subpopulations

NPC cultures were treated overnight with 5 μ g/mL R848, Poly I:C, or cGAMP; cells treated with 1 μ g/mL LPS served as a positive control. CD86 and CD80 expression was evaluated by flow cytometry in KCs, DCs and LSECs. Data are the means $\pm$ SEM ($n = 3$). Significantly different from the control (untreated): ns, not significant; * $p < 0.05$; ** $p < 0.01$ (one-way ANOVA, Dunnett's multiple comparison test). MFI, mean fluorescent intensity.

cultured with the adjuvant alone. The levels of IFN- γ , TNF- α , and IL-6 produced in cultures treated with R848 and Poly I:C, for example, were 50%–60% greater than those produced in cultures treated with R848 only; the concentrations of IFN- β and IFN- α were nearly 2-fold and 5-fold greater, respectively. Similarly, modest increases in the levels of IFN- γ , TNF- α , IFN- α , and IFN- β were determined in cultures treated with a combination of R848 and cGAMP relative to cultures treated with either adjuvant alone; RANTES, IP-10, and MCP-1 concentrations were also increased. cGAMP combined with Poly I:C stimulated slight increases in IFN- α , IFN- β , IL-6, TNF- α , RANTES, and MCP-1 relative to NPCs cultured in the presence of either adjuvant separately.

Adjuvant-stimulated T cell proliferation

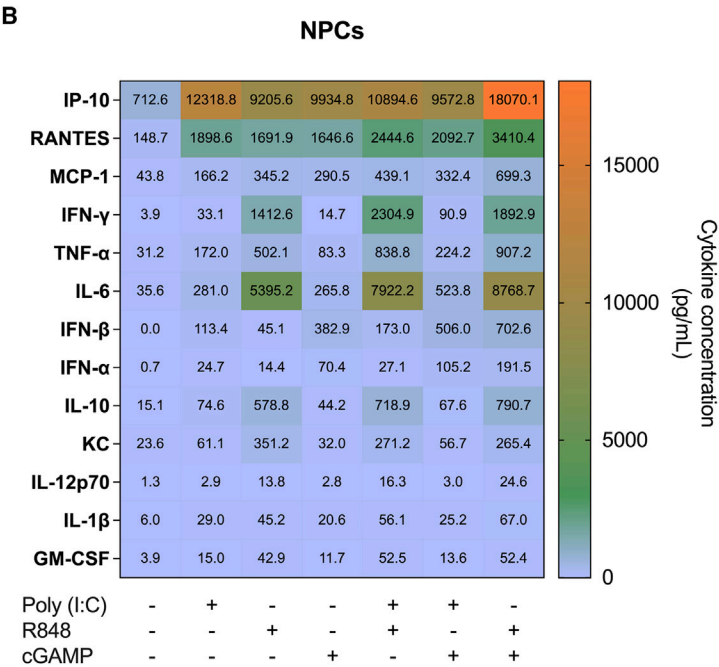
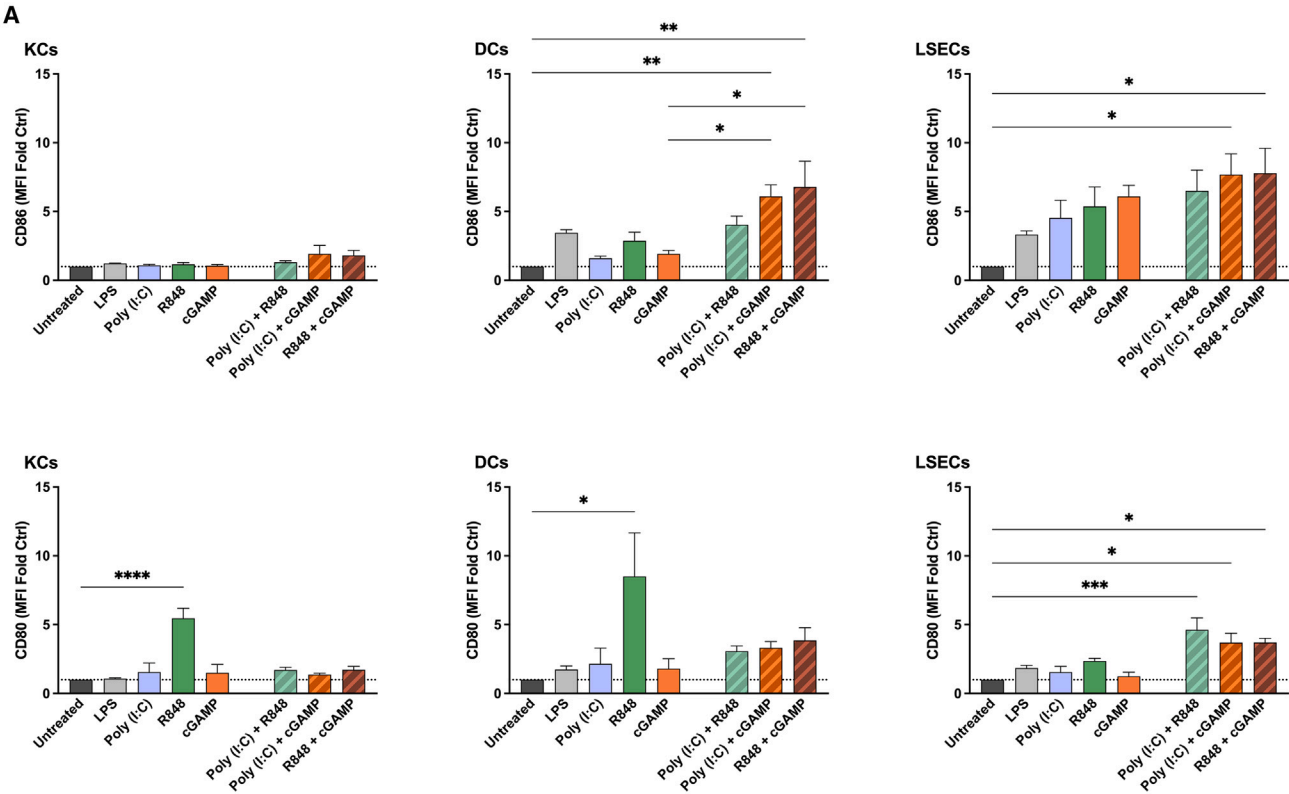
To evaluate the effects of R848, cGAMP, and Poly I:C on the capacity of liver NPCs to stimulate T cell proliferation, ovalbumin (OVA)-responsive CD8 $^{+}$ and CD4 $^{+}$ T cells were co-cultured with OVA protein- or peptide-pulsed NPCs (Figure 3A). Cell proliferation rates were evaluated and compared upon addition of adjuvants alone or in combination. It is noteworthy that OVA-stimulated liver NPC cultures exhibited higher CD8 $^{+}$ T cell proliferation when pretreated with R848 plus cGAMP compared with cultures treated with a combination of R848 and Poly I:C (Figure 3B). However, none of the adjuvant

combinations conferred stronger CD8 $^{+}$ T cell proliferation by SIINFEKL-pulsed NPC than pretreatment with individual adjuvants. A significant increase in the proliferation of OVA-responsive CD4 $^{+}$ T cells was also found in OVA protein-pulsed NPC cultures treated with both R848 and cGAMP. Similarly, the combinations of R848 with either cGAMP or Poly I:C stimulated the proliferation of CD4 $^{+}$ T cells co-cultured with the GREY-pulsed NPCs; however, proliferation was not significantly greater than that determined in co-cultures treated with R848 alone.

The secretion of cytokines and chemokines was increased by the addition of R848 and cGAMP or R848 and Poly I:C to co-cultures composed of purified, antigen-specific CD8 $^{+}$ and CD4 $^{+}$ T cells and OVA- or peptide-pulsed NPCs (Figure 3C). For the most part, cytokine/chemokine release in co-cultures treated with adjuvants containing antigen-specific CD8 $^{+}$ T cells and peptide- or OVA-pulsed NPCs was greater than that observed in antigen-specific CD4 $^{+}$ T cell co-cultures.

Mouse non-parenchymal liver cells respond to OVA mRNA-LNPs administered with adjuvants

As an initial approach to determine the impact of adjuvants on the response to mRNA-based vaccines, NPC activation was studied in



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mice injected intravenously (i.v.) with nucleoside-modified OVA-encoding mRNA formulated into LNPs co-applying R848, cGAMP or a combination of both (Figure 4A). OVA mRNA-LNPs alone (LNPs-OVA) failed to upregulate the expression of either CD86 or CD80 co-stimulatory molecules by KCs, DCs, or LSECs when assessed 24 h after single injection (Figure 4B). LNPs-OVA administered with cGAMP alone or in combination with R848 up-regulated the expression CD80 or CD86 by KCs, DCs, and LSECs compared with NPCs derived from mice that have been administered cGAMP alone. However, cGAMP did not partition into the LNP structure (Figure S2). Meanwhile, R848 increased activation markers to a similar extent either when administered alone or with LNPs-OVA.

Additionally, NPCs derived from mice vaccinated with LNPs-OVA with or without adjuvants were cultured. The supernatants were collected after 24 h of incubation, and cytokine/chemokine production was quantified by cytometric bead array (CBA). The administration of LNPs-OVA alone had a negligible effect on cytokine/chemokine secretion. Cytokine/chemokine release by NPCs derived from control (PBS) and LNPs-OVA-treated mice was comparable (Figure 4C). LNPs-OVA administered in combination with adjuvants, however, promoted cytokine/chemokine secretion. The according concentrations were particularly elevated in supernatants collected from cultures of NPCs derived from mice vaccinated with LNPs-OVA and a combination of cGAMP and R848. Notably, the increase in IFN- α concentration was marginal.

R848 impairs LNP-encapsulated mRNA translation

To determine the adjuvant effects on mRNA translation, the former were mixed rapidly with *Luc* mRNA-LNPs and administered i.v. into mice. Luciferase activity was measured *in vivo* and *ex vivo* at 6 h after treatment (Figure 5A). *Luc* mRNA-LNPs (LNPs-Luc) control mice expressed a strong signal localized mainly in the liver (Figure 5B). LNPs-Luc administered in combination with cGAMP also showed a marked signal around the liver, though no fluorescence was detected in 2 animals. The cohorts that had been administered LNPs-Luc with R848 or with R848 and cGAMP exhibited marked decreases in Luc expression as compared with the control group. Immediately after tissue collection, the luciferase signal was measured *ex vivo* as shown in Figure 5C. Quantitation of the fluorescence detected in the liver determined comparable values in mice administered LNPs-Luc and those administered LNPs-Luc with cGAMP. However, fluorescence was significantly diminished in the livers of mice inoculated with LNPs-Luc and R848 with or without cGAMP. In this context, no interaction between cGAMP and the LNPs could be detected (Figure S1).

Repetitive administration of Ova mRNA-LNP in combination with adjuvants led to distinct immune responses

Experimental design and safety

Based on the obtained results on cell-specific activation and antigen expression, an immunization study using LNPs-OVA with adjuvants was performed in order to assess how these formulations modulate antigen-specific immunity. Mice were injected i.v. three times, 5 days apart, as depicted in Figure 6A. The experimental endpoint occurred on day 11, where the liver and spleen tissues were collected for cell isolation and study. Of note, treatment-induced liver toxicity was evaluated by the activity of liver transaminases (alanine aminotransferase and aspartate aminotransferase) after treatment and appearance of the extracted organs. No acute toxic effects were observed in any of the treated animals, reflected by the absence of abnormal behavior and no significant differences in bodyweight compared with a PBS control (Figures S3 and S4).

Immune cell activation

In contrast with the single-injection experiment, no significant up-regulation of activation markers on liver NPCs was observed after repetitive injections compared with LNPs-OVA alone (Figure 6B). Moreover, mRNA-LNP without adding adjuvants increased the frequencies of CD86⁺ cells for LSEC, DC, and KC populations. In the case of liver DCs, LNPs-OVA significantly up-regulated the levels of the MHC II marker (Figure 6B). Previous observations showed an absence in cell activation to LNPs-OVA after a single injection (Figure 4B). Notably, the co-application of R848 with LNPs-OVA arrested the increment in activation markers observed by LNPs-OVA alone.

However, splenocytes showed a higher response to adjuvant treatments. For DC or macrophage, R848 significantly increased the frequencies of CD80⁺, CD86⁺, and MHC II⁺ cells (Figure 6C). Concurrently, cGAMP showed stimulation on DCs and macrophages at CD86 level. Moreover, the expression of CD80 was increased by cGAMP treatment in macrophages. Co-application of R848/cGAMP resulted in no significant differences in comparison to R848 treatment alone (Figure 6C).

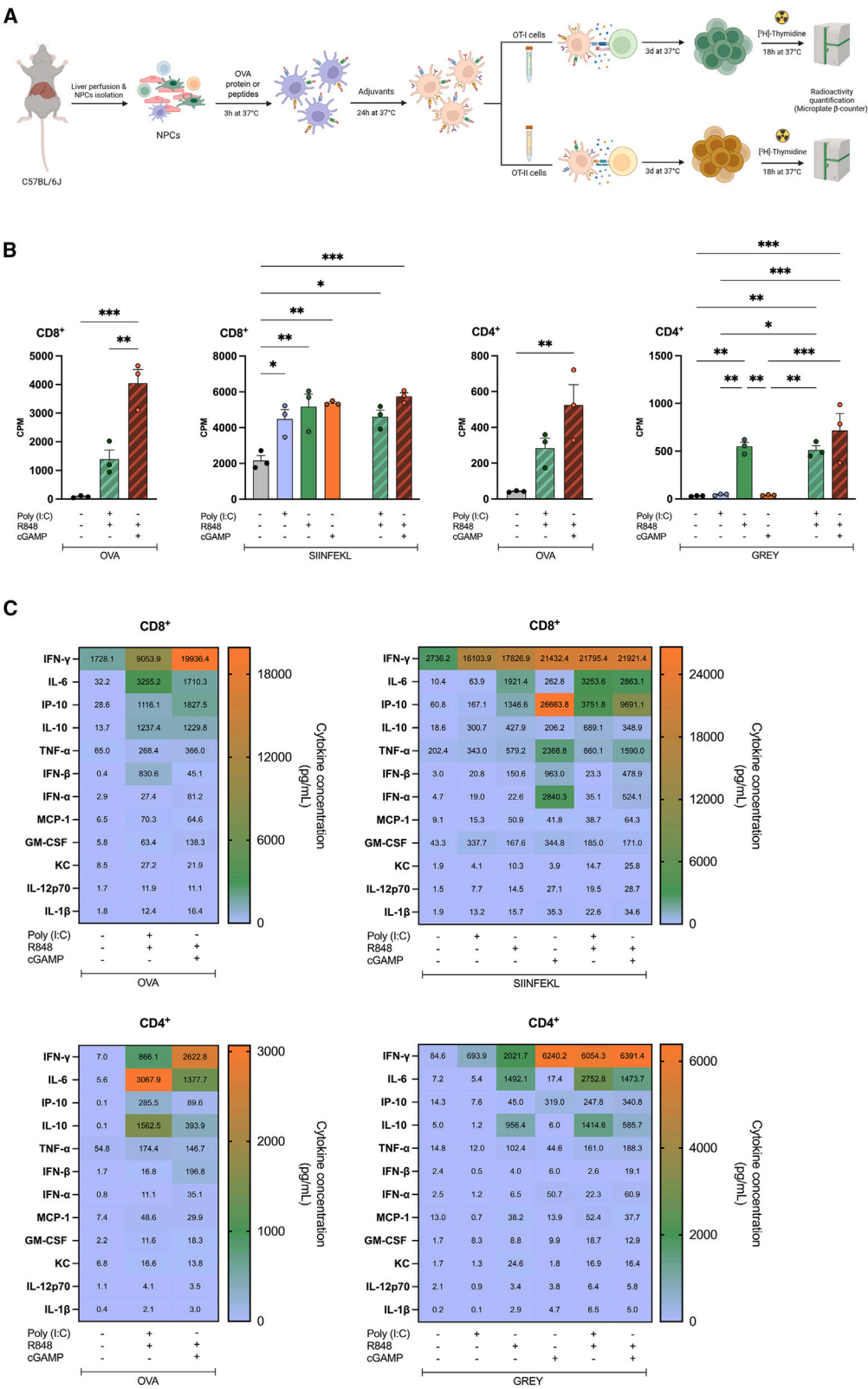
Antigen-specific immune responses

Antigen-specific cell-mediated immune responses were characterized by evaluating levels of cytokine-secreting CD4⁺ and CD8⁺ T cells (Figure 6D). After stimulation using MHCI-restricted (SIINFEKL) or MHCII-restricted (GRAY) peptides, T cells secreting IFN- γ , IL-2, or TNF- α were identified. Co-administration with R848/cGAMP led to the highest levels of cytokine-secreting CD4⁺ T cells when stimulated with SIINFEKL. In contrast, when T cells were incubated with GREY, no additive effect was observed by adjuvants in

Figure 2. Individual or a combination of adjuvants stimulates the expression of CD86 and CD80 by NPCs

NPC cultures were treated overnight with 5 μ g/mL of each adjuvant; cells treated with 1 μ g/mL LPS served as positive controls. CD86 and CD80 were evaluated by flow cytometry in KCs, DCs and LSECs. Data are the means $\pm$ SEM ($n = 3$).

Significantly different between groups: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (one-way ANOVA, Tukey's multiple comparison test) (A). Cytokines in the supernates of NPCs culture treated with adjuvants were quantified by CBA (B). MFI, mean fluorescent intensity.



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comparison with the positive effect generated by LNPs-OVA alone (Figure 6D). Cytokine secretion by CD8⁺ T cells was stronger when R848 was administered along with LNPs-OVA. These effects were observed only with R848 co-applied alone with LNPs-OVA. Cytokine levels collected from culture supernates paralleled this observation (Figure S5).

In addition, antigen-specific T cell proliferation was evaluated. Both CD4⁺ and CD8⁺ T cells displayed elevated proliferation rates when immunized with LNPs-OVA administered with R848 alone (Figure 6E). Noteworthy, R848/cGAMP also led to increased proliferation but only in CD4⁺ T cells.

Humoral immune response

A pertinent finding was observed regarding the production of anti-OVA antibodies. All treated groups displayed significant antibody titers (Figure 6F). However, no differences were observed between the groups.

T_{reg} cell response

Elevated T_{reg} levels are associated with the failure of anti-cancer immunotherapies.²⁶ Here, the percentage of T_{reg} cells after co-application of adjuvant and LNPs-OVA was assessed by the frequency of CD4⁺ CD25⁺ FoxP3⁺ T cells. Treatment with LNPs-OVA alone led to a significant increase in T_{reg} cells at the experimental endpoint. Treatment with cGAMP exerted similar effects. Conversely, single or combine treatment with R848 did not show a significant increase in T_{reg} cells compared with the untreated control group (Figure 6G).

DISCUSSION

The fragile balance between immunity and tolerance in the liver is mediated, in part, by the NPCs, i.e., KCs, DCs, and LSECs.^{1,2} Reversing tolerance to induce therapeutic responses to persistent viral infections and cancers is a frequent topic of discussion. An improved understanding of the response of NPCs to adjuvants would help to optimize the design of liver-directed therapies that include mRNA-based technologies. Three well-known adjuvants, cGAMP, R848, and Poly I:C, were selected for the current study based on their immunologic potencies and distinct structural characteristics and mechanisms of action.

cGAMP is a cyclic dinucleotide that participates in the oligomerization and subsequent activation of the stimulator of IFN genes protein. It promotes cancer immunity by inducing type I IFN production, including the expansion of antigen-specific CD8⁺ T cells.^{27,28} R848

is an imidazoquinoline that activates TLR7/8 and thus stimulates DC maturation, up-regulates co-stimulatory molecule expression, and promotes pro-inflammatory cytokine secretion.²⁹ R848 increases the number of infiltrating CD8⁺ T cells and decreases T_{reg} cell frequencies when used in cancer treatment.³⁰ Poly I:C is a double-stranded RNA molecule that triggers TLR3, MDA-5, and RIG-I receptors resulting in type I IFNs production, Th1-polarizing cytokine secretion and overall improved T cell responses.²⁸

In the current study, liver NPCs were treated with cGAMP, R848, and Poly I:C in parallel settings, and the responses were assessed and compared. R848 treatment up-regulated CD86 and/or CD80 expression by all three monitored NPC populations: KCs, DCs, and LSECs. KCs and liver DCs, however, were unresponsive toward other adjuvants. Furthermore, treatment with either cGAMP or Poly I:C, as well as R848, up-regulated the expression of the cell-surface costimulatory molecules by LSECs. Notably, LSECs display many characteristics typical of APCs and exhibit the capacity to cross-present antigens.⁶ Indeed, LSEC stimulation was demonstrated to improve T cell-mediated immunity in mice.³¹

Remarkably, R848 combined with cGAMP or Poly I:C synergized, up-regulating stronger CD80 and CD86 expression by liver DCs and LSECs, but not KCs, than treatment with any single adjuvant. As expected, combining adjuvants that exert their effects by different mechanisms maximized immunostimulatory potency. The adjuvant systems developed by GlaxoSmithKline constitute a rational combination of classical adjuvants like alum with novel immunostimulators like TLR agonists. This proved to be a potent and reliable approach to optimizing immune responses.³²

Cheng et al.³³ explored adjuvant combinations in a nanoformulation. R848 and cGAMP combined with immune checkpoint inhibitors reduced immunosuppression in a tumor microenvironment, and elicited anti-tumor immunity in a number of mouse models.³³ Reportedly, the combination of R848 and Poly I:C also offers a successful strategy to activating DC subsets circulating in the blood. In this regard, Hänel et al.³⁴ found that activating human DCs in the circulation stimulated natural killer cells and potent antigen-specific T cell immunity. Similarly, Anfray et al.³⁵ reported that in a mouse lung cancer model treatment with a nano-formulated combination of R848 and Poly I:C reprogrammed tumor-associated macrophages by increasing the M1 (F4/80⁺,CD86⁺) by M2 (F4/80⁺,Arg1⁺) ratio and suppressing tumor progression. To the best of our knowledge, the effects of adjuvant combinations on liver NPC have not been studied to date.

Figure 3. NPCs pre-treated with R848, cGAMP and Poly I:C exert a distinctive capability to stimulate T cell proliferation

Experimental scheme. NPCs were isolated from C57BL/6J mice, pulsed with OVA or peptides (SIINFEKL or GRAY), and stimulated with adjuvants. Then, the NPCs were cocultured with SIINFEKL-specific CD8⁺ T cells (OT-I) or GREY-specific CD4⁺ T cells (OT-II); proliferation was quantified by the incorporation of H³-thymidine (A). Proliferation of OVA-specific CD4⁺ and CD8⁺ T cells cocultured with OVA- or peptide-pulsed NPCs in the presence of individual or combined adjuvants. Data are the means ± SEM (n = 3). Significantly different between groups: *p < 0.05; **p < 0.01; ***p < 0.001; (one-way ANOVA, Tukey's multiple comparison test). (B). Cytokine secretion in cocultures comprised of OVA-specific CD4⁺ or CD8⁺ T cells, and NPCs pulsed with OVA, SIINFEKL, or GRAY in the presence of individual or combined adjuvants (C). Data are the means ± SEM (n = 3). Significantly different from the control: ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (one-way ANOVA).

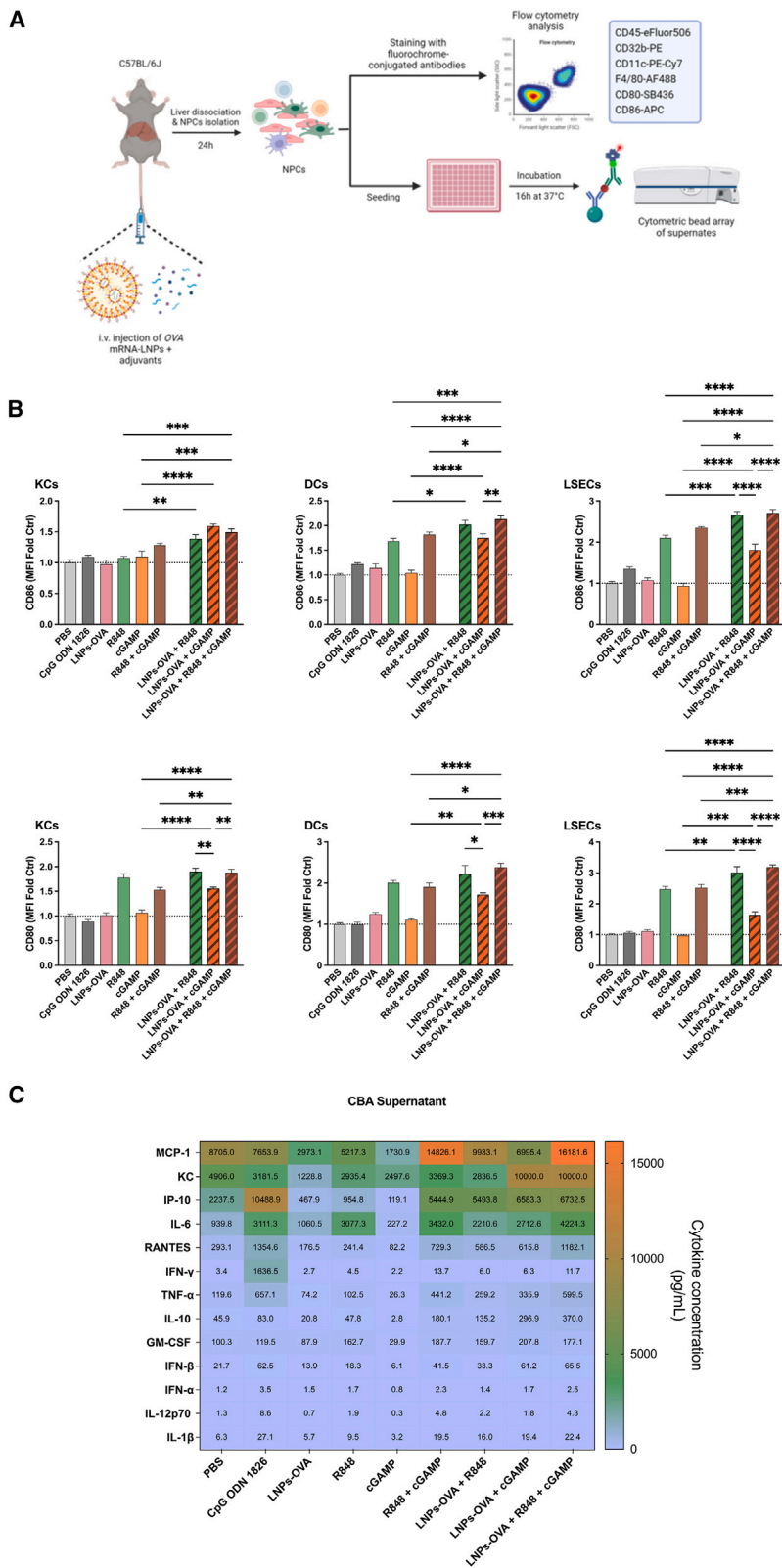
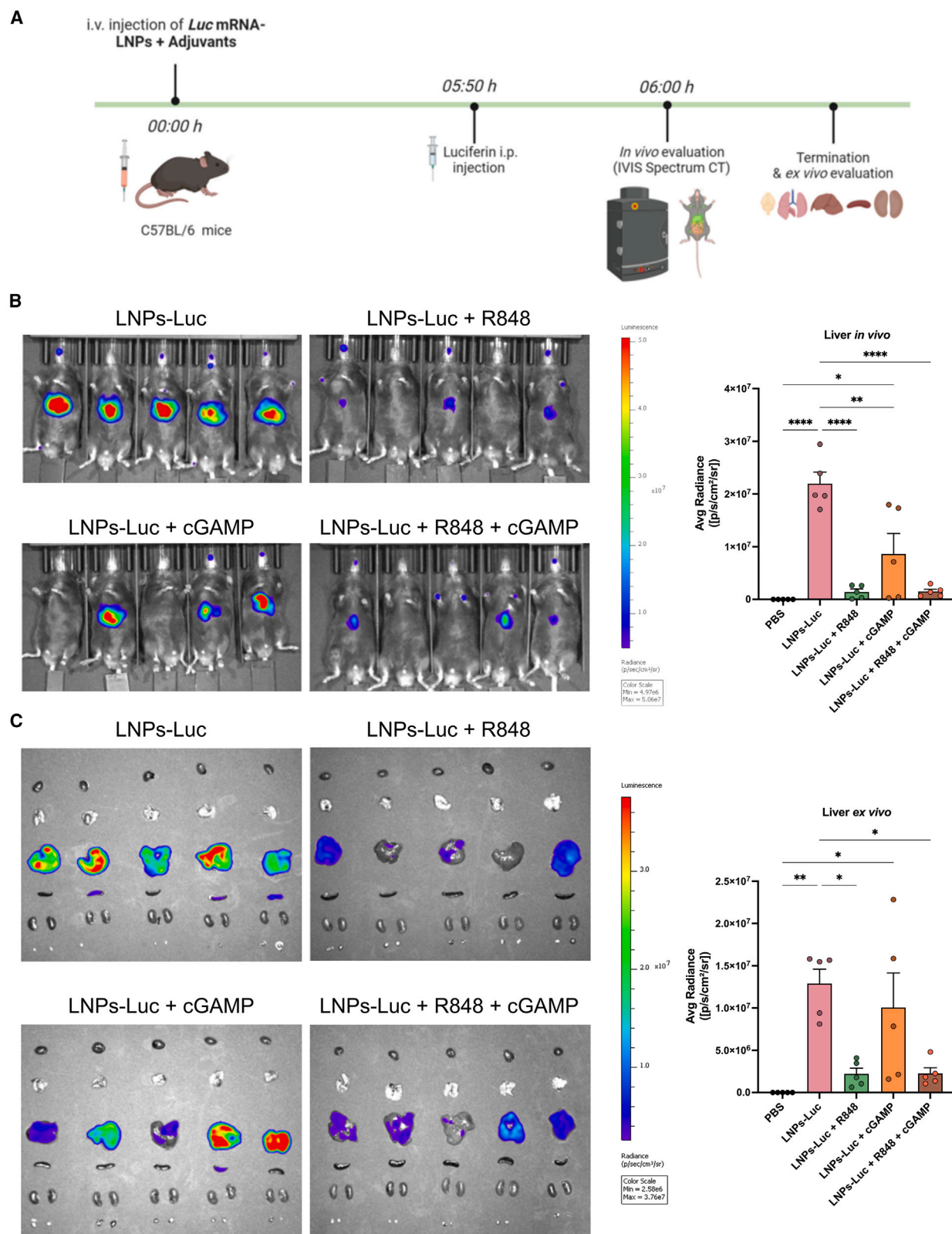


Figure 4. Adjuvants co-administered with mRNA-LNPs lead to the up-regulation of co-stimulatory markers in liver NPCs, after single i.v. injection in mice

Experimental scheme. C57BL/6J mice were injected i.v. with LNPs-OVA and adjuvants. The NPCs were isolated and separated into two fractions. The NPC activation in one fraction was quantified by flow cytometric analysis. The NPCs in the second fraction were cultured; the supernates were collected and the cytokine/chemokine profile was evaluated (A). CD86 and CD80 expression by NPC subpopulations; i.e., KCs, DCs, and LSECs; isolated from mice inoculated with OVA mRNA-LNPs and adjuvants alone or in combination was assessed. Data are the means $\pm$ SEM ($n = 5$). Significantly different between groups: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (one-way ANOVA, Tukey's multiple comparison test) (B). Cytokines and chemokines in the NPCs culture supernates were quantified by CBA (C).



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To clarify the adjuvant effect of these agents on the liver, NPC loaded with OVA-derived antigen were cocultured with OVA antigen-specific CD8⁺ and CD4⁺ T cells. Cocultures composed of liver NPCs pre-treated with R848, cGAMP, or Poly I:C alone or in combination and T cells stimulated comparable increases in CD8⁺ T cell proliferation. In the case of CD4⁺ T cells, however, increased proliferation occurred only in cocultures containing liver NPCs pre-treated with R848 and either cGAMP or Poly I:C.

The use of synthetic mRNA-LNP formulations in therapeutic approaches to prevent or treat diseases has strongly expanded over the past years. The LNPs in these formulations perform two primary functions at the same time: transport mRNA molecules inside of cells and provide adjuvant effects.²⁰ These effects are not well understood from a mechanistic point of view.²⁴ The co-application of well-known adjuvants into mRNA-LNP formulations provides a rational approach to predict the effects of adjuvants *in vivo* and to potentiate innate immunity. The latter is extremely relevant in situations where reversing immune tolerance is paramount, e.g., cases of liver pathologies. While various adjuvants have been shown to stimulate liver NPCs, none have been co-applied with mRNA-LNPs to treat liver diseases.^{21,36}

In the current study, LNP-encapsulated OVA mRNA combined with cGAMP and R848 were injected into mice to determine the effects of these adjuvants on liver NPC activity. Liver tissues were targeted rapidly by simply mixing adjuvants with mRNA-LNPs and injecting i.v. Similar to the results obtained *in vitro*, adjuvants administered alone failed to activate KCs. KCs were activated, however, when either R848 or cGAMP was administered in combination with LNPs-OVA, indicative of a co-activator property of the transfection complexes. R848 inoculated alone stimulated both DCs and LSECs; stimulation increased perceptibly when R848 was administered in combination with mRNA-LNPs. cGAMP, in contrast, only exerted a strong stimulatory effect on either NPC population monitored when co-applied with LNPs-OVA. Notably, LNPs-OVA administered alone as a control never activated NPCs.

Upon systemic administration, blood proteins are adsorbed onto the surface of LNPs forming a corona.³⁷ As such, it is a matter of conjecture whether the observations reported herein result from the interaction of adjuvants with LNPs or with the protein corona. Other investigators report that the encapsulation of cGAMP in LNPs improved its stability and immunomodulating effects.^{38–40} The simple combination of cGAMP with mRNA-LNPs in the study reported here was sufficient to promote KCs, DCs, and LSECs activation.

A delicate balance exists between adjuvant-activated innate immunity and the effects of adjuvants on mRNA translation. Indeed, activation of the innate immune response may impair mRNA translation.^{20,41} Here, the effects of cGAMP and R848 on mRNA translation *in vivo* were tested. Our data indicate that R848 exerts a strong negative impact on mRNA translation; cGAMP affected translation to a far lesser extent. A number of previous studies reported improved immune responses to mRNA-LNP formulations that incorporated either R848 or cGAMP; the effects of these adjuvants on mRNA translation, however, were not considered.^{25,42,43}

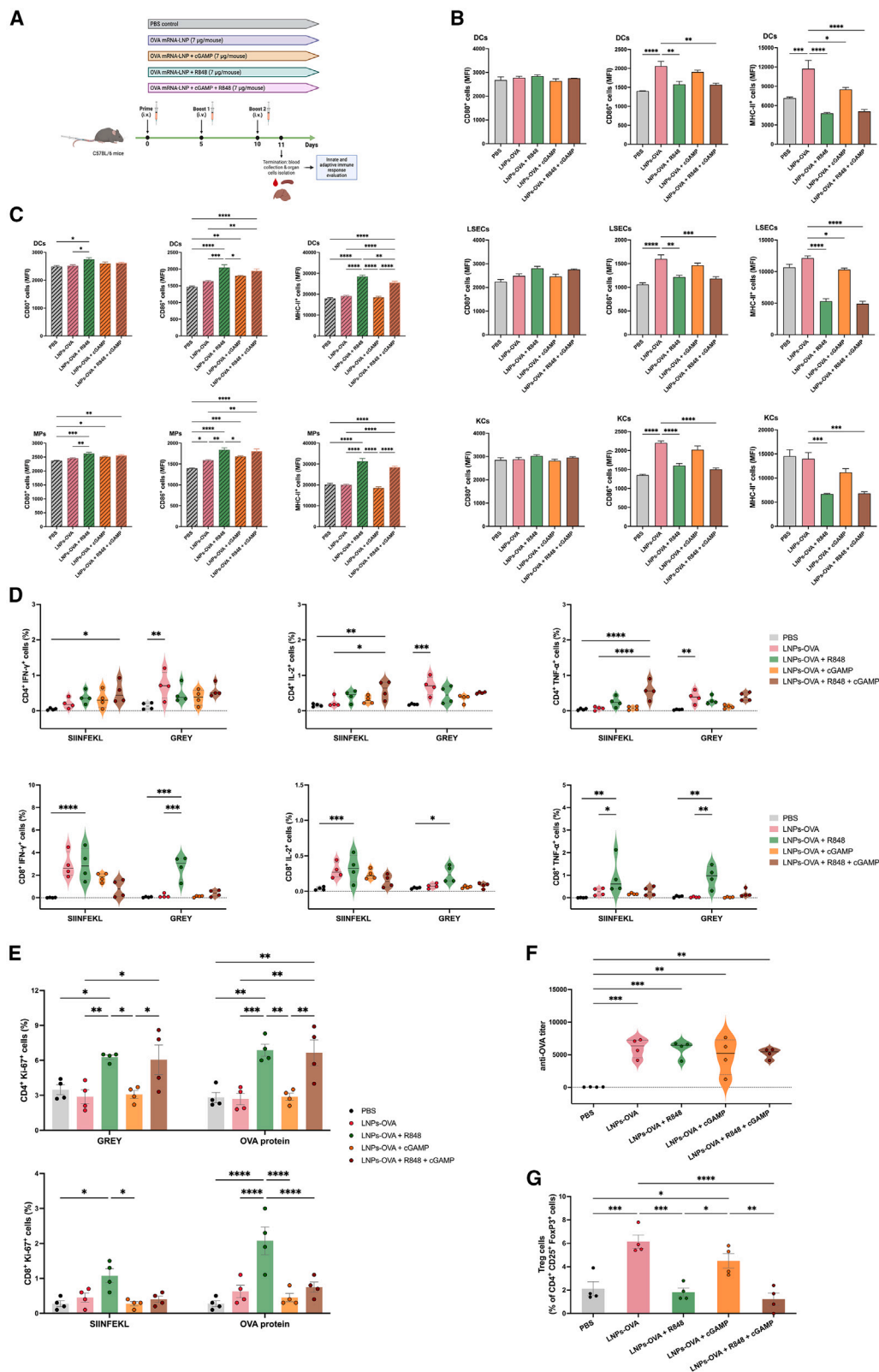
To further investigate the effects of these adjuvants in mRNA-based immunization, a repetitive administration scheme was designed to mimic strategies frequently used in cancer immunotherapy.^{44,45} Interestingly, the state of activation of liver DCs, LSECs, and KCs was not affected by the presence of adjuvants. However, the administration of LNPs-OVA elicited up-regulation of the measured activation markers. This observation was counterintuitive, considering the results obtained after single injection, where mRNA-LNP did not show any stimulatory effect. Conceivably, the repetitive administration induced adjuvant-like effects by the LNPs on liver NPCs. It remains a matter of conjecture whether LNPs can exert an effect strong enough to overcome liver tolerance. Further investigation would need to be conducted to understand these events. In contrast, DCs and macrophages isolated from the spleen responded mainly to R848 co-treatment. These findings provided evidence regarding the complexity in the induction of immune activated state by liver NPCs through adjuvant treatments. Notably, cell-mediated immunity was improved by R848 and, to a lesser extent, by the R848/cGAMP combination, evidenced by an increase in the number of cytokine-secreting CD4⁺ and CD8⁺ T cells. A similar pattern was observed when the capacity of T cells to proliferate after antigen stimulus was evaluated.

Intriguingly, the production of anti-OVA antibodies did not seem to be affected by the co-application of the adjuvants used in this study. Due to the observed negative impact on mRNA translation by R848, a decrease in total IgG titers was expected. Conceivably, the level of antigenic protein obtained in R848-adjuvanted LNPs-OVA was enough to mount a humoral response similar to the LNPs-OVA alone. Moreover, the boosting effects of the repetitive administrations might have additionally contributed to reaching similar systemic antibody levels between groups. The connection between antigen dose and antibody production responds to a complex relationship and requires further investigations.^{46,47}

T_{reg} cells play a crucial role in homeostasis and the induction of tolerance. However, the abundant presence of T_{reg} cells in the tumor

Figure 5. R848 and cGAMP negatively impact mRNA translation after mRNA-LNP i.v. injection in mice

Biodistribution of LNPs-Luc with or without adjuvants was performed as shown in the experimental scheme. Six hours after injection of the formulations, luciferase-associated bioluminescence was measured (A). *In vivo* images were collected in all mice cohorts and luminescence in the region of the liver was quantified (B). The mice were then dissected, and the hearts, lungs, livers, spleens, kidneys, and inguinal lymph nodes were collected for *ex vivo* imaging. Luminescence localized in the liver *ex vivo* was quantified (C). Data are the means $\pm$ SEM ($n = 5$). Significantly different between groups: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (one-way ANOVA, Tukey's multiple comparison test).



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microenvironment leads to huge challenges in cancer therapies.⁴⁸ Modulation of T_{reg} frequencies, e.g., with adjuvants, is a promising approach to overcome immune tolerance. Remarkably, R848 and R848/cGAMP co-treatments did not induce an increase in the numbers of T_{reg} cells, while mRNA-LNP without adjuvantation showed an effect. Recently, Zhou et al.⁴⁹ reported that continuous R848 treatment led to a decrease in the number of T_{reg} cells in tumor-bearing mice. Primarily, the CTL: T_{reg} ratio is often associated with the therapeutic index of a given anti-tumor treatment.⁵⁰ The expansion of $CD8^+$ T cells together with a low number of T_{reg} cells indicate a potentially strong anti-tumor effect. Nevertheless, more in-depth studies using R848-adjuvanted mRNA-LNP will need to be carried out to confirm this observation.

The inoculation of mice with mRNA-based vaccines induces production of type I IFNs, which function as a double-edged sword, stimulating T and B cell responses on the one hand, while inhibiting mRNA translation as an anti-viral effector mechanism on the other.^{23,25,41,51,52} The results of the present study suggest that an informed selection of adjuvants with an equilibrated impact on mRNA translation may in parallel promote APC activation and the production of antigenic proteins. This is particularly relevant for immunotherapies that target liver tissue, e.g., hepatocellular carcinoma, to overcome intrinsic liver- as well as tumor-induced tolerance and to elicit antigen-specific effector $CD4^+$ and $CD8^+$ T cells collectively contribute to successful therapies.

Conclusions

The intricate balance between immunity and self-tolerance in the liver is governed, in part, by NPCs. Approaches to reverse liver tolerance for therapies to treat viral infections or cancer remain a matter of debate. Understanding the response of NPCs to adjuvants should serve to optimize liver-directed therapies. Adjuvant combinations, especially R848 in combination with cGAMP or Poly I:C, synergized to enhance immune responses. Despite these findings, all adjuvant combinations failed to activate KCs. Co-application of adjuvants with mRNA-LNP formulations potentiated innate immune activation. Notably, a single injection of cGAMP and R848 co-administered with LNPs-OVA activated NPCs and promoted OVA peptide-specific $CD8^+$ and $CD4^+$ T cell proliferation. R848 impacted mRNA translation negatively, however, emphasizing the requirement to balance immune stimulation and translation efficiency. Repetitive injections of adjuvanted LNPs-OVA led to distinct immune responses

with a rather poor activation of NPCs by adjuvants. Cellular responses in splenocytes were significantly enhanced by the co-administration of R848 or R848/cGAMP together with the mRNA-LNP formulation. In contrast with the results obtained from LNPs-OVA alone, these adjuvanted treatments did not provoke an increase in tolerogenic T_{reg} cells.

Furthermore, the reduced mRNA translation by R848 seems to be negligible to the immunity generated after a repetitive injection scheme. In summary, the selection of adjuvants for co-administration with mRNA-LNPs relies on the capacity to exert minimal impact on mRNA translation while preserving stimulatory properties. Such a strategy may enhance liver-directed immunotherapies like those designed to treat hepatocellular carcinoma or chronic liver infections, e.g., hepatitis B.

MATERIALS AND METHODS

Mice

Wildtype male C57BL/6Jrj mice were purchased from Janvier (Le Genest-Saint-Isle, France) and Charles River Laboratories (Wilmington, MA, USA) were maintained in the Central Animal Facilities of the University Medical Center of Mainz under pathogen-free conditions. Five mice/cage were housed in a dedicated vivarium (18°C–23°C ambient temperature, 40%–60% humidity, 14 h:10 h light cycle) with clean food, water, and bedding. Similarly, male and female transgenic OT-I and OT-II mice on a C57BL/6 background were bred and maintained in the Central Animal Facilities. All animal procedures were approved by the local authorities (Landesuntersuchungsamt Rheinland-Palatinate). Ethical approval was granted by the Landesuntersuchungsamt LUA, Koblenz, AK G20-1-123 and G20-1-130.

LNP-encapsulated mRNA

Ova-mRNA and luciferase-mRNA were synthesized by TriLink Biotechnologies (San Diego, CA, USA). The mRNA was encapsulated into LNPs using a NanoAssemblr Ignite machine for formulation (Precision Nanosystems, Vancouver, Canada). All procedures were conducted according to the manufacturer's protocols. Briefly, Genvoy-ILM lipid mix (Precision Nanosystems), containing 50% ionizable lipid, 10% DSPC, 37.5% cholesterol, and 2.5% PEG-lipid was mixed with mRNA in 50 mM citrate buffer, pH 4.0, using an N/P ratio of 4. The aqueous and organic phases were mixed at a total flow rate of 12 mL/min and flow rate ratio of 3:1 (aqueous:organic). After synthesis, LNPs were 1:20 diluted or 1:40 in PBS for *in vitro*

Figure 6. Immunization using repetitive LNPs-OVA i.v. injections provide insights in the improvement of cell-mediated immune responses by co-administration with adjuvants

Experimental scheme. Mice were i.v. injected, three times, 5 days apart either with LNPs-OVA alone, with R848 or cGAMP and with both adjuvants combined. The following day after the second booster, whole blood was collected, and spleen and liver were dissected for further analysis (A). Activation of NPCs (B) and splenocytes (C) subpopulations was measure by flow cytometry through up-regulation of the activation markers CD80, CD86 and MHC II. MP, macrophages. Peptide-specific $CD4^+$ and $CD8^+$ T cell responses were measure by the release of intracellular cytokines: IFN- γ , IL-2, and TNF- α . Cells percentages were normalized to their respective $CD3^+$ T cell population (D). T cell proliferation of the $CD4^+$ and $CD8^+$ T cells after 48-h peptide- or OVA-stimulated splenocytes measured by the up-regulation of the intranuclear protein Ki-67. Cells percentages were normalized to their respective $CD3^+$ T cell population (E). Total anti-OVA IgG titers at termination (day 11) of the immunization study (F). T_{reg} response after 48-h GREY-stimulated splenocytes. T_{reg} cells are gated as $CD4^+CD25^+FoxP3^+$ cells (G). Data are the means $\pm$ SEM ($n = 4$). Significantly different between groups: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (one-way and two-way ANOVA, Tukey's multiple comparison test).

or *in vivo* purposes, respectively. Samples were concentrated in an Amicon centrifugal filters of 50,000 MWCO (Merck KGaA, Darmstadt, Germany) at $2,000\times g$ for 5 min per fraction. The final solution was filter sterilized, stored at 4°C and used within 3 days. Encapsulation efficiency and encapsulated mRNA concentration were determined using the RiboGreen RNA Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).⁵³

The mean hydrodynamic size and distribution (PDI) of the LNP formulations were measured by dynamic light scattering in a Nano ZS Zetasizer (Malvern Instruments Corp., Malvern, UK) LNPs loaded with OVA or Luc-mRNA (EE >90%) and mean diameter in the range of 90–100 nm with low PDI (<0.05) were successfully obtained (Table S1).

Mouse NPCs

For the adjuvant testing and co-culture studies liver perfusion was used as method for NPCs isolation. Ten-week-old mice were anesthetized (i.p., 100 mg/kg ketamine and 5 mg/kg xylazine) and the livers were perfused via the portal vein with 20 mL perfusion medium (Ca^{2+} - and Mg^{2+} -free HBSS, Thermo Fisher Scientific) containing 100 U/L collagenase (Sigma-Aldrich, Burlington, MA, USA), 5% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 10 $\mu\text{g/mL}$ DNase I (Sigma-Aldrich). The livers were dissected and incubated in 5 mL perfusion medium at 37°C for 15 min. Afterward, the livers were teased through 70 μm nylon cell strainers and washed with 50 mL wash medium (RPMI 1640 medium supplemented with 5% FBS, and 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin purchased from Sigma-Aldrich), and centrifuged at $30\times g$, 4°C for 15 min to pellet the parenchymal cells. The supernatants were collected and centrifuged at $300\times g$, 4°C for 10 min to pellet the NPCs. Cells in the pellet were re-suspended in ice-cold 30% Histodenz (Sigma-Aldrich) in Ca^{2+} - and Mg^{2+} -free HBSS, overlaid with cold HBSS, and centrifuged at $1,500\times g$ at 4°C for 20 min without braking during deceleration. The NPCs were collected at the HBSS/Histodenz interface, washed with 12 mL wash medium and centrifuged at $300\times g$ at 4°C for 10 min. The cells in the pellet were re-suspended in 1 mL RPMI 1640 culture medium (Life Technologies Ltd.; Scotland, UK) containing 10% FBS, 1% L-glutamine, 1% HEPES, and 1% essential and nonessential amino acids, 1% Na-pyruvate, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin and 50 μM 2-mercaptoethanol (Gibco), counted, and cultured as described below.

The liver dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to isolate NPCs for *ex vivo* studies. Dissociation mix was prepared in accordance with the supplier's protocol. The livers were dissected from euthanized mice, rinsed with DMEM (Thermo Fisher Scientific), cut into small pieces and transferred into gentleMACS C tubes containing dissociation mix. The tubes were then attached to the gentleMACS dissociator and the liver dissociation program was run. After the program terminated, the samples were re-suspended and transferred to 50-mL Falcon tubes by passing through a 100- μm nylon cell strainer. The samples were centrifuged

at $300\times g$ at 4°C for 10 min. Supernatants were aspirated, and the red blood cells (RBCs) were lysed by treating with 2 mL RBC lysis buffer (Thermo Fisher Scientific) at room temperature (RT) for 3 min. The RBC-free cells were centrifuged at $400\times g$ at 4°C for 10 min. The supernatants were aspirated, and the cell pellets were re-suspended in 1 mL HBSS. The cells were then mixed with 2 mL freshly prepared 30% Histodenz, 1 mL cold HBSS was layered on top, and the samples were centrifuged at $1,500\times g$ at 4°C for 20 min without braking during deceleration. Cells at the Histodenz interface were collected, washed, and centrifuged at $300\times g$ at 4°C for 10 min. Cells in the pellet were re-suspended in culture medium and counted.

NPC stimulation *in vitro*

The NPCs were transferred to 96-well flat-bottom plate containing RPMI 1640 culture medium supplemented with 10% FBS, 1% L-glutamine, 1% HEPES, 1% essential and nonessential amino acids, 1% Na-pyruvate, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 50 μM 2-mercaptoethanol (400,000 cells/200 μL) and incubated overnight with 1 $\mu\text{g/mL}$ bacterial endotoxin (LPS) or 5 $\mu\text{g/mL}$ R848, Poly I:C or cGAMP, when combined 10 $\mu\text{g/mL}$ final adjuvant concentration (InvivoGen, San Diego, CA, USA) at 37°C and 5% CO_2 .

T cell proliferation

NPCs were transferred to 96-well U-bottom plates (400,000 cells/100 μL) and incubated with 5 $\mu\text{g/mL}$ OVA; after 3 h, 5 $\mu\text{g/mL}$ R848, Poly I:C or cGAMP (InvivoGen) was added and NPCs were incubated at 37°C in 5% CO_2 . On the following day, cell samples were collected, washed, resuspended in culture medium, and transferred to triplicate wells in 96-well plates (Greiner Bio-One). Splenic CD8^{+} T cells specific for OVA_{257–264} peptide in the context of H-2K^b, and CD4^{+} T cells specific for OVA_{323–339} peptide in the context of H-2 I-A^b and I-A^d were isolated by immunomagnetic sorting (Miltenyi Biotec) from OT-I and OT-II mice, respectively, and cultured at 5×10^4 cells/100 μL /well with the NPCs. After 3 days, 10 μL culture supernatant was collected from each well and stored at -20°C for subsequent CBA analysis. ³H-thymidine (0.5 μCi /well) was added during the last 18 h incubation. Subsequently, cell lysates were transferred onto glass fiber filter mats (Harvester 96; TomTec, Hamden, CT, USA), and the incorporated radioactivity was quantified using a microplate β -counter (1450 MicroBeta Trilux; PerkinElmer, Waltham, MA, USA).

NPC activation *in vivo*

Nine groups of wildtype C57BL/6J mice ($n = 5$) were injected i.v. with 100 μL of one of the following: (i) 7 μg LNPs-OVA; (ii) 25 μg R848; (iii) 5 μg cGAMP; (iv) 7 μg LNPs-OVA and 25 μg R848; (v) 7 μg LNPs-OVA and 5 μg cGAMP; (vi) 7 μg LNPs-OVA, 25 μg R848 and 5 μg cGAMP; (vii) 25 μg R848 and 5 μg cGAMP; or (viii) PBS control. Whole blood was collected on the day following injection by intracardiac puncture under anesthesia (i.p., 100 mg/kg ketamine and 5 mg/kg xylazine) and the mice were euthanized by cervical dislocation. The livers and spleens were dissected. Single cell suspensions were prepared and cultured as described above. A 150- μL aliquot of the supernatant was collected from each well and

stored at -20°C for CBA analysis; and the cells were stained and analyzed by flow cytometry.

Biodistribution and protein expression

Four groups of C57BL/6 mice ($n = 5$) were injected *i.v.* with 100 μL of (i) 7 μg LNPs-Luc; (ii) 7 μg LNPs-Luc and 25 μg R848; (iii) 7 μg LNPs-Luc and 5 μg cGAMP; or (iv) 7 μg LNPs-Luc, 25 μg R848 and 5 μg cGAMP. The substrate, luciferin, was injected *i.p.* after 6 h and the mice were anesthetized with isoflurane mixed with oxygen 5 min later. After an additional 5 min, the anesthetized mice (3 vol. % isoflurane) were monitored by bioluminescence imaging with an IVIS Spectrum CT (PerkinElmer) using a 3- or 5-s exposure time. The mice were euthanized after imaging by cervical dislocation and the organs (heart, lungs, liver, kidneys, spleen, and auxiliary lymph nodes) were dissected, imaged, and weighed. Images were analyzed with Living Image Software (PerkinElmer).

Mouse immunization and single-cell isolation

The immune response generated after repetitive injections with the LNPs-OVA with and without adjuvants was evaluated. Five groups of C57BL/6J mice ($n = 4$) were injected *i.v.*, three times, 5 days apart with a final volume of 100 μL . The cohorts were injected as follows: (i) 7 μg LNPs-OVA; (ii) 7 μg LNPs-OVA and 25 μg R848; (iii) 7 μg LNPs-OVA and 5 μg cGAMP; (iv) 7 μg LNPs-OVA, 25 μg R848 and 5 μg cGAMP; and (v) PBS control. One day after the last boost, the animals were anesthetized (*i.p.*, 100 mg/kg ketamine and 5 mg/kg xylazine), weighed and blood was collected by intracardiac puncture and sera was isolated and stored for further serological analysis and liver transaminases quantitation. Mice were then euthanized by cervical dislocation and livers and spleen were dissected for single-cell isolation. Mouse NPCs were isolated as explained before and spleen single-cell suspension was obtained by spleen disaggregation through a 70- μm strainer. After a washing step, splenocyte suspensions were treated with RBC lysis buffer (Thermo Fisher Scientific) for 5 min at RT and counted for seeding and *ex vivo* stimulation. In contrast, NPCs and splenocytes were stained and analyzed by flow cytometry to evaluate the innate immune response by the activation state of the different cell subpopulations after repetitive injections (Figures S6–S8).

Splenocyte specific stimulation

To evaluate the specific-adaptive immune response against OVA after the immunization scheme, splenocytes were seeded into 96-well U-bottom plates (2×10^6 cells/200 μL /well) in RPMI-1640 medium supplemented with 2% L-glutamine, 10% iFBS and 1% penicillin-streptomycin. Cells were then either unstimulated (only culture medium) or stimulated with 2 $\mu\text{g}/\text{mL}$ of the MHC I-restricted SIINFEKL or the MHC II-restricted GRAY peptides (JPT Peptide Technologies GmbH, Berlin, Germany) and incubated at 37°C overnight for intracellular cytokine staining (IFN- γ , IL-2, and TNF- α) and 24 h for quantification of the cytokines in supernatants (Figure S9). Splenocytes were also stimulated with 10 $\mu\text{g}/\text{mL}$ of OVA protein (InvivoGen) for 48 h to evaluate the CD4 $^{+}$ as well as the CD8 $^{+}$ T cell-specific proliferation by intranuclear staining of Ki-67 and to

estimate the specific response of T $_{\text{reg}}$ cells upon MHC II-restricted GRAY peptide stimulation (Figure S10).

Flow cytometry

To assess the activation of the NPC subpopulations after the *in vitro* incubation with adjuvants and upon a single *i.v.* LNPs-OVA injection with and without adjuvants, cells were washed with 150 μL DPBS, 100 μL 0.05 mM EDTA was added, and the cells were detached by incubation on ice for 30 min. The detached cells were collected; single NPC suspensions were washed with washing buffer (2% FBS in DPBS) and Fc receptors were blocked by the addition of CD16/CD32-specific antibody (clone 2.4G2, Invitrogen) at 4°C for 15 min. The cells were stained with fluorescent dye-conjugated monoclonal antibodies (Table S2) specific for: mouse CD45 (eFlour 506) purchased from Invitrogen; F4/80 (AF 488), CD86 (APC) purchased from BioLegend; CD32b (PE), CD11c (PE-Cy7), CD80 (SuperBright 436), and CD86 (APC) purchased from eBioscience (San Diego, CA, USA) for 30 min at 4°C in the dark. The stained cells were washed, and viability was assessed by incubation with 7-AAD (BD Biosciences, Franklin Lakes, NJ, USA) at RT for 5 min in the dark. The cells were analyzed by flow cytometry (Figures S6 and S7) using an LSRII flow cytometer (BD Biosciences) and FlowJo software v10.8.0 (BD Biosciences).

CBA

Cell culture supernatants were collected and stored at -20°C prior to cytokine/chemokine analysis. The cytokine and chemokine concentrations were quantified using the LEGENDplex mouse anti-virus response panel kit, 13-plex(BioLegend) that includes the following murine cytokines/chemokines: IFN- γ , KC, TNF- α , MCP-1, IL-12p70, RANTES, IL-1 β , IP-10, GM-CSF, IL-10, IFN- β , IFN- α , and IL-6. The assay was performed as recommended by the manufacturer (BioLegend). Samples were acquired with an LSRII flow cytometer (BD Biosciences). Results were analyzed using Qognit Legendplex Analysis Software (BioLegend). The cytokines released from the 24 h peptide-stimulated splenocytes isolated in the immunization study were quantified using the LEGENDplex mouse Th Cytokine Panel kit (BioLegend) including IFN- γ , TNF- α , IL-2, IL-6, IL-10, IL-4, and IL-22. The samples were acquired with a Symphony A3 flow cytometer (BD Biosciences) and the results were analyzed using the Qognit Legendplex Analysis Software (BioLegend).

Serological analysis

The sera were isolated from whole blood in the immunization study and anti-OVA IgG titers were determined by ELISA. Briefly, 96-half area well plates were coated with 10 $\mu\text{g}/\text{mL}$ OVA protein at 4°C in humid chamber overnight. Plates were washed, blocked with 3% skim milk-PBS and the sera were incubated in serial dilutions at RT for 1 h. Anti-mouse IgG H&L (HRP) (ab6789; Abcam) was applied as secondary antibody and plates were developed with TMB ELISA substrate and read at 450 nm in an ELISA plate reader after adding Stop solution. Titers were calculated using regression analysis.

Statistical analysis

All graphs and statistical analyses were performed with GraphPad Prism software v10.1.2 (GraphPad Software, San Diego, CA, USA). One-way ANOVA followed by Dunnett's or Tukey's multiple comparison test and two-way ANOVA followed by Tukey's multiple comparison test was used to compare three or more groups. Data are the means $\pm$ SEM. Significantly different between groups: ns, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

DATA AVAILABILITY

Data supporting the findings of this study are available from the corresponding author, M.L.C., upon request. The data are not publicly available due to restrictions that could compromise proprietary information.

ACKNOWLEDGMENTS

This research was funded by Deutsche Forschungsgemeinschaft (DFG), grant number SFB1066-3 and the subprojects B15 (M.S. and S.G.) and B17 (P.S. and L.K.). The animal study protocols were approved by the local authorities (Landesuntersuchungsamt Rheinland-Palatinat). Ethical approval was granted by the Landesuntersuchungsamt LUA, Koblenz, AK G20-1-123 and G20-1-130. All graphical abstract and figure schemes were created with Biorender software (<https://BioRender.com/d35a436>). The authors are grateful to Stephen H. Gregory (Providence, RI, USA) for editing this manuscript.

AUTHOR CONTRIBUTIONS

M.L.C., S.G., M.B., and M.J.L. designed and planned experiments. L.K. provided oversight on the study design. M.S. and M.J.L. performed *in vitro* and *in vivo* experiments. K.H., S.F.E., and R.G. assisted with *in vitro* experiments. R.G., L.P., Y.Z., and P.S. assisted with *in vivo* experiments. G.A.I. and I.R.B. prepared LNP formulations. M.S. and M.J.L. processed animal samples and tissues. M.S. and M.J.L. analyzed data and performed statistical analyses. M.S. and M.J.L. drafted figures. M.L.C. wrote and edited the manuscript. S.G. and M.B. provided financial support for the study. All authors performed critical revision and approved final manuscript text and figures.

DECLARATION OF INTERESTS

M.L.C. is currently an employee at BioNTech SE (Mainz, Germany); however, the contributions from M.L.C. were made prior to his employment at BioNTech SE. The remaining authors declare no conflict of interest. L.K. declares receiving travel expenses and speaker honoraria from Gilead science (Foster City, CA, USA) and Takeda (Doshomachi, Japan).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtm.2025.101427>.

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6.3. Publication 3

“Lipoamino bundle LNPs for efficient mRNA transfection of dendritic cells and macrophages show high spleen selectivity”

Published in: *European Journal of Pharmaceutics and Biopharmaceutics*. **2024** Jan;194:95-109.

DOI: 10.1016/j.ejpb.2023.11.025

In this paper, lipoamino xenopeptide structures were incorporated into mRNA lipid nanoparticles (LNPs) for nucleic acid-based therapies. These lipoamino xenopeptides consist of polar synthetic amino acids and lipophilic tertiary lipoamino fatty acids, and are arranged in different 3D topologies like bundles or U-shapes. These optimized LNPs demonstrated efficient mRNA encapsulation, enhanced cellular uptake, and improved endosomal escape. Further, *in vitro* studies showed fast transfection and high potency in various tumor cell lines, macrophages, and dendritic cells, even at low mRNA doses and in the presence of serum. *In vivo* experiments with a highly efficient lipoamino xenopeptide structure incorporated into LNP showed that intravenous administration resulted in strong mRNA expression with selectivity for dendritic cells and macrophages, particularly in the spleen.

Contribution:

As co-author of this paper, I performed analysis and visualization of *in vivo* experiments.

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Haase F, Pöhmerer J, Yazdi M, Grau M, **Zeyn Y**, Wilk U, Burghardt T, Höhn M, Hieber C, Bros M, Wagner E, Berger S. Lipoamino bundle LNPs for efficient mRNA transfection of dendritic cells and macrophages show high spleen selectivity. *Eur J Pharm Biopharm*. 2024 Jan;194:95-109. doi: 10.1016/j.ejpb.2023.11.025. Epub 2023 Dec 6.



Research paper

Lipoamino bundle LNPs for efficient mRNA transfection of dendritic cells and macrophages show high spleen selectivity

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ARTICLE INFO

Keywords:

mRNA
LNPs
Xenopeptides
Amino ethylene
Lipoamino fatty acid
Dendritic cells
Macrophages
Spleen

ABSTRACT

Messenger RNA (mRNA) is a powerful tool for nucleic acid-based therapies and vaccination, but efficient and specific delivery to target tissues remains a significant challenge. In this study, we demonstrate lipoamino xenopeptide carriers as components of highly efficient mRNA LNPs. These lipo-xenopeptides are defined as 2D sequences in different 3D topologies (bundles or different U-shapes). The polar artificial amino acid tetraethylene pentamino succinic acid (Stp) and various lipophilic tertiary lipoamino fatty acids (LAFs) act as ionizable amphiphilic units, connected in different ratios via bisamidated lysines as branching units. A series of more lipophilic LAF₄-Stp₁ carriers with bundle topology is especially well suited for efficient encapsulation of mRNA into LNPs, facilitated cellular uptake and strongly enhanced endosomal escape. These LNPs display improved, faster transfection kinetics compared to standard LNP formulations, with high potency in a variety of tumor cell lines (including N2a neuroblastoma, HepG2 and Huh7 hepatocellular, and HeLa cervical carcinoma cells), J774A.1 macrophages, and DC2.4 dendritic cells. High transfection levels were obtained even in the presence of serum at very low sub-microgram mRNA doses. Upon intravenous application of only 3 µg mRNA per mouse, *in vivo* mRNA expression is found with a high selectivity for dendritic cells and macrophages, resulting in a particularly high overall preferred expression in the spleen.

1. Introduction

Over the last decades, lipid nanoparticles (LNPs) have evolved to become the most advanced nonviral platform for safe and highly effective nucleic acid delivery *in vivo*, since they can encapsulate and deliver a wide variety of therapeutic agents. The first FDA approved small-interfering RNA (siRNA) drug, Onpattro (Patisiran), is an LNP formulation that after intravenous application delivers siRNA into liver hepatocytes for the treatment of hereditary transthyretin amyloidosis [1]. More recently, messenger RNA (mRNA) LNPs have gained importance as intramuscularly applied vaccines in the context of the COVID-19

pandemic [2] and for other vaccination directions, such as antitumoral immunotherapy [3]. In addition, other work uses LNPs for the co-delivery of different nucleic acid molecules, such as CRISPR-Cas9 mRNA and single guide RNA to the liver, as evaluated in a recent clinical study for the treatment of transthyretin amyloidosis [4].

LNPs typically consist of four lipidic components: cholesterol, phospholipids, polyethylene glycol (PEG)-lipids, and cationizable lipids [5]. These components enable monodisperse particle formation and high encapsulation efficiency of nucleic acid, improve particle stability, and promote endosomal escape of nucleic acid after cellular uptake via endocytosis. The main focus in LNP research is the optimization of

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<https://doi.org/10.1016/j.ejpb.2023.11.025>

Received 28 August 2023; Received in revised form 23 November 2023; Accepted 30 November 2023

Available online 6 December 2023

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ionizable lipids since these are mainly responsible for efficient complexation of the negatively charged nucleic acid and for endosomal escape after cellular uptake. Most recently, LNP optimization through variation of phospholipids, PEG lipids, replacement of cholesterol as well as ratio optimization of the individual components has come to the fore [6,7]. For example, by replacing zwitterionic 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) with anionic 1,2-distearoyl-*sn*-glycero-3-phosphoglycerol (DSPG) in Onpattro (Patisiran), the LNP surface charge can be switched from neutral to anionic and thus target the reticuloendothelial system (RES) [8]. Furthermore, the combination of appropriate helper lipids is crucial. By changing the head group of DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) to ethanolamine (DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine), the LNP formulation containing cationic lipid DODAP (1,2-dioleoyl-3-dimethylammonium propane) facilitates a transition from the LDL (low density lipoprotein) receptor-mediated hepatocyte delivery via apolipoprotein E (ApoE) to predominant spleen targeting via complement receptors [9,10]. Currently, the most advanced LNP formulations base on standard cationizable lipids with a single positive charge or lipidoids with several positive charges [11,12]. *In vivo*, the LNP composition dictates protein corona formation in the bloodstream, which in turn affects bio-distribution and cellular uptake. Due to ApoE-mediated liver targeting and good blood perfusion, the liver represents an ideal organ for LNP delivery and a major site of drug metabolism [13]. In addition to that, the LNP composition influences the physico-chemical properties (particle size, charge, surface shielding), which enables organ-selective targeting in the absence of receptor targeting moieties. For example, particle size can be regulated by altering the PEG-lipid concentration to reach hepatocytes by passing through liver fenestrae (100–160 nm depending on species) [14]. Further, negatively charged particles show high spleen preference as reported for lipoplexes [15]; and larger particle sizes, ranging from 200 to 500 nm, are beneficial for targeting splenic dendritic cells [16].

Despite the potent delivery of LNPs to the liver and the overall established medical usefulness of hepatocyte-directed nucleic acid therapies, it may be desirable to redirect the particles to other therapeutically relevant tissues. A new approach to minimize liver accumulation is selective organ targeting (SORT). This method involves the addition of a fifth, “sorting” molecule to the lipid composition that determines the tissue-specific delivery and activity of the LNPs [17–19]. Most recently, peptide ligands specifically binding receptors/cell types were identified by *in vivo* phage peptide biopanning and conjugated to mRNA LNPs for photoreceptor targeting of the neutral retina after sub-retinal injection [20]. Additionally, there are strategies to address leukocytes; e.g., conjugation of a fusion protein, which addresses $\alpha\beta\gamma$ integrin expressed on gut-homing leukocytes, to the surface of the LNPs resulted in interferon γ silencing in the gut and therapeutic efficacy in experimental colitis [21]. Another approach is the design of new libraries of ionizable amino lipids based on ethanolamine, hydrazine, or hydroxylamine linkers for efficient siRNA delivery into leukocytes [22]. Library screening and evaluation with Fast Identification of Nanoparticle Delivery (FIND), a high-throughput DNA barcode-based *in vivo* LNP screening, facilitates the characterization of chemically distinct LNPs for efficient RNA delivery to T-lymphocytes or splenic immune cells *in vivo* via chemical targeting [23,24]. Also, the additional incorporation of the bioactive phospholipid phosphatidylserine into LNPs promotes mRNA transfection efficiency in secondary lymphoid organs both *in vitro* and *in vivo* [25,26].

A critical issue in successful nucleic acid delivery is the endosomal escape. Due to their ionizable character, LNPs are neutral at physiological pH, but become protonated in the acidic environment of endosomes. This aids in the fusion of their lipids with the endosomal membrane and facilitates nucleic acid delivery to the cytosol [27,28]. Nevertheless, most of the cargo remains inactive by accumulation in late endosomes and lysosomes [5,29]. There are several factors and limitations to endosomal escape that can hinder the efficiency of nanocarrier-

mediated drug delivery, resulting in a cargo escape rate of only 1–2% [30,31]. Overcoming these barriers is critical for the efficiency of therapeutic agents [32,33].

The present study aimed at the evaluation of mRNA LNP formulations with improved intracellular cargo release. For this purpose, recently developed double pH-responsive lipo-xenopeptides [34] were evaluated as cationizable components in mRNA LNPs.

These sequence-defined xenopeptides were synthesized via solid phase-assisted peptide synthesis (SPPS). The double pH-responsiveness was implemented by combining cationizable polar aminoethylene units in form of the artificial amino acid succinoyl tetraethylene pentamine (Stp) [35], with cationizable apolar units in form of lipoamino fatty acids (LAFs) [34], yielding different 2D sequences and 3D topologies. These LAF-Stp carriers show a molecular chameleon character due to the pH-dependent switch in polarity and high endosomolytic activity. Selected library members were already found highly effective in standard mRNA polyplexes [34]. To identify the best suitable LAF-Stp carriers for mRNA LNPs in comparison to standard ionizable lipids that contain only single tertiary amines (i.e., SM-102, and DLin-MC3-DMA) [2,5,36,37], our multi-protonatable carriers with 6–10 protonatable amines were tested in different molar lipid, molar charge, and N/P ratios.

The LAF-Stp carriers formulated as LNPs turned out to be very potent, mediating high mRNA expression *in vitro* and *in vivo*, with an interesting specificity for the spleen as a central immune organ, displaying a far higher spleen/liver expression ratio than established standard SM-102 and DLin-MC3-DMA LNPs. All in all, our novel LAF-Stp carriers proved to be an interesting alternative to ionizable lipids in LNPs.

2. Materials and methods

2.1. Materials

Chemically modified CleanCap® FLuc mRNA (5moU), CleanCap® mCherry mRNA (5moU), and CleanCap® eGFP (5moU) encoding firefly luciferase, fluorescent protein mCherry, or enhanced green fluorescent protein (eGFP), respectively, were obtained from Trilink Biotechnologies (San Diego, CA, USA). EZ Cap™ Cy5 Firefly Luciferase mRNA (5-moUTP) was ordered from Apexbio Technology LLC (Houston, USA). Cholesterol was purchased from Sigma-Aldrich (Munich, Germany). 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2-dimyristoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG 2000) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The ionizable lipid DLin-MC3-DMA was ordered from MedChemExpress (Monmouth Junction, NJ, USA) and SM-102 from Biosynth Carbosynth (Staad, Switzerland). HEPES was purchased from Biomol (Hamburg, Germany), hydrazinium hydroxide, glucose, and disodium ethylenediaminetetraacetic acid (EDTA) from Merck (Darmstadt, Germany). Agarose BioReagent – low EEO was purchased from Sigma Aldrich (Munich, Germany), and GelRed 10,000 × as well as ethanol absolute from VWR (Darmstadt, Germany).

Antibodies for flow cytometric analyses (immunostaining experiment, see section 2.6.3). eFl450-labeled anti-F4/80 (clone BM8), SB702-CD45 (30-F11), PE-CD32b (AT130-2), PE-Cy7-CD11c (N418), eFl506-CD3 (145-2C11), SB600-CD11b (M1/70), PE-CD11c (N418), PE-eFl610-Ly6G (1A8-L6g), and eFl780-FVD (fixable viability dye for live/dead cell discrimination) were purchased from ThermoFisher (Waltham, MA, USA). APC-CD19 (1D3) was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

All cell culture consumables were obtained from Faust Lab Science (Klettgau, Germany). Cell culture media, fetal bovine serum (FBS), antibiotics, trypsin/EDTA as well as paraformaldehyde (PFA) were purchased from Sigma Aldrich (Munich, Germany) and PAN-Biotech (Aidenbach, Germany). Cell culture 5 × cell culture lysis buffer, and D-luciferin sodium salt were ordered from Promega (Mannheim,

Germany); 6-(p-toluidino)-2-naphthalenesulfonyl chloride (TNS), β -mercaptoethanol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), dithiothreitol (DTT), adenosine 5'-triphosphate (ATP) disodium salt trihydrate, coenzyme A trilithium salt, bafilomycin A1, and protease and phosphatase inhibitor cocktail from Sigma-Aldrich (Munich, Germany).

Polypropylene syringe microreactors were obtained from Multi-syntech (Witten, Germany). Fmoc-Stp(Boc)₃-OH was synthesized in house according to published protocols [34]. Fmoc-protected amino acids Fmoc-L-Lys(Dde)-OH and Fmoc-L-Lys(Fmoc)-OH, 2-chlorotrityl-chloride polystyrene resin, dimethylformamide and piperidine were acquired from Iris Biotech (Marktredwitz, Germany). Diisopropylethylamine (DIPEA), 1-hydroxybenzotriazole hydrate (HOBt), triton X-100, triisopropylsilane (TIS) were purchased from Sigma-Aldrich (Munich, Germany). (Benzotriazol-1-yloxy)-tripyrrolidinophosphonium hexafluorophosphate (PyBOP) was procured from Millipore (Oakville, Canada). Oleic acid was bought from ThermoFisher (Kandel, Germany). Trifluoroacetic acid was obtained from Acros Organics (Geel, Belgium). Sephadex G10 was acquired from GE Healthcare (Uppsala, Sweden).

2.2. Synthesis of cationizable carriers

The library of ionizable LAF-Stp carriers (Table 1) was synthesized via solid phase-assisted peptide synthesis (SPPS) as described in Thal-mayr et al. [34]. The synthesis of OleA carrier **1829** was likewise carried out as described in the Supporting Information, replacing the terminal LAF units with oleic acid (OleA) analogously as previously described [38–40].

2.3. mRNA LNP formulation

LNPs were formulated by mixing an acidic aqueous phase containing mRNA with an EtOH phase containing ionizable and helper lipids (v/v

3:1) by rapid pipetting. The aqueous phase was prepared with mRNA in citrate buffer (10 mM, pH 4.0). The EtOH phase includes a mixture of cholesterol, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dimyristoyl-*rac*-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG 2000) and an ionizable lipid/carrier at predetermined molar and nitrogen/phosphate (N/P) ratios shown in Table 2. The N/P ratio represents the molar ratio of all protonatable nitrogens of the carrier to phosphates of the nucleic acid. All secondary amines of the Stp units, terminal amines, and the tertiary amines of the LAF units were considered in this N/P ratio calculation. The aqueous and EtOH solutions were rapidly mixed by pipetting for 30 sec and then incubated at room temperature (RT) for 10 min to allow LNP assembly. The formulated LNPs were diluted with HBG (HEPES buffer 20 mM with 5% (w/v) glucose; pH 7.4) to a final ethanol concentration below 5% for *in vitro* experiments and dialyzed against HBG in 1 kDa MWCO tubes at 4 °C for 2 h for subsequent *in vivo* studies. The final concentration of nucleic acid in the LNP solution was 3 μ g/mL, unless indicated differently.

2.4. Physicochemical characterization of LNPs

2.4.1. Particle size and zeta potential measurement

LNPs were formulated in HBG as described in section 2.3 at a concentration of 3 μ g/mL FLuc mRNA. Measurements of size and zeta potential were performed with a Zetasizer Nano ZS (Malvern Instruments, Malvern, Worcestershire, U.K.) in a folded capillary cell (DTS1070) by dynamic and electrophoretic laser-light scattering (DLS, ELS). Size and polydispersity index were measured in 80 μ L of the above described nanoparticle solutions using the following instrument settings: equilibration time 30 sec, temperature 25 °C, refractive index 1.330, viscosity 0.8872 mPa*s. Samples were measured three times with six sub runs per measurement. For measurement of the zeta potential, all samples were diluted to 800 μ L with HBG directly before measurement. Parameters were identical to the size measurement apart from an equilibration time of 60 sec. Three measurements with 15 sub runs lasting 10 sec each were performed, and zeta potentials were calculated by the Smoluchowski equation.

2.4.2. Agarose gel shift assay

A 1% (w/v) agarose gel was prepared by dissolving agarose in 1x TBE buffer (10.8 g trizma base, 5.5 g boric acid, 0.75 g disodium EDTA, in 1 L of water) and heating it up to 100 °C. Then, 1x GelRed was added, and the agarose solution was poured in the electrophoresis chamber and left for 40 min at RT to form a gel. mRNA LNPs were prepared as described above (see section 2.3) containing 60 ng of FLuc mRNA in 20 μ L of HBG. 4 μ L of loading buffer (6 mL glycerol, 1.2 mL 0.5 M EDTA, 2.8 mL H₂O, 0.02 g bromophenol blue) was added to each sample before filling it into the gel pockets. Electrophoresis was performed in 1x TBE buffer at 120 V for 70 min.

2.4.3. TNS dye binding assay

For the TNS assay, 20 mM citric acid/NaOH buffer (with 150 mM NaCl; pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5), 20 mM sodium dihydrogen phosphate/NaOH buffer (with 150 mM NaCl; pH 6.0, 6.4, 6.8, 7.2, 7.6, 8.0), and 20 mM Tris/HCl buffer (with 150 mM NaCl; pH 8.5, 9.0, 9.5, 10.0) were prepared. TNS was dissolved at 0.6 mM in water as a stock solution. In a black 96-well plate, 2 μ L of the TNS solution, 12 μ L of the LNP solution containing 250 ng of FLuc mRNA in 20 μ L of HBG and 186 μ L of each of the buffers above were mixed. After shaking the incubation mixture (400 rpm, 10 min), the fluorescence of the TNS was measured using a Spark Tecan microplate reader (Spectrafluor Plus, Tecan, Männedorf, Switzerland) at an excitation wavelength of 360 nm and an emission wavelength of 465 nm. The apparent pK_a of the surface was calculated as the pH at which the LNP showed 50% of the maximum fluorescence.

Table 1

LAF-Stp carriers with bundle and U-shape topologies.

ID	sequence (N→C)	Stp/LAF ratio	protonatable amines
			with/without LAF
Bundles			
1613	K[K(12Oc) ₂] ₂ -Stp	1:4	7 / 3
1621	K[K(8Oc) ₂] ₂ -Stp	1:4	7 / 3
1713	K[K(12Oc) ₂] ₂ -Stp ₂	2:4	10 / 6
1730	K[K(8Oc) ₂] ₂ -Stp ₂	2:4	10 / 6
1752	K[K(12Bu) ₂] ₂ -Stp	1:4	7 / 3
1753	K[K(16Bu) ₂] ₂ -Stp	1:4	7 / 3
1754	K[K(12He) ₂] ₂ -Stp	1:4	7 / 3
1755	K[K(14He) ₂] ₂ -Stp	1:4	7 / 3
1762	K[K(10Oc) ₂] ₂ -Stp	1:4	7 / 3
1829	K[K(OleA) ₂] ₂ -Stp	–	3 / 3
U-shapes			
1611	K(12Oc)-Stp-K(12Oc)	1:2	6 / 4
1612	K(12Oc) ₂ -Stp-K(12Oc)-K(12Oc)	1:4	7 / 3
1620	K(8Oc) ₂ -Stp-K(8Oc)-K(8Oc)	1:4	7 / 3
1716	K(12Oc) ₂ -Stp-K[K(12Oc) ₂]	1:4	7 / 3
1718	[K(12Oc)] ₂ -Stp-[K(12Oc)] ₂	1:4	8 / 4
1720	K[K(12Oc) ₂]-Stp-K[K(12Oc) ₂]	1:4	8 / 4
1722	K(12Oc) ₂ -Stp ₂ -K(12Oc)-K(12Oc)	2:4	10 / 6

K, lysine; OleA, oleic acid; Stp, succinyl tetraethylene pentamine; 12Bu, 16 Bu: LAF (lipophilic fatty acid) based on 4-aminobutanoic acid modified with two dodecyl chains or two hexadecyl chains; 12He, 14He: LAF based on 6-aminohexanoic acid and two dodecyl or tetradecyl chains; 8Oc, 10Oc, 12Oc: LAF based on 8-aminooctanoic acid and two octyl, decyl, or dodecyl chains, respectively. **1829** presents a OleA-based bundle control sequence. The number of protonatable amines includes Stp and N-terminal amino groups and is listed alternatively with and without lipophilic LAF tertiary amines that are not considered to be protonated at physiological pH.

Table 2
Molar (mol%) and w/w (weight%) ratios of the lipids and ionizable carrier used in mRNA LNPs, and ratios between cationizable carrier to mRNA.

ID	ratios		ratios				
	Chol/DSPC/PEG-DMG/ionizable carrier		w/w (total lipids + carrier)/mRNA	N/P (ionizable carrier/mRNA)			
	molar [mol%]	w/w [weight%]		N with LAF	N/P	N without LAF	alternative N/P
Positive controls							
MC3	38.5/10/1.5/50	25.4/13.5/6.4/54.7	10		3	–	–
SM-102	38.5/10/1.5/50	24/12.7/6.1/57.2	22		6	–	–
Bundles							
1613	59.9/15.6/2.3/22.2	22.2/11.8/5.6/60.5	17	4 + 3	9	3	4.1
1621	59.9/15.6/2.3/22.2	24.5/13/6.2/56.3	16	4 + 3	9	3	4.1
1713	64.2/16.7/2.5/16.7	25.4/13.5/6.4/54.7	15	4 + 6	9	6	5.6
1730	64.2/16.7/2.5/16.7	27.5/14.6/6.9/51	14	4 + 6	9	6	5.6
1752	59.9/15.6/2.3/22.2	23.3/12.4/5.9/58.5	17	4 + 3	9	3	4.1
1753	59.9/15.6/2.3/22.2	21.2/11.2/5.3/62.3	18	4 + 3	9	3	4.1
1754	59.9/15.6/2.3/22.2	22.7/12.1/5.7/59.5	17	4 + 3	9	3	4.1
1755	59.9/15.6/2.3/22.2	21.7/11.5/5.5/61.4	18	4 + 3	9	3	4.1
1762	59.9/15.6/2.3/22.2	21.7/11.5/5.5/61.4	17	4 + 3	9	3	4.1
1829	46.2/12/1.8/40	16.9/9/4.3/69.9	23	0 + 3	9	3	9
U-shapes							
1611	57.8/15/2.2/25	27/14.3/6.8/51.9	14	2 + 4	9	4	6.3
1612	59.9/15.6/2.3/22.2	22.2/11.8/5.6/60.5	17	4 + 3	9	3	4.1
1620	59.9/15.6/2.3/22.2	24.5/13/6.2/56.3	16	4 + 3	9	3	4.1
1716	59.9/15.6/2.3/22.2	22.2/11.8/5.6/60.5	17	4 + 3	9	3	4.1
1718	61.6/16/2.4/20	23.2/12.3/5.9/58.6	17	4 + 4	9	4	4.7
1720	61.6/16/2.4/20	23.2/12.3/5.9/58.6	17	4 + 4	9	4	4.7
1722	64.2/16.7/2.5/16.7	25.4/13.5/6.4/54.7	15	4 + 6	9	6	5.6

The N/P ratio represents either the molar ratio of all protonatable nitrogens (LAF + Stp + any terminal amine) of the carrier to phosphates of the mRNA, or as an alternative N/P calculation without considering the lipophilic tertiary LAF amines. LAF; lipoamino fatty acid; N, protonatable nitrogen; Stp, succinoyl tetraethylene pentamine.

2.4.4. RiboGreen assay
mRNA encapsulation efficiency [ee(%)] was measured using QUANT-iTTM RiboGreen RNA Assay-Kit (ThermoFisher Scientific). LNPs were formulated in HBG as described in section 2.3. In a black 96-well plate, 40 µL of HBG (blank) or LNP solution were added to 210 µL of 1x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5 in RNase-free water) per well. 50 µL of the obtained solutions was added to either 50 µL 1x TE buffer for non-lysed LNPs or 50 µL triton/1x TE buffer (2% (v/v) triton X-100 in TE buffer) for lysed LNPs. 96-well plates were incubated for 10 min at 37 °C to ensure complete lysis of triton incubated LNPs and afterwards cooled down for 5 min at RT. RiboGreen reagent was diluted 1:100 in 1x TE buffer and 100 µL were added to each well. After 5 min incubation, the fluorescent signal was measured in duplicates in a Spark Tecan microplate reader (Spectrafluor Plus, Tecan, Männedorf, Switzerland) at 485/535 nm excitation/emission wavelength. After blank subtraction, the encapsulation efficiency (in percent) was calculated with following equation:

$$ee(\%) = \left(1 - \frac{\text{mean emission}_{\text{non-lysedLNPs}}}{\text{mean emission}_{\text{lysedLNPs}}}\right) \times 100$$

2.5. In vitro characterization

2.5.1. Cell culture

The murine neuroblastoma cell line Neuro2A (N2a) (ATCC, American Type Culture Collection Manassas, Virginia, USA), the human adenocarcinoma cell line HeLa (DSMZ, German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany), and HeLa-Gal8-mRuby3 cells (stably expressing Galectin8-mRuby3 fusion protein) [41–43] were cultured in Dulbecco’s modified Eagle’s medium (DMEM)-low glucose (1 g/L glucose). Human hepatocellular carcinoma cell lines Huh7 (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan) and HepG2 (ATCC, American Type Culture Collection Manassas, Virginia, USA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM)/Nutrient Mixture F12-Ham. The murine dendritic cell (DC)-like cell line DC2.4 (Merck Millipore, Darmstadt, Germany) was grown in Iscove’s Modified Dulbecco’s Medium (IMDM). The murine

macrophage (MAC) model cell line J-774A.1 (DSMZ, German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) was cultured in DMEM-high glucose (4.5 g/L glucose). All cell culture media were supplemented with 10% (v/v) FBS, 4 mM of stable L-glutamine, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and in case of DC2.4 cells, additionally with 50 µM of β-mercaptoethanol. The cell lines were cultured at 37 °C and 5% CO₂ in an incubator at a relative humidity of 95%.

2.5.2. Cellular transfection efficiency of FLuc mRNA LNPs determined via luciferase reporter assay

One day prior to mRNA delivery, 10,000 N2a, 8000 Huh7, 8000 HepG2, 8000 HeLa, 5000 DC2.4, and 10,000 J774A.1 cells/well were seeded in 96-well plates. Before the treatment, cell culture medium was replaced by 80 µL of fresh medium containing 10% (v/v) FBS. LNPs were added in 20 µL HBG, unless indicated differently. mRNA LNP treatments were performed in triplicate in 96-well plates. The transfection efficiency of LNPs was evaluated for different doses of FLuc mRNA per well. HBG was used as negative control. DLIN-MC3-DMA (as used in Patisiran) and SM-102 (as used in the Moderna COVID-19 vaccine) LNPs served as positive controls for mRNA LNPs. After 24 h incubation at 37 °C, the medium was removed, and cells were treated with 100 µL of 0.5x cell culture lysis buffer (Promega). Prior to measurement of luciferase activity, plates were incubated for 1 h at RT. Luciferase activity was measured in 35 µL cell lysate for 10 sec in a Centro LB 960 plate reader luminometer (Berthold Technologies, Bad Wildbad, Germany) after addition of 100 µL/well of LAR buffer (20 mM glycylglycine, 1 mM MgCl₂, 0.1 mM EDTA, 3.3 mM DTT, 0.55 mM ATP, 0.27 mM coenzyme A; pH 8–8.5) supplemented with 5% (v/v) of a mixture of 10 mM luciferin and 29 mM glycylglycine. When the measured values overshoot the detector limit of the plate reader luminometer, the cell lysate was 10- or 100-fold diluted in PBS (phosphate buffered saline; 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄; pH 7.4) and mixed thoroughly before measurement. Transfection efficiency was presented as relative light units (RLU) per well after background subtraction (HBG-treated control cells).

2.5.3. Viability of transfected cells assessed via MTT assay

Transfections were performed as described above (see [section 2.5.2](#)). At 24 h after transfection, 10 μ L MTT (5 mg/mL) were added to each well, reaching a final concentration of 0.5 mg/mL. After incubation for 2 h at 37 °C and 5% CO₂, the supernatant was removed, and the plates were stored at –80 °C for at least 1 h. Then, the purple formazan product was dissolved in 100 μ L DMSO by constant shaking for 30 min at 37 °C. Quantification was done photometrically using a spark Tecan microplate reader (Spectrafluor Plus, Tecan, Männedorf, Switzerland). Absorbance was measured at wavelength λ = 590 nm with background correction at λ = 630 nm. Experiments were carried out in triplicates. Relative metabolic activity normalized to control wells (HBG-treated cells) was calculated by the equation: $[A]_{\text{test}}/[A]_{\text{control}}$.

2.5.4. Cellular transfection efficiency of mRNA LNPs determined by flow cytometry

mRNA LNP treatments were performed in triplicate in 96-well plates. N2a cells were seeded 24 h prior to transfection (10,000 cells/well). On the next day, the medium was replaced with 80 μ L of fresh pre-warmed medium containing 10% (v/v) FBS. The LNPs were prepared as described in [section 2.3](#), and volumes of 20 or 5 μ L of LNP solution containing 60 or 15 ng mCherry mRNA were added per well. HBG was added to reach a final volume of 100 μ L per well. 20 μ L HBG per well was used as negative control. After 24 h of treatment, cells were trypsinized and resuspended in PBS solution containing 10% (v/v) FBS (FACS buffer) for flow cytometric evaluation. All samples were analyzed by flow cytometry using a CytoFLEX S Flow Cytometer (Beckman Coulter, Brea, CA, USA). Before measurement, 1 ng/ μ L 4',6-diamidino-2-phenylindole (DAPI) was added to discriminate between viable and dead cells. The cellular fluorescence was assayed by excitation of DAPI at 405 nm and detection of emission at 450 nm. Only viable cells were considered. Gates were set compared to control measurements with HBG-treated cells. The percentage of mCherry positive cells represented efficient mRNA transfection. Flow cytometry data were analyzed using FlowJo™ v10.8 flow cytometric analysis software by FlowJo, LLC (Becton, Dickinson and Company, U.S.). The cellular mCherry expression was assayed by excitation at 561 nm, the detection of emission at 610 nm for mCherry. Only isolated viable cells were evaluated. The transfection efficiency was determined as the percentage of mCherry positive cells.

2.5.5. Confocal laser scanning microscopy (CLSM)

HeLa-Gal8-mRuby3 cells [41–43] (18,000 per well) were seeded into ibidi μ -slide 8-well chamber slides (ibidi GmbH, Germany) and cultured overnight at 37 °C. LNPs were formulated as described in [section 2.3](#) at a total mRNA concentration of 6.75 μ g/mL, whereby 80% of FLuc mRNA and 20% of Cy5-labeled FLuc mRNA were mixed. Medium was changed before transfection, and 20 μ L of LNP solution was added to the cells to a final volume of 300 μ L per well. After incubation at 37 °C for 4 h, cells were washed twice with PBS, fixed with 4% (w/v) paraformaldehyde in PBS for 45 min at RT in the dark and washed with PBS again. Nuclei were stained with DAPI (1 μ g/mL in PBS) for 20 min at RT in the dark. After removal of the staining solution, cells were washed twice with PBS and stored in 300 μ L fresh PBS. Imaging was performed with a Leica TCS-SP8 CLSM equipped with an HC PL APO 63 $\times$ 1.4 objective and images were processed with the LAS X software from Leica.

2.5.6. Bafilomycin A1 assay

N2a cells were seeded in 96-well plates as described above (see [section 2.5.2](#)) one day prior to transfection. Medium was changed to either fresh medium or medium supplemented with bafilomycin A1 (BafA1) (0.1 μ g/ μ L in DMSO) to reach a final concentration of 200 nM BafA1 after LNP addition and then incubated for 2 h. LNPs were formed as described in [section 2.3](#) at a concentration of 3 μ g/mL FLuc mRNA and transfected with 20 μ L per well. Cells were incubated for 4 h and then lysed. Luciferase activity was measured as described above (see

[section 2.5.2](#)). Transfections were performed in triplicates.

2.5.7. Serum dilution assay

One day prior to transfection, N2a, Huh7, DC2.4, and J774A.1 cells were seeded in 96-well plates as described above (see [section 2.5.2](#)). Medium was replaced with 80 μ L fresh medium containing 10% (v/v) FBS before the treatment. LNPs were formulated as described above (see [section 2.3](#)) at a final FLuc mRNA concentration of 3 μ g/mL. Directly before transfection, LNPs were diluted appropriately with FBS, and 20 μ L of each dilution was added to the cells at indicated mRNA doses ranging from 0.15 to 30 ng/well. As a control, HBG-diluted LNPs were transfected at an mRNA dose of 30 ng per well. Read-out was performed at 24 h after transfection via a luciferase reporter assay as described above (see [section 2.5.2](#)). Transfections were carried out in triplicates.

2.6. In vivo activity of mRNA LNPs in mice

2.6.1. In vivo application of mRNA LNPs

In vivo experiments were performed according to the guidelines of the German Animal Welfare Act and were approved by the animal experiments ethical committee of the Government of Upper Bavaria (accreditation number Gz. ROB-55.2-2532.Vet.02-19–20). For the evaluation of luciferase expression ([section 2.6.2](#)), N2a cells (10⁶ cells in 150 μ L PBS) were inoculated subcutaneously into the left flank of 6-week-old female A/J mice (Envigo RMS GmbH, Düsseldorf, Germany). For the immunostaining experiment ([section 2.6.3](#)), tumor-free 6-week-old female A/J mice were used. Mice were randomly divided into groups of three (tumor-bearing mice) or five (tumor-free mice), respectively, and were housed in isolated ventilated cages under specific pathogen-free conditions with a 12 h day/night interval, and food and water ad libitum. Weight and general well-being were monitored continuously. Tumor size was measured with a caliper and determined by the formula: $\frac{a \cdot b^2}{2}$ (a = longest side of the tumor; b = widest side vertical to a). When tumors reached a size of 250–500 mm³, LNPs formulated with FLuc mRNA were injected intravenously via the tail vein at a dose of 3 μ g mRNA in 150 μ L injection volume per mouse. The same application (3 μ g mRNA; 150 μ L HBG; i.v.) was done in the tumor-free 6-week-old mice with eGFP mRNA LNP formulations. At 6 h post LNP injections, blood samples were taken for evaluation of plasma parameters, and the mice were euthanized. The organs (lungs, liver, spleen, kidneys, heart, muscle (biceps femoris), brain, and tumor) were dissected and washed with PBS. The luciferase or eGFP expression, respectively, were determined as described below.

2.6.2. Ex vivo luciferase reporter assay of organs and tumors

Organs and tumor tissues were homogenized in Luciferase Cell Lysis Lysis Reagent 1x, supplemented with 1% (v/v) protease and phosphatase inhibitor cocktail using a tissue and cell homogenizer (FastPrep®-24, MP Biomedicals, USA). Then, the samples were frozen overnight at –80 °C to ensure full lysis. In a next step, the samples were thawed and centrifuged for 10 min at maximum speed (~13,000 rpm) and 4 °C. Luciferase activity in 25 μ L supernatant was measured in a Centro LB 960 plate reader luminometer (Berthold Technologies, Bad Wildbad, Germany) for 10 sec as described above (see [section 2.5.2](#)). Luciferase activity was presented as relative light units (RLU) per gram (g) tumor or organ after background subtraction (lysis buffer)..

2.6.3. Ex vivo immunostaining

Preparation of single cell suspensions from dissected organs. Spleens were mashed through a 40 μ m cell strainer (Greiner Bio-One, Frickhausen, Germany) with a pestle to produce a single-cell suspension. Spleen cells (2×10^6 in 500 μ L) were resuspended in medium (IMDM containing 5% (v/v) FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 50 μ M β -mercaptoethanol), placed in FACS tubes and kept overnight in the incubator (37 °C, 7.5% CO₂). Liver

dissociation was performed to yield murine liver non-parenchymal cells (NPCs) following a previously described method [44]. Briefly, an enzyme-dependent dissociation mixture was pre-incubated for 15 min at 37 °C (Liver Dissociation Kit; Miltenyi Biotec, Bergisch-Gladbach, Germany). The liver tissue was dissected into small pieces and transferred into prepared C tubes (Miltenyi Biotec). The C tubes were subjected to a gentle MACS Dissociator using program m_liver_03. The resulting cell suspension was incubated under continuous shaking for 30 min at 37 °C, followed by another round of gentle MACS-mediated cell dissociation (m_liver_04). Subsequently, liver NPCs were enriched by using density centrifugation with 30% Histodenz-HBSS (both from Sigma-Aldrich, Munich, Germany). To obtain lung cell suspensions, the Lung Dissociation Kit (Miltenyi Biotec) was used, following the manufacturer's protocol.

Flow cytometry and gating strategy. To stain cells, cells were washed in antibody incubation buffer containing PBS, 2% (v/v) FBS, and 0.5 mM EDTA. Subsequently, the samples were incubated with Fc receptor blocking antibody (clone 2.4-G2) for 15 min at 4 °C. Afterwards, the samples were incubated with fluorescence-labeled antibodies (see section 2.1) for 20 min at 4 °C. Then, cells were washed with PBS and incubated with eF1780-FVD to identify living cells. Samples were measured using an Attune NxT flow cytometer, and data were analyzed by using Attune NxT software, both by ThermoFisher (Waltham, MA, USA). Gating was performed according to the strategies described in Fig. S1 and S2.

2.7. Statistical analysis

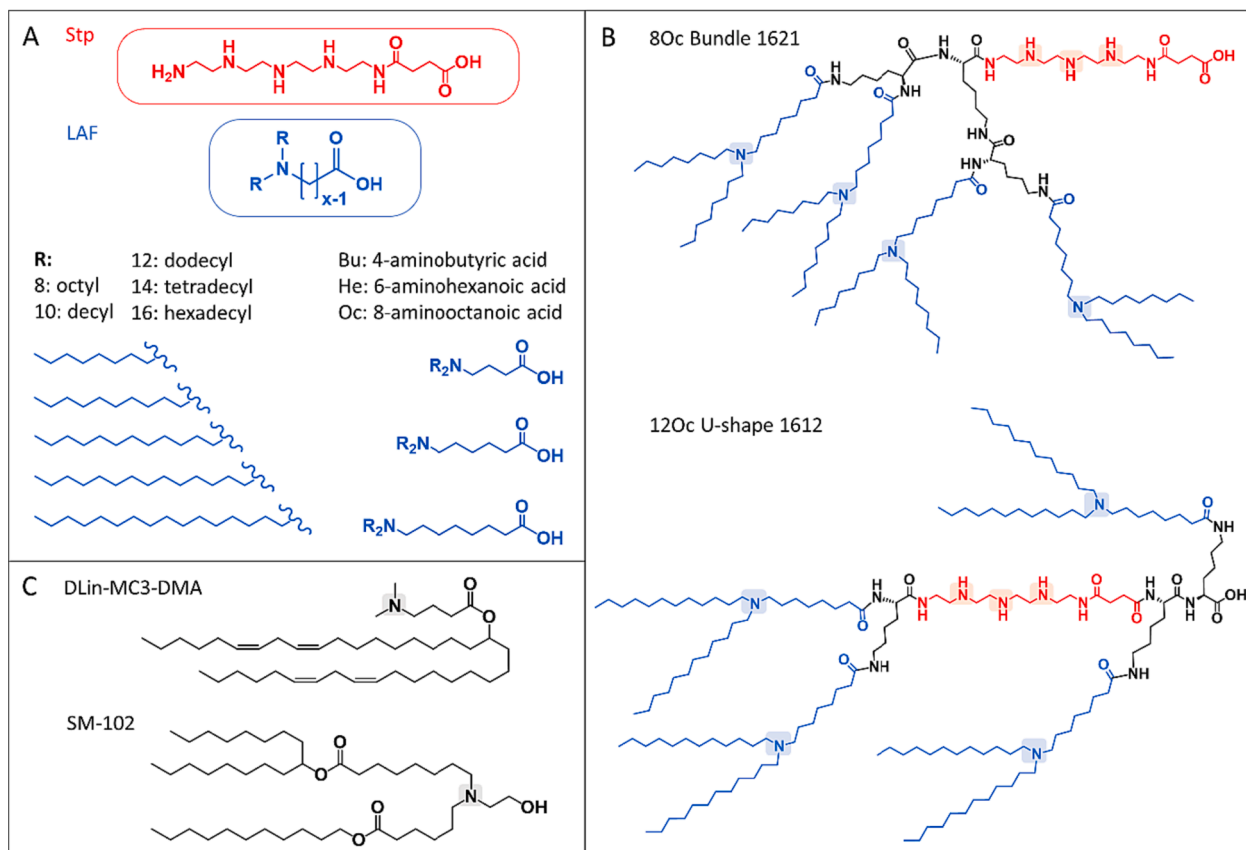
All data were analyzed at least in triplicates and presented as

arithmetic mean $\pm$ standard deviation (SD), if not otherwise stated. In order to analyze statistical significances, an unpaired Student's two-tailed t-test with Welch's correction, or an ordinary one-way ANOVA (multiple comparison, Tukey test), respectively, were carried out using GraphPad Prism™ 9.5.1. Statistical significance was indicated by p values ≤ 0.05 . The indication “ns” was used to display non-significant differences ($p > 0.05$).

3. Results and discussion

3.1. mRNA LNP formulation with novel ionizable LAF-Stp carriers

Recently, novel LAF-Stp carriers for nucleic acid delivery were generated by a sequence-defined combination of succinoyl tetraethylene pentamine (Stp) units as cationizable polar domain and lipoamino fatty acids (LAF) as cationizable lipidic domain, containing a central tertiary amine between hydrocarbon chains [34]. The LAFs were generated by reductive amination of different amino fatty acids with fatty aldehydes of various lengths. By this, the position of the tertiary amine was shifted within the LAF molecule, resulting in different LAF types. In the displayed LAF nomenclature, the carbon chain lengths of the terminal alkyl chains are indicated by digits (8, 12, 14, 16), and the amino fatty acids of the LAF building block by two-letter abbreviations (Bu, He, Oc) (Scheme 1A). Lysines connect the polar (Stp) and apolar (LAF) units into diverse topologies, for example bundles (N-terminal branched lipophilic domains), and U-shapes with hydrophilic center and lipophilic ends at the N- and C-terminus (Scheme 1B, Table 1). The hydrophilic/lipophilic balance within the carriers was varied by the adjustment of the ratio of



Scheme 1. LAF-Stp carriers as cationizable lipids for LNP formulations. (A) Succinoyl tetraethylene pentamine (Stp; red) and lipoamino fatty acid (LAF; blue) as building blocks for mRNA LNP carriers. Different LAFs comprise ω -amino fatty acids of different carbon backbone length (Bu, He, Oc) with amines disubstituted with alkyl chains of different carbon lengths (8, 10, 12, 14, 16). By this variation, the position of the tertiary amine can be shifted within the LAF lipidic acid, resulting in different LAF types. (B) Different topologies of LAF-Stp carriers, exemplarily shown for sequences of 8Oc bundle **1621** and 12Oc U-shape **1612**. (C) Commercially available gold standards DLin-MC3-DMA and SM-102.

Sfp to LAF units. Lipophilic tertiary LAF amino groups of the LAF-Sfp carriers are commonly not protonated at physiological pH, therefore the protonatable nitrogens are listed alternatively with and without consideration of lipophilic tertiary amines of the LAF units (Table 1).

So far, the LAF-Sfp carriers were used in polyplexes for pDNA, mRNA, and siRNA delivery [34]. In the current study their use as cationizable lipidic component within mRNA LNPs should be investigated. Hereto, a subset of the LAF-Sfp carrier library was selected. The more lipophilic LAF-Sfp carriers with only one polar Sfp unit per molecule and an Sfp/LAF ratio of 1:4 (bundle or U-shape topology) were considered to be most suitable for LNP formulations. For comparison, cationizable lipids DLin-MC3-DMA (MC3, as used in the Patisiran formulation) and SM-102 (as used in the Moderna COVID-19 vaccine) were included as commercially available gold standards. As a further control, the OleA-Sfp bundle structure **1829** (Fig. S3, Table 1) was synthesized as analog to LAF-Sfp bundle **1621**. Here, the LAF units were substituted by standard oleic acid. Since oleic acid is known to be efficient in lipoligomers [38] and ionizable lipids for LNP formulation and nucleic acid delivery [45], it was considered as a suitable control motif. All LAF-Sfp carriers used in the current study were synthesized and characterized as described in Thalmayr et al [34].

LNPs were prepared by flash mixing (rapid pipetting) of the acidic aqueous phase (citrate buffer 10 mM, pH 4.0) containing FLuc mRNA with the ethanol lipid phase composed of cholesterol, DSPC, PEG-DMG, and an ionizable LAF-Sfp carrier or standard lipid (Scheme 2A). LNPs were formulated at different molar and weight/weight (w/w) lipid ratios (Scheme 2B). Table 2 also lists the N/P ratio, which corresponds to the molar ratio of all protonatable nitrogens of the carrier (secondary amines of Sfp, tertiary amine of LAF, N-terminal amines) to negatively charged mRNA phosphates. All novel cationizable LAF-Sfp carriers contain at least six amines, whereas DLin-MC3-DMA and SM-102 contain only one tertiary amine. At an identical N/P ratio, this would

result in LAF-Sfp LNPs with a strongly reduced total lipid/mRNA mass ratio compared to the DLin-MC3-DMA and SM-102 LNPs. Hence, the N/P ratio of LAF-Sfp carriers was raised to 9 and similar w/w ratios of cholesterol/DSPC/PEG-DMG/ionizable carrier were used as in the state-of-the-art formulations, resulting in different molar ratios of lipids (Scheme 2B). As mentioned above, not all LAF-Sfp amines are protonated at physiological pH. Hence, an additional alternative calculation of the N/P ratio is provided in Table 2, without considering the lipophilic tertiary LAF amines (N/P ratio only including secondary amines of Sfp and N-terminal amines). For additional information, the ratio of total lipids plus carrier to mRNA in weight% is presented in Table 2.

3.2. Physicochemical characterization of mRNA LNPs

To figure out the requirements on the LAF-Sfp carriers for LNP formulation, particles were physicochemically characterized regarding size distribution, zeta potential, apparent surface pK_a , mRNA encapsulation efficiency, and stability of corresponding LNP formulations. DLS and ELS measurements revealed defined, homogeneous nanoparticles with hydrodynamic diameters of around 120–200 nm and an almost neutral zeta potential for the mRNA LNPs formulated with the novel LAF-Sfp carriers, by this exhibiting comparable particle properties like the standard LNPs formulated with DLin-MC3-DMA and SM-102 (Fig. 1). Only in the case of U-shape **1620** (8Oc, Sfp/LAF = 1:4), nanoparticle formation was problematic, resulting in larger particles with a z-average > 270 nm. Moreover, the tested LAF-Sfp LNPs successfully encapsulated mRNA (encapsulation efficiency $ee \geq 80\%$, except **1613**), as determined via RiboGreen assay (Table S1). This was confirmed by a gel shift assay (Fig. S4).

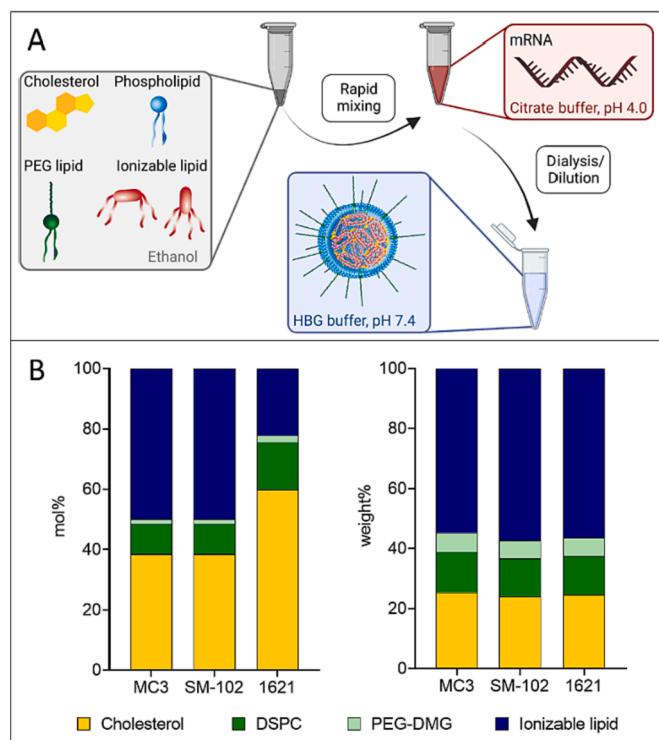
Additionally, the apparent surface pK_a of selected LNP formulations was determined by a TNS dye binding assay (Table S2). According to literature, the optimal pK_a of the LNP's surface is in the range of 6.2 to 6.5, resulting in neutral particles at physiological pH and positively charged particles in acidic endosomes, by this promoting endosomal disruption [5]. The results of the TNS dye binding assay were in accordance with the trend in zeta potential for the evaluated carriers, as 12Oc U-shape **1612** had the lowest apparent surface pK_a ($pK_a = 5.1$) and the lowest zeta potential of < -4 mV, followed by **1613** and **1716**. Bundle **1621** seemed to be most beneficial with an apparent pK_a of 6.4 (Table S2).

The replacement of LAF by oleic acid strongly affected particle formation. Comparing 8Oc bundle **1621** with its OleA analog **1829**, lack of aqueous solubility and aggregation in case of the latter was a severe issue (160 vs 1760 nm; Fig. 1). Therefore, **1829** was excluded from further physicochemical and biological characterization.

Bundles were additionally characterized at N/P 18. Compared to N/P 9, significantly increased particle sizes were obtained at this N/P ratio, except for bundles **1613** (12Oc) and **1762** (10Oc) (Fig. S5). Noteworthy, 8Oc bundle **1621** still formed monodisperse particles, yet size increased by 25 nm at the higher N/P ratio.

3.3. Biological evaluation of LAF-Sfp carriers in mRNA LNPs

The LAF-Sfp carriers were tested for their capability to deliver mRNA LNPs to three different tumor cell lines (N2a, HepG2, and Huh7) using a luciferase reporter assay (Fig. 2). A standard luciferase mRNA dose (250 ng/well) [40] and a fourfold lower dose (62.5 ng/well) were chosen. Lipidic carriers with a Sfp/LAF ratio of 1:4 (Fig. 2) were significantly more efficient than more hydrophilic carriers with a Sfp/LAF ratio of 1:2 or 2:4 (Fig. S6). High luciferase expression for several LAF-Sfp carriers as well as carrier- and cell line-dependent differences in luciferase expression were observed. In N2a cells (Fig. 2A), all LAF-Sfp carriers outperformed DLin-MC3-DMA LNPs. LAF-Sfp bundle **1621** (8Oc, Sfp/LAF = 1:4) even outperformed SM-102 LNPs with 5-fold higher RLU values. **1621** analogs **1752** (12Bu) and **1755** (14He) as well as U-shape carriers **1716** (8Oc, Sfp/LAF = 1:4) and **1720** (12Oc, Sfp/LAF = 1:4)



Scheme 2. mRNA LNP formulations. (A) LNPs were formulated by flash mixing an ethanol phase of cholesterol, phospholipids, PEG-lipids and cationizable lipids (or LAF-Sfp carriers) with an aqueous phase of mRNA using rapid pipetting, followed by dilution with or dialysis against HBG. (B) Molar and weight ratios of standard LNPs (DLin-MC3-DMA, MC3; SM-102) and LAF-Sfp LNPs (exemplarily shown for **1621**).

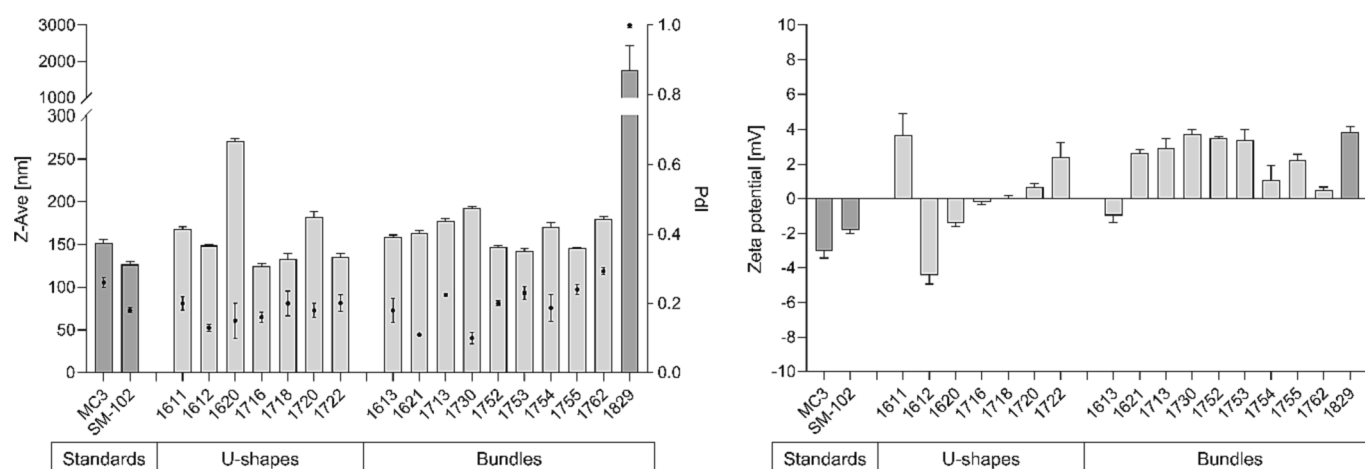


Fig. 1. Particle characterization by dynamic and electrophoretic light scattering. Physicochemical characterization of mRNA LNPs formed with different LAF-Stp carriers (N/P 9) in comparison to standard LNPs DLin-MC3-DMA (MC3, N/P 3) and SM-102 (N/P 6) containing 3 $\mu\text{g}/\text{mL}$ FLuc mRNA, regarding particle size (Z-Ave), polydispersity index (PdI), and zeta potential in indicated molar ratios (LNP compositions see Table 2). Data are presented as mean $\pm$ SD ($n = 3$). Statistical analysis was done by unpaired Student's two-tailed t-test with Welch's correction; GraphPad PrismTM 9.5.1. Significance between **1621** and OleA-Stp control carrier **1829**: * $p \leq 0.05$.

showed similar luciferase expression levels as SM-102. In HepG2 cells (Fig. 2B), all carriers were comparable or superior to DLin-MC3-DMA, with SM-102 LNPs providing the highest transfection activity. In Huh7 cells (Fig. 2C), **1755** and SM-102 containing LNPs ranked top. Also, bundle carrier **1621** (8Oc) and its LAF analogs **1752** (12Bu) and **1754** (12He) could reach high expression levels (10-fold higher than DLin-MC3-DMA). Noteworthy, LAF 8Oc bundle **1621** reached six log units higher RLU values than its OleA analog **1829** (Fig. S6). This emphasizes the benefit of the cationizable LAF motif over a standard OleA fatty acid for mRNA transfection.

mRNA that encodes for fluorescent mCherry reporter protein and flow cytometry was utilized to assess the transfection efficiency at the cellular level (Fig. 3). Hereby, the mean fluorescence intensity (MFI) correlates with mCherry protein expression of the total cell population. Carriers that mediated high luciferase expression also displayed high mCherry expression in almost 100% of cells, even at a low mRNA dose of 15 ng/well. Top-performing structures identified in the luciferase activity assay (8Oc bundle **1621**, and 12Oc U-shape **1716**) also proved to be highly effective carriers in the flow cytometry study. They outperformed the gold standards DLin-MC3-DMA and SM-102 regardless of the applied mRNA dose. Only for **1612** and **1755**, a discrepancy between the mean fluorescence intensity and the results of the luciferase activity assay was observed. 12Oc U-shape **1612** was highly efficient with almost 100% transfected cells, but performed less effective in the luciferase reporter assay. Compared to 8Oc bundle **1621**, 12Oc analog **1613** yielded < 90% mCherry positive cells, which also matched with the lower MFI and RLU values.

The bundle carriers with different LAF motifs were further tested at an additional N/P ratio of 18 in two different cell lines (N2a, HeLa) at low mRNA concentrations of 15 ng/well (Fig. 4A). In N2a cells, all bundles, except 12Oc carrier **1613**, could reach RLU levels as high as SM-102 and clearly outperformed DLin-MC3-DMA up to sixfold. In HeLa cells, terminal alkyl chains consisting of dodecyl and hexadecyl were less favorable. Nevertheless, 12Oc **1613** was at least comparable to SM-102, and 16Bu **1753** showed even higher RLU values. All other bundles reached even higher expression levels than SM-102.

To figure out the best time point for the detection of reporter mRNA expression, the transfection kinetics of a selection of LAF-Stp carriers with different topologies and LAF domains were evaluated (Fig. 4B) in two different cell lines (N2a, HeLa). Generally, the highest transfection efficiencies were reached after 24 h of incubation, except for SM-102 being most efficient after 48 h. Nevertheless, the LAF-Stp carriers,

especially those with an Stp/LAF ratio of 1:4 (8Oc bundle **1621**, 12Oc U-shapes **1612** and **1720**), showed remarkably fast transfection kinetics. At 6 h post transfection, they already reached at least 10-fold higher RLU levels than SM-102 in both cell lines. They could outperform the gold standards DLin-MC3-DMA and SM-102 by far. Especially 8Oc bundle **1621** maintained high transfection efficiency after 12–24 h in both cell lines, and 14He bundle **1755** showed the highest mRNA expression in HeLa cells after 24 and 48 h overall. 12Oc U-shape **1611** (Stp/LAF = 1:2) was only moderately efficient. All in all, the LAF-Stp carriers displayed faster transfection kinetics than DLin-MC3-DMA and SM-102 and were superior at early time points (e.g., **1621** vs SM-102 at 6 h: 37-fold higher RLU values, ** $p = 0.0020$ in N2a cells, and 70-fold higher RLU values, ** $p = 0.0017$ in HeLa cells).

In addition to N2a and Huh7 cells, a selection of carriers was tested in the DC-like cell line DC2.4 and in the macrophage model cell line J774A.1 using different low mRNA concentrations. Only best performing carriers with a Stp/LAF ratio of 1:4, different topologies and LAF motifs were chosen. Biological evaluation was carried out using a luciferase reporter assay (Fig. 5), and cytotoxicity was assessed by measuring the metabolic activity of transfected cells via an MTT assay (Fig. S7). High luciferase expression was observed for several LAF-Stp carriers. At the highest concentration (30 ng/well), the difference between the carriers did not stand out as much as at lower concentrations. Especially for the best performing LAF-Stp carrier **1621** (8Oc, Stp/LAF = 1:4), very high luciferase expression was observed even at the lowest dose (3 ng/well) in all cell lines. DLin-MC3-DMA was outperformed by far in N2a, DC2.4 and J774A.1 cells. Especially in immune cells (DC2.4 and J774A.1), there did not appear to be any significant dose dependency for **1621**, as 30 and 3 ng/well yielded similar RLU levels. **1621** analog **1755** (14He) showed comparable levels of transfection efficiency to SM-102 in N2a, Huh7 and J774A.1 cells, whereas it was less efficient in DC2.4 cells. Dose reduction maintained high transfection efficiencies but reduced toxicity, which was otherwise observed at higher doses (30 ng/well) (Fig. S7).

In terms of cytotoxicity, the critical dose was cell line-dependent. In N2a cells, relatively high mRNA doses of 30 ng/well were still well tolerated, whereas in Huh7, DC2.4 and J774A.1 cells a reduced dose of 15 ng/well was required. Especially LAF-Stp U-shapes showed rather high cytotoxicity at higher mRNA dose compared to bundle carriers in tumor cell lines (N2a, Huh7). It can be assumed that mechanisms for effective mRNA delivery, such as destabilization of the lipid membrane, may cause toxic effects. Nevertheless, this can be managed by lowering

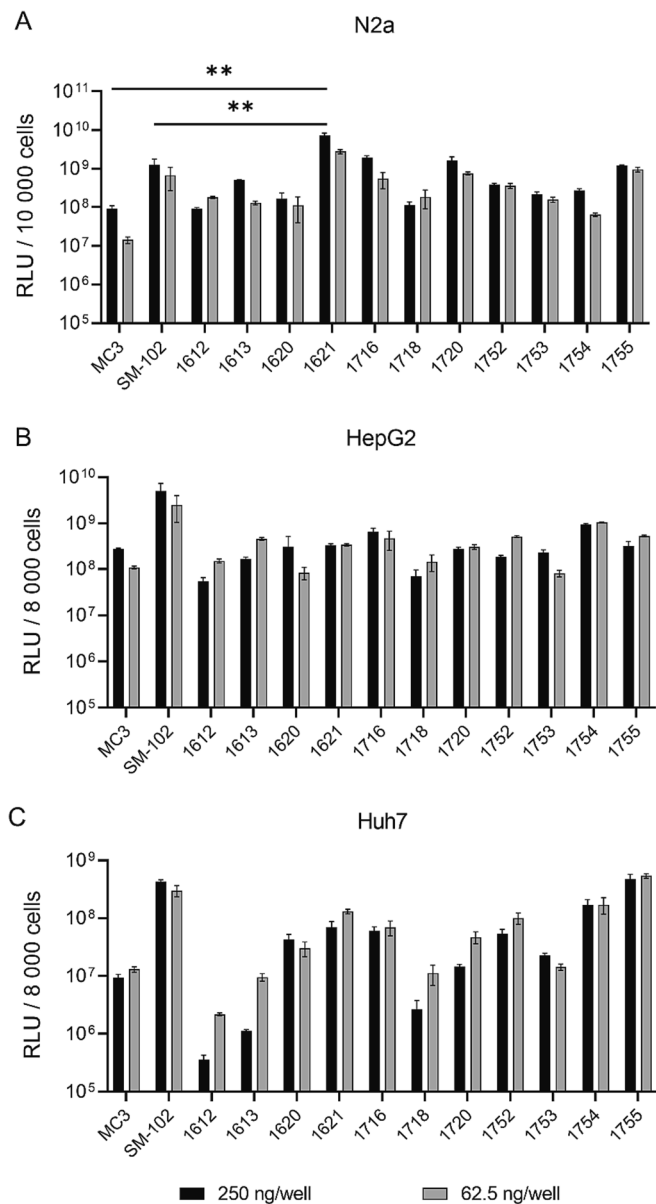


Fig. 2. Luciferase expression induced by mRNA LNPs in different tumor cell lines. N2a (A), HepG2 (B), and Huh7 (C) cells were transfected with mRNA LNPs at 250 ng and 62.5 ng FLuc mRNA per well. LNPs were prepared at N/P 9 and at different molar ratios and were compared to DLin-MC3-DMA (MC3, N/P 3) and SM-102 (N/P 6) LNPs serving as gold standards (LNP compositions see Table 2). Luciferase activity was measured 24 h after transfection and is shown as RLU values ($n = 3$; mean $\pm$ SD) after background subtraction (HBG-treated control cells). Statistical analysis was done by unpaired Student's two-tailed t-test with Welch's correction; GraphPad Prism™ 9.5.1. Significant differences of **1621** vs MC3 and SM-102 at 250 ng mRNA/well: ** $p \leq 0.01$.

the mRNA dose and choosing a suitable carrier.

3.4. Mechanistic studies on endosomal escape of mRNA LNPs

To gain a better understanding of the delivery process of LAF-Stp LNPs, mechanistic studies on the endosomal escape were carried out. Only carriers with a preferable Stp/LAF ratio of 1:4, different topologies and LAF motifs were evaluated. Due to the pH-dependent reversibly protonatable LAF building blocks and the previously described fast transfection kinetics (Fig. 4B) of the LAF-Stp carriers, a fast endocytosis upon destabilizing effects on the endosomal membrane was expected.

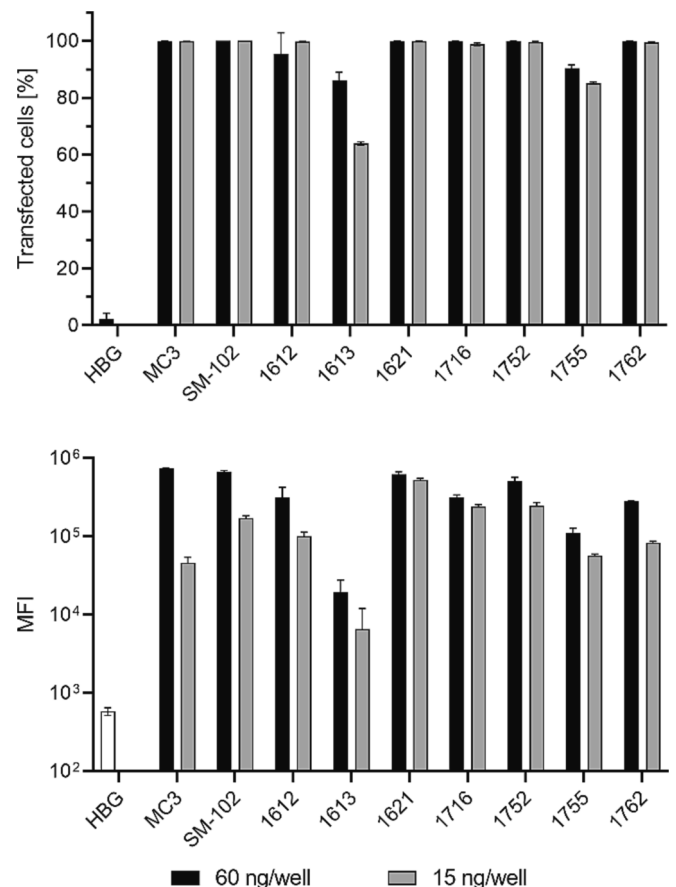


Fig. 3. Transfection efficiency of mRNA LNPs in N2a cells as assessed by flow cytometry. (A) Transfection of 60 ng and 15 ng mCherry mRNA per well. LNPs were prepared at N/P 9 and at different molar ratios and were compared to DLin-MC3-DMA (MC3, N/P 3), and SM-102 (N/P 6) LNPs serving as positive controls (LNP compositions see Table 2). mCherry expression was measured 24 h after transfection ($n = 3$; mean $\pm$ SD) and is determined by the percentage of cells expressing mCherry protein 24 h post treatment.

The following evidence supports this hypothesis. Intracellular uptake of Cy5-labeled mRNA LNPs into endolysosomal vesicles and destabilization/disruption of endosomal membranes was monitored by CLSM at 4 h after transfection of HeLa cells that stably expressed the Galectin-8 mRuby3 fusion protein (HeLa-Gal8-mRuby3) [41–43] (Fig. 6A). When endosomes are disrupted, the Gal8-mRuby3 protein interacts with the glycans present on the inner surface of the endosomal membranes, leading to green-fluorescent spots. As observed in HBG-treated control cells, the cells exhibited a dispersed pattern of Gal8-mRuby3 distribution in the cytosol, when endosomal membrane destabilization was not present. All LAF-Stp carriers predominantly facilitated cellular uptake of the corresponding mRNA LNP through endocytosis, as visualized by Cy5-labeled mRNA (red dots), and mediated endosomal membrane destabilization, resulting in membrane disruption (green dots). Certain LAF-Stp carriers exhibited more notable cellular internalization than others. Especially for the 8Oc bundle carrier **1621** and 12Oc U-shape **1716**, cellular internalization via endocytosis was most pronounced as indicated by large, partly overlapping Gal8 and mRNA positive areas (yellow). 8Oc bundle **1621** was most effective in promoting endocytosis with a greater quantity of endosomal mRNA (red dots) compared to the other carriers. LAF-Stp carriers showed higher endosomal escape after 4 h than standard LNPs DLin-MC3-DMA and SM-102. At that early time point and relatively low mRNA dose (135 ng in 300 μ L medium per well), both the mRNA uptake and the endosomal disruption mediated by the standard formulations was hardly detectable. This is in accordance

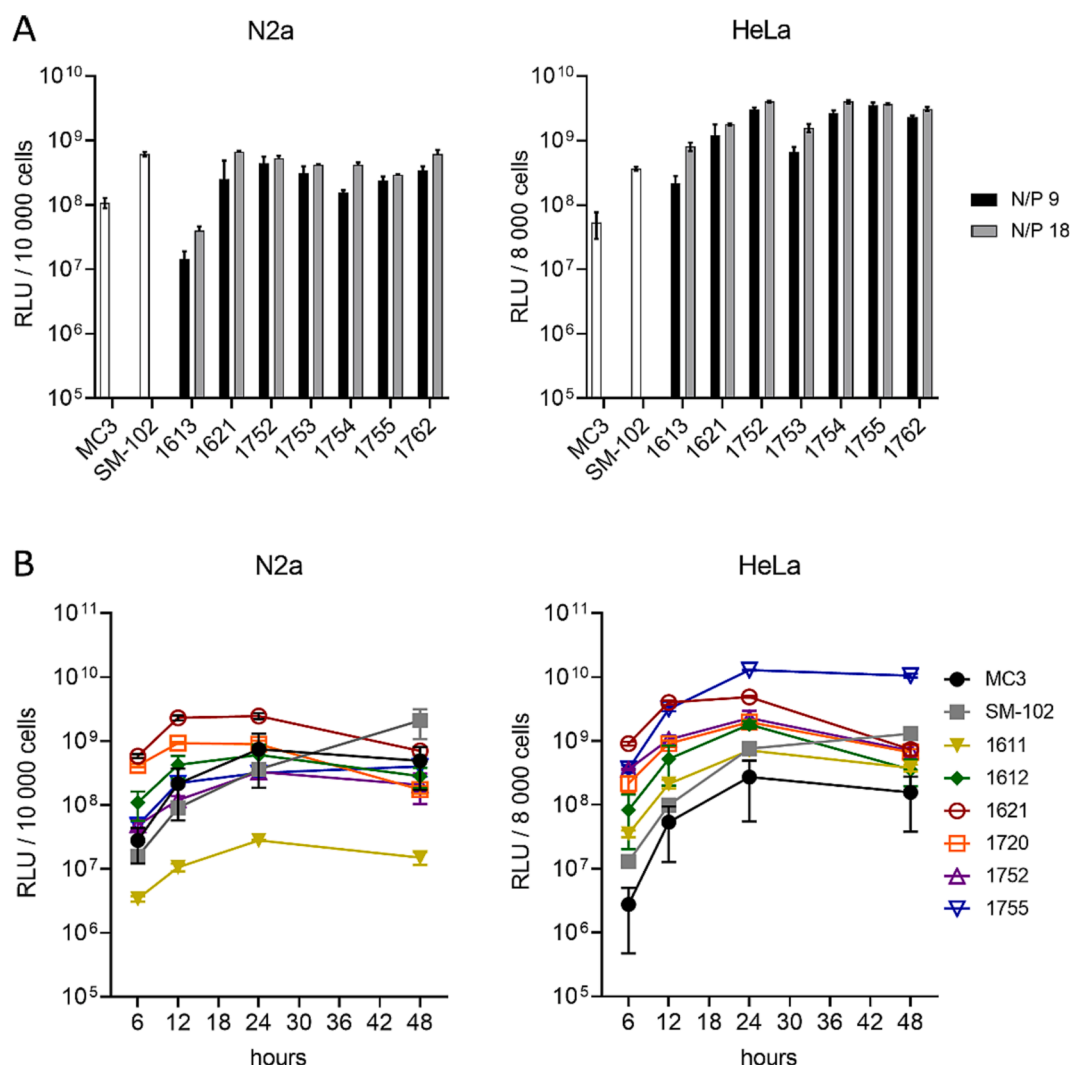


Fig. 4. Luciferase expression induced by mRNA LNPs in N2a (left) and HeLa cells (right). (A) Transfection of 15 ng FLuc mRNA per well. LNPs were prepared at N/P 9 and N/P 18 and at different molar ratios and were compared to DLin-MC3-DMA (MC3, N/P 3) and SM-102 (N/P 6) LNPs serving as gold standards (LNP compositions see Table 2). Luciferase activity was measured 24 h after transfection and is shown as RLU values ($n = 3$; mean $\pm$ SD) after background subtraction (HBG-treated control cells). (B) Transfection kinetics. Time-dependent luciferase expression after transfection of 60 ng FLuc mRNA per well on N2a and HeLa cells. LNPs were prepared at N/P 9 and at different molar ratios and were compared to DLin-MC3-DMA (MC3, N/P 3) and SM-102 (N/P 6) LNPs serving as gold standards (LNP compositions see Table 2). Luciferase activity was measured at 6, 12, 24, and 48 h after transfection and is shown as RLU values ($n = 3$; mean $\pm$ SD) after background subtraction (HBG-treated control cells).

with the results of the transfection kinetic studies (Fig. 4B), where the standard formulations (DLin-MC3-DMA and SM-102) showed slower transfection kinetics and needed longer incubation times to reach similar high transfection rates than LAF-Stp LNPs.

Furthermore, the pH-dependence of endosomolytic activity was evaluated in a bafilomycin A1 (BafA1) assay in N2a cells (Fig. 6B). BafA1 specifically hinders endosomal acidification by inhibition of the endosomal proton pump (vacuolar H^+ ATPase). When cells were treated with BafA1 prior to transfection, the transfection efficiencies of the mRNA LNPs were significantly decreased. Especially the standard LNPs showed a strong BafA1 effect, resulting in a massive decrease in RLU values (52-fold for DLin-MC3-DMA, 144-fold for SM-102). Also for the LAF-Stp U-shapes, a BafA1 effect was visible, but less distinctive (12-fold for 1612, and 22-fold for 1716). In the case of bundles, the BafA1 effect was less pronounced; the RLU values decreased only by sevenfold for 1755 (14He) and by approx. threefold for 1621 (8Oc).

In summary, the results of the BafA1 assay indicate that endosomal acidification was crucial for efficient mRNA delivery in the case of U-shaped LAF-Stp carriers, such as 1612 and 1716, and especially for the

standard DLin-MC3-DMA and SM-102 LNPs (Fig. 6B). However, it was not absolutely necessary for 8Oc bundle 1621, which also showed the highest amount of cytosolic mRNA release and endosomal destabilization in the CSLM images (Fig. 6A). Here, fast endosomal escape seemed to be mediated largely regardless of endosomal acidification. Possibly, in addition to endocytosis into acidifying endosomes, other uptake mechanisms may exist, such as fast passage through various lipid membranes at neutral pH, which would be consistent with the fast kinetics of mRNA expression already after 4 h.

3.5. Screen of mRNA LNPs in presence of full serum

The translation from *in vitro* to the *in vivo* setting poses several challenges since *in vitro* models cannot accurately mimic the physiological environment. When LNPs are administered intravenously (*i.v.*), they come into contact with various blood components such as plasma proteins, which adsorb onto the nanoparticle surface and can affect their cellular interaction [46,47]. Thus, the transfection efficiency of mRNA LNPs was evaluated in the presence of serum in four different cell lines

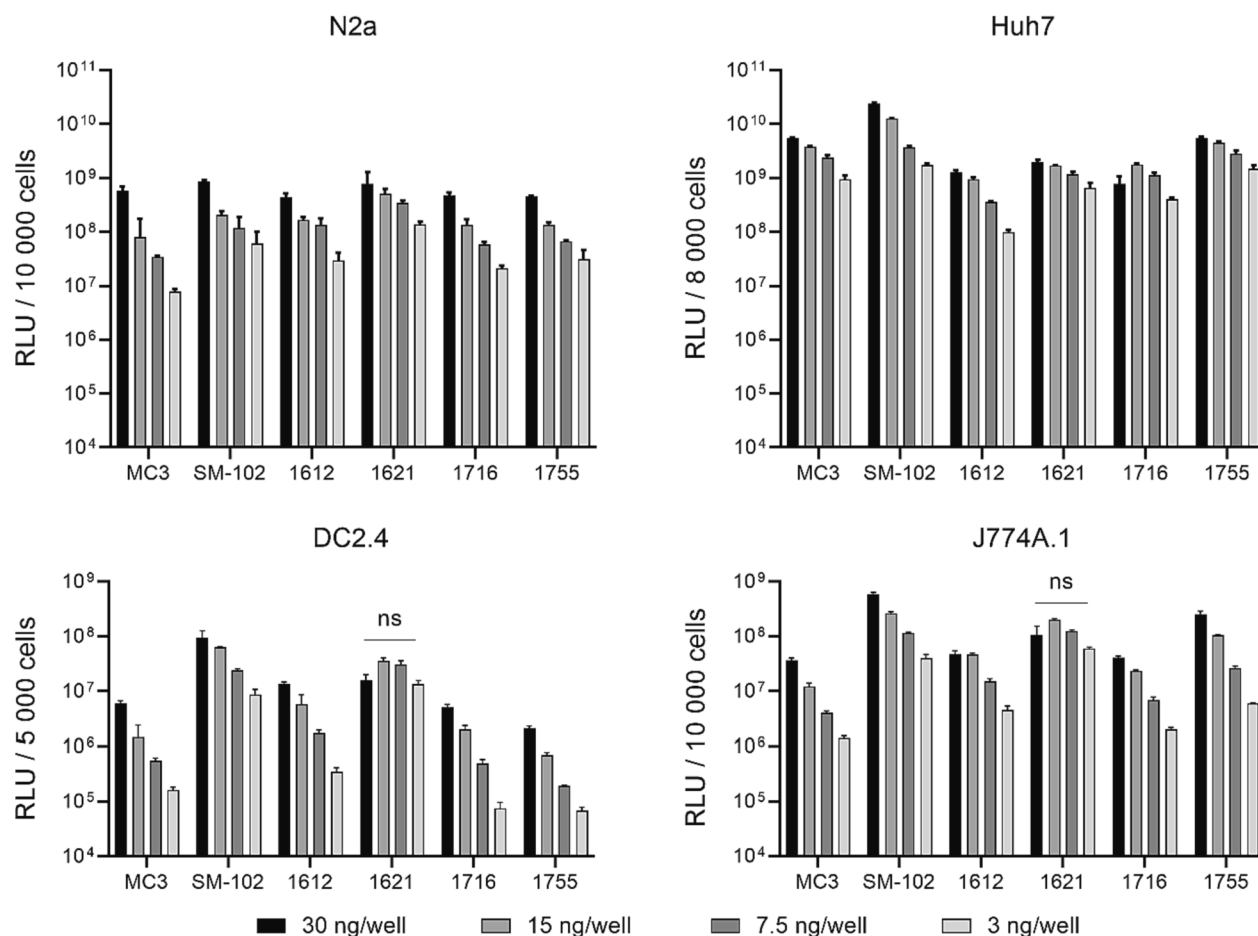


Fig. 5. Luciferase expression induced by mRNA LNPs in N2a, Huh7, DC2.4 and J774A.1 cells. Cells were transfected with mRNA LNPs at FLuc mRNA doses of 30, 15, 7.5, and 3 ng/well. LNPs were prepared at N/P 9 at different molar ratios and were compared to DLin-MC3-DMA (MC3, N/P 3) and SM-102 (N/P 6) LNPs serving as gold standards (LNP compositions see Table 2). Luciferase activity was measured at 24 h after transfection and is shown as RLU values ($n = 3$; mean + SD) after background subtraction of HBG-treated control cells. Statistical analysis was done by unpaired Student's two-tailed t-test with Welch's correction; GraphPad PrismTM 9.5.1. Statistical differences for **1621** LNPs at highest vs lowest applied mRNA dose: ns, not significant.

(N2a, Huh7, DC2.4, and J774A.1) as a pre-screening for *in vivo* studies. Additionally, the mRNA dose was further titrated down to 150 pg/well.

Generally, in all cell lines incubation in full (up to 99%) serum resulted in a comparable transfection efficiency as obtained in standard 10% FBS containing cell culture medium (Fig. 7). All tested LAF-Stp carriers were highly active in the presence of serum at higher mRNA doses. Especially 8Oc bundle **1621** was extremely efficient, outperforming DLin-MC3-DMA by far and showing high RLU values in all cell lines comparable (DC2.4, J774A.1) or superior (N2a, Huh7) to SM-102. Noteworthy, at the very low mRNA dose of 150 pg/well, **1621** reached around one log unit higher RLU levels than SM-102 in N2a and Huh7 cells. 14He bundle **1755** was very potent and even superior to **1621** at higher mRNA doses (30 and 15 ng/well), particularly in Huh7 and J774A.1 cells. U-shapes (**1612**, **1716**) were inferior to bundles in the presence of full serum and showed lower transfection efficiency.

3.6. *In vivo* application of mRNA LNPs

In sum, cell culture experiments identified bundles such as **1621** and its LAF analogs as most effective and superior to U-shape carriers (Figs. 2–5, Fig. 7). Nevertheless, to evaluate one representative of each topology class, U-shape **1612** and bundle **1621** were chosen for intravenous application of 3 μ g FLuc mRNA per mouse in an N2a tumor model. LNPs were dialyzed against HBG for 2 h to completely remove residual ethanol for *in vivo* studies. At 6 h post injection, organs were retrieved; the quantitated luciferase expression per gram organ is shown

in Fig. 8. No visible toxicity was observed; standard plasma parameter (ALT, alanine transaminase; AST, aspartate aminotransferase; Crea, creatinine; BUN, blood urea nitrogen) are displayed in Fig. S8. The well-established SM-102 LNPs mediated maximal expression in the liver by being > 10-fold higher than DLin-MC3-DMA LNPs and > 100-fold higher than the LAF-Stp LNPs. SM-102 LNPs also mediated the highest expression in the subcutaneous N2a tumors. However, despite their far lower potency in the liver and tumor, 12Oc U-shape **1612** and 8Oc bundle **1621** LNPs elicited RLU values as high as DLin-MC3-DMA and SM-102 in the spleen, thereby showing a much higher spleen selectivity. In the case of **1612**, luciferase activity per gram organ in the spleen was 60-fold higher than in the liver (* $p = 0.0338$), and for **1621** even 350-fold (** $p = 0.0021$).

Based on their high transfection activity in the spleen, SM-102 and **1621** LNPs were applied for eGFP mRNA delivery in tumor-free A/J mice to delineate cell type-specific transfection efficacies of immune cells within the liver, the spleen, and the lungs (Fig. 9). Standard plasma parameter (ALT, AST, Crea, BUN) are displayed in Fig. S9, and demonstrated good biocompatibility of the LNPs.

Expression within splenic and liver dendritic cells (DCs) was found for both LNPs, with SM-102 highest in spleen DCs. **1621** displayed highest expression in liver macrophages (Kupffer cells, KCs), whereas SM-102 transfected more spleen macrophages (MACs). Both formulations showed moderate expression levels in liver sinusoidal endothelial cells (LSECs; liver) and B-cells (spleen). In the lungs, eGFP gene expression in DCs and MACs was insignificant within the range of assay

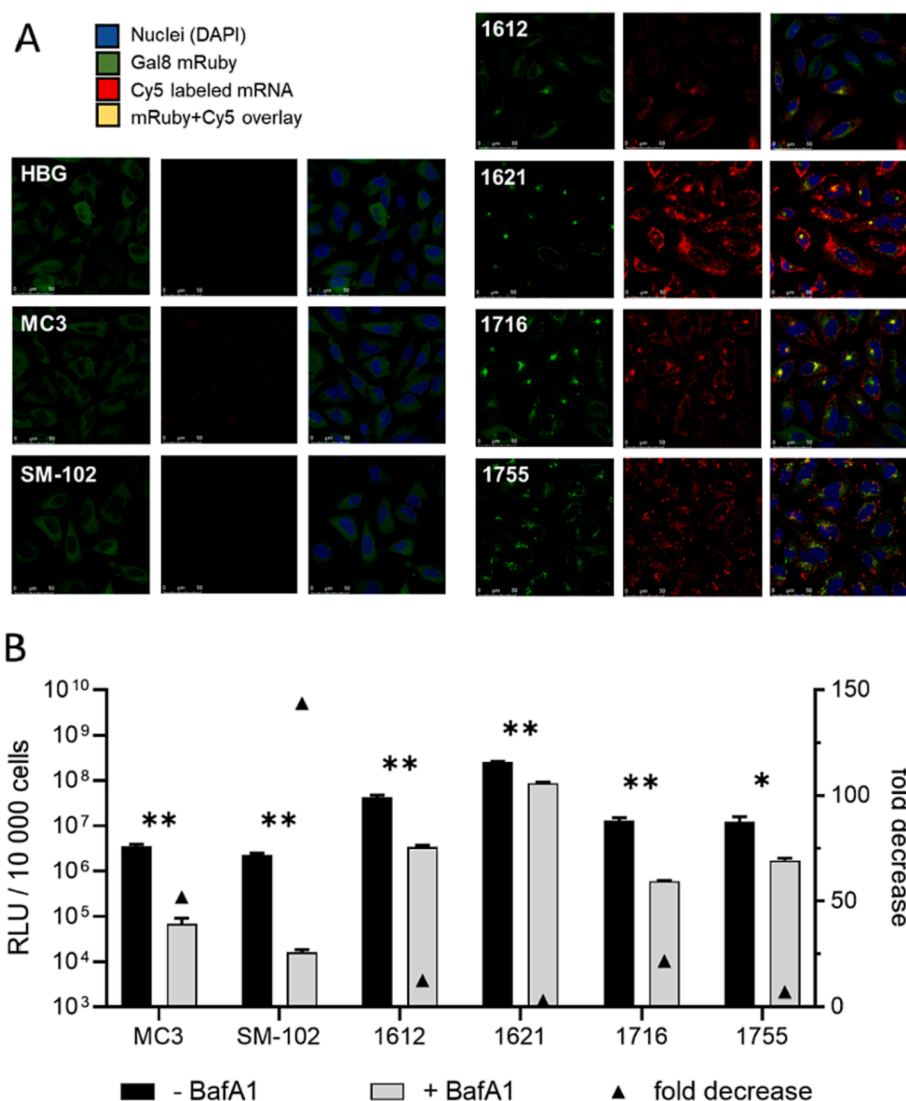


Fig. 6. Mechanistic studies. (A) Rupture of endosomes in HeLa Gal8-mRuby3 cells (green) transfected with LNPs containing 135 ng/well mRNA (20% Cy5-labeled; red) after 4 h as assessed by CLSM. The nuclei were stained with DAPI (blue). (B) Influence of endosomal protonation on mRNA LNP transfection efficiency using bafilomycin A1 (BafA1) as an H⁺-ATPase inhibitor. Transfection of 60 ng FLuc mRNA per well of N2a cells untreated (“-BafA1”, black) or treated with 200 nM BafA1 (“+BafA1”, grey) 2 h before transfection. LNPs were prepared at N/P 9 and at different molar ratios and were compared to DLin-MC3-DMA (MC3), N/P 3 and SM-102, N/P 6 LNPs serving as gold standards (LNP compositions see Table 2). Luciferase activity was measured 4 h after transfection and is shown as RLU values (n = 3; mean + SD) after background subtraction (HBG-treated control cells). Statistical analysis was done by unpaired Student’s two-tailed t-test with Welch’s correction; GraphPad Prism™ 9.5.1. Significance between “-BafA1” and “+BafA1”: * p ≤ 0.05; ** p ≤ 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sensitivity (MFI) for both LNPs, whereas a moderate expression could be detected for both LNPs in polymorphonuclear neutrophils (PMNs). In other immune cell types, no eGFP expression was monitored (*data not shown*).

It should be noted that the evaluated immune cell types represent only a marginal fraction of the total cell population of the distinct organs (< 4–5%). Thus, the results of eGFP expression obtained on the single cell level cannot be directly compared with the results of the *ex vivo* luciferase activity assay of the whole organ (Fig. 8). For example, the apparent discrepancy of a slightly higher eGFP expression of **1621** LNPs in liver immune cells (Fig. 9A) despite an approx. 100-fold higher luciferase expression of SM-102 LNPs in the liver (Fig. 8) can be explained by the different assay. In contrast to the *ex vivo* luciferase reporter assay, eGFP expression was only recorded for immune cells, and hepatocytes and other non-immune cells were not analyzed. As known from previous work, SM-102 mRNA LNPs express highly in liver hepatocytes, and to some extent also in liver immune cells, whereas the eGFP

expression signal of **1621** LNPs reflects predominant transfection of immune cells and not hepatocytes. This hypothesis could be supported by looking at the eGFP expression in CD45 (lymphocyte common antigen)-negative (i.e., non-immune cells) vs CD45-positive cells (i.e., immune cells) in the liver (Fig. S10), which was slightly higher in non-immune cells for SM-102 than for **1621**. SM-102 may transfect at low extent both CD45⁻ hepatocytes (Fig. S10, left panel), albeit below statistical significance, and CD45⁺ immune cells (Fig. S10, right panel). However, since hepatocytes account for about 95% of all liver cells, even low transfection of this cell population may account at large part for the strong luciferase signal of this organ (Fig. 8). Since **1621** transfected the quantitatively minor CD45⁺ liver-resident immune cell population at similar extent as SM-102 (Fig. S10, right panel), the overall much lower luciferase activity in livers of **1621**-treated mice might be due to an inability of **1621** to transfect hepatocytes *in vivo* (Fig. S10, left panel).

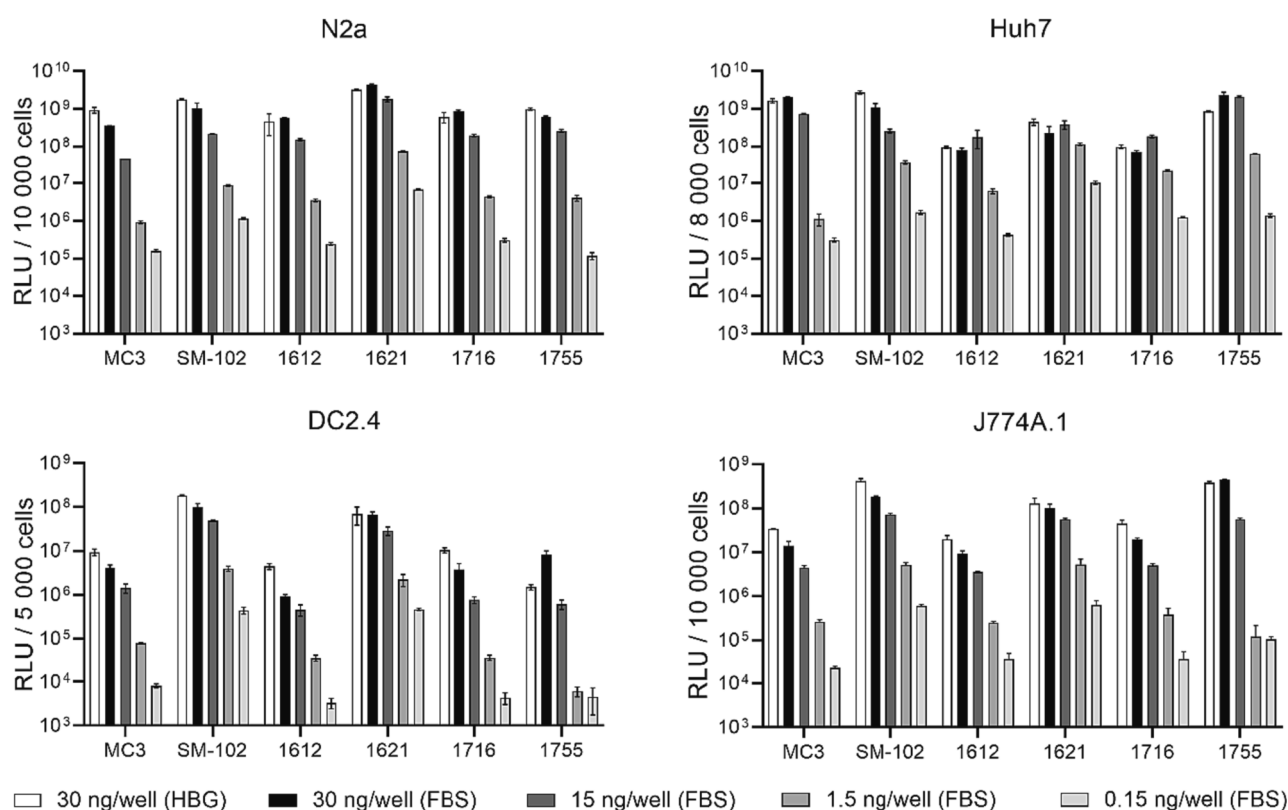


Fig. 7. Luciferase activity after transfection with mRNA LNPs in the presence of high serum in different cell lines. Transfection efficiency of fetal bovine serum (FBS)-diluted mRNA LNPs at the indicated FLuc mRNA doses in N2a, Huh7, DC2.4, and J774A.1 cells in comparison to LNPs diluted in HBG (30 ng/well). Prior to transfection, LNPs were diluted in serum or HBG (30 ng/well containing 50% serum or HBG, 15 ng/well containing 75% serum, 1.5 ng/well containing 97.5% serum, and 0.15 ng/well containing 99.75% serum) and then transfected in standard 10% FBS containing cell culture medium. Luciferase activity was evaluated at 24 h after transfection and is shown as RLU values ($n = 3$; mean $\pm$ SD) after background subtraction (HBG-treated control cells). DLin-MC3-DMA (MC3), N/P 3; SM-102, N/P 6; **1612**, **1621**, **1716** and **1755**, N/P 9 (LNP compositions see Table 2).

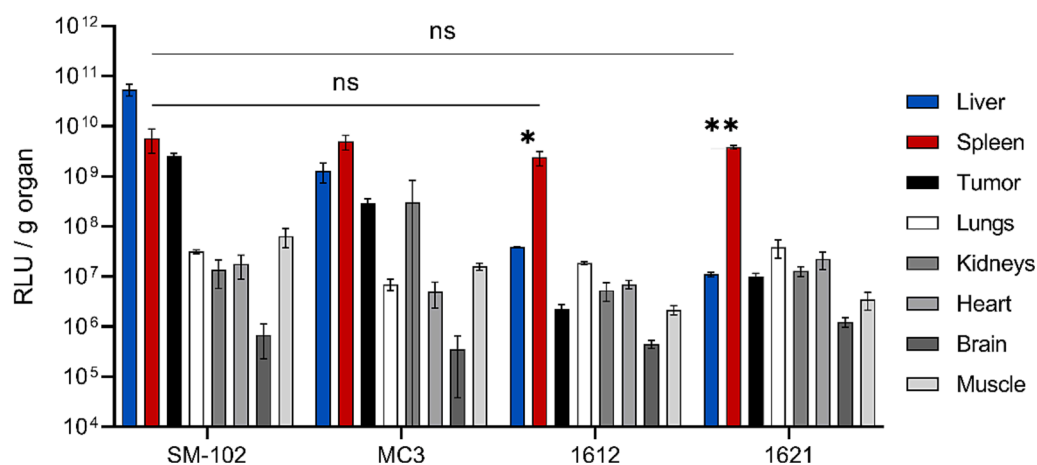


Fig. 8. Luciferase expression of mRNA LNPs in different organs. N2a tumor-bearing A/J mice were intravenously injected with LNP formulations containing 3 μ g FLuc mRNA in 150 μ l HBG per mouse. LNPs were prepared at N/P ratios 3 (DLin-MC3-DMA, MC3), 6 (SM-102), or 9 (**1612**, **1621**) at different molar ratios (LNP compositions see Table 2). Luciferase activity in RLU was determined ex vivo per gram (g) organ at 6 h post injection ($n = 3$; mean $\pm$ SD). Statistical analysis was done by unpaired Student's two-tailed t-test with Welch's correction; GraphPad Prism™ 9.5.1. Significance between liver and spleen expression: * $p \leq 0.05$ for **1612**, ** $p \leq 0.01$ for **1621**. Significance between SM-102 and **1612** or **1621**, in spleen expression: ns, statistically not significant.

4. Conclusion

A chemical evolution design of xenopeptides, defined in a precise 2D sequence and 3D architecture made of lipoamino fatty acids (LAFs), succinoyl tetraethylene pentamine (Stp) and α,ϵ -amido-linked lysines, identified new potent mRNA carriers. Here, we demonstrated the high

potential of this novel LAF-Stp class as cationizable carriers in mRNA LNP formulations. We identified LAF 8Oc carrier **1621** with a Stp/LAF ratio of 1:4 and bundle 3D topology as the most efficient carrier, with impressive dynamics of re-distribution from the lipidic to the aqueous phase by 100-fold upon endosomal acidification [34 48]. The incorporation of the LAF building block provided efficient nanoparticle

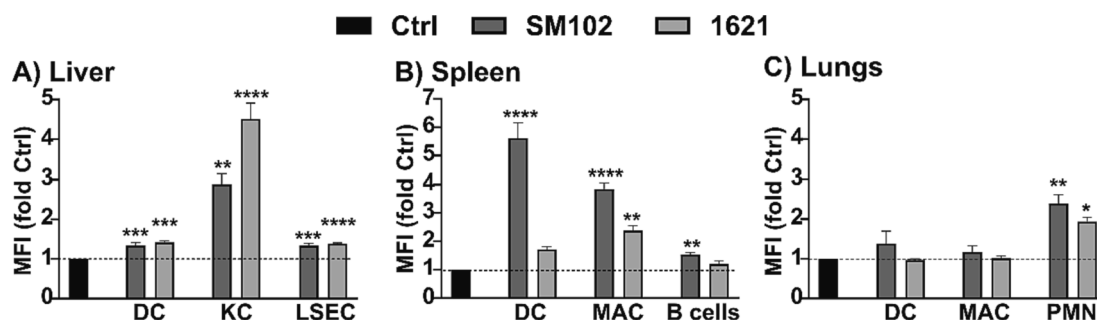


Fig. 9. eGFP expression in immune cells of liver, spleen, and lungs at 6 h after intravenous administration of LNP formulations containing 3 µg eGFP mRNA in 150 µL HBG per mouse ($n = 5$; mean + SEM). SM-102 (N/P 6); **1621** (N/P 9); LNP compositions see Table 2. Gating was performed according to the gating strategies displayed in Fig. S1 and S2. eGFP expression presented as mean fluorescence intensity (MFI) was normalized to HBG-treated controls (Ctrl). Statistical analysis was done by one-way ANOVA, Tukey test; GraphPad Prism™ 9.5.1. Significant differences vs Ctrl: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. DC, dendritic cells; MAC, macrophages; KC, Kupffer cells; B cells; PMN, polymorphonuclear leukocytes/neutrophils; and LSEC, liver sinusoidal endothelial cell.

formation, cellular uptake, and potent endosomal escape even in the presence of serum, resulting in fast and high reporter expression kinetics at very low mRNA doses (150 pg/well) in antigen-presenting cells and tumor cell lines. Upon intravenous administration of 3 µg mRNA per mouse, LAF-Stp mRNA LNPs exerted predominant mRNA expression in the spleen, far higher compared to other organs such as the liver, which was observed as the main organ of reporter expression by established standard mRNA LNPs (DLin-MC3-DMA and SM-102). Cellular analysis of eGFP mRNA expression in liver, lungs, and spleen revealed reporter expression in dendritic cells, macrophages, Kupffer cells, liver sinusoidal endothelial cells, and neutrophils.

Current LNP library formulation was performed by simple flash mixing of standard ethanol/pH 4 citrate buffer solutions at small scale. LNP characteristics can be further refined by standardized formulation procedures including microfluidics [49]. Additionally, optimizing ratios of helper lipids, PEG-lipids and ligand targeting of LNPs could help in "chemical and biological targeting", to modulate organ selectivity and efficacy [18,50,51].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors acknowledge support by the German Research Foundation (DFG) SFB1032 (project-ID 201269156) sub-project B4 (to E.W.), SFB1066 subproject B15 (to M.B.), and BMBF Cluster for Future 'CNATM - Cluster for Nucleic Acid Therapeutics Munich' (to E.W.). We thank i) Dr. Yi Lin and Prof. Ulrich Lächelt for providing the HeLa-Gal8-mRuby3 cells, ii) Johanna Seidl and Ricarda Steffens (both Pharmaceutical Biotechnology, LMU Munich) for support in LAF synthesis, iii) Carolina Medina-Montano and Evelyn Montermann (both Department of Dermatology, University Medical Center of the JGU Mainz) for their help in the immunostaining experiment, and iv) Wolfgang Rödl, Olga Brück, and Markus Kovac (all Pharmaceutical Biotechnology, LMU Munich) for their practical support.

The Graphical Abstract as well as Scheme 2A were created with BioRender.com.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejpb.2023.11.025>.

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6.4. Publication 4

“Glycogen-Inspired Trimannosylated Serum Albumin Nanocarriers for Targeted Delivery of Toll-like Receptor 7/8 Agonists to Immune Cells and Liver”

Published in: *Journal of Controlled Release*. **2025**

DOI: 10.1016/j.jconrel.2025.113705

Innovative nanoparticles were generated by conjugating branched trimannose (TM) to human serum albumin (HSA). These TM-HSA particles form compact structures that are smaller than 40 nm and specifically target dendritic cells (DCs) and other liver non-parenchymal cells through DC-SIGN receptor. Targeting DC-SIGN enabled rapid and selective uptake into DCs and cells expressing homologues. The nanoparticles were also functionalized with a TLR7/8 agonist to achieve immune activation. *In vitro*, TM-HSA-TLR7/8 particle uptake led to stronger cytokine production of bone marrow-derived DCs compared to soluble agonists and further promoted proliferation of CD8⁺ and CD4⁺ T-cells. *In vivo* experiments showed rapid bloodstream clearance and accumulation of TM-HSA in the liver. This approach represents a significant advancement in nanomedicine by combining specific targeting together with controlled immune activation.

Contribution:

As co-first author of this paper, I performed *in vitro* experiments and the *in vivo* experiment. I also contributed to data analysis, visualization, manuscript draft preparation, as well as review and editing.

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Lantzberg B#, **Zeyn Y#**, Forster R#, Jian L, Schauenburg D, Hieber C, Nuhn L, Zhou T, Silva MJSA, Koynov K, Jiang HL, Kuan SL*, Bros M*, Opatz T*, Weil T*. Glycogen-inspired trimannosylated serum albumin nanocarriers for targeted delivery of toll-like receptor 7/8 agonists to immune cells and liver. *Journal of Controlled Release*. 2025. doi: 10.1016/j.jconrel.2025.113705.

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Journal Pre-proof

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PII: S0168-3659(25)00325-6

DOI: <https://doi.org/10.1016/j.jconrel.2025.113705>

Reference: COREL 113705

To appear in: *Journal of Controlled Release*

Received date: 20 September 2024

Revised date: 20 March 2025

Accepted date: 4 April 2025

Please cite this article as: B. Lantzberg, Y. Zeyn, R. Forster, et al., Glycogen-inspired trimannosylated serum albumin nanocarriers for targeted delivery of toll-like receptor 7/8 agonists to immune cells and liver, *Journal of Controlled Release* (2024), <https://doi.org/10.1016/j.jconrel.2025.113705>

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Glycogen-Inspired Trimannosylated Serum Albumin Nanocarriers for Targeted Delivery of Toll-like Receptor 7/8 Agonists to Immune Cells and Liver

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Abstract

Nanocarriers can improve the therapeutic efficiency of small molecule immunomodulators or inhibitors, which is important for immunotherapy of liver diseases or cancer. Macromolecular protein carriers, such as human serum albumin (HSA), could provide better penetration compared to large nanoparticles (>50 nm) but are hampered by systemic biodistribution. To overcome these limitations, inspired by the natural glycogen structure, we have designed the

HSA nanocarrier (<40 nm) consisting of multiple trimannose (TM) ligands attached to the protein surface, to target mannose or dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptors in immune cells or immunological organs such as the liver. Capitalizing on the chemical reactivity of different amino acids present in HSA, we have incorporated multiple copies of a cargo relevant for immunotherapy, i.e. the toll-like receptor (TLR) 7/8 agonist. The resulting TM-HSA conjugates exhibit excellent and specific uptake *ex vivo* in various immune cells and liver-specific uptake *in vivo*, opening access to protein nanocarriers with rapid and efficient *in vivo* targeting with great potential for immune-related diseases.

Keywords

Immunotherapy, nanomedicine, nano-immunotherapy, biomacromolecular therapeutics, albumin-carrier, trimannose targeting

1. Introduction

Immunotherapy plays a critical role in the modern strategy for treating fatal diseases such as cancer and liver diseases and is experiencing rapid growth to address these global health threats (9.7 million cancer-related deaths in 2022¹, two million deaths annually due to liver diseases).² It has become state of the art to formulate immunotherapeutics in nanosized delivery systems to enhance their therapeutic efficacy and to improve their pharmacokinetic and –dynamic profile. To improve delivery, different targeting strategies, divided into passive and active targeting, are used for better biodistribution. At the same time, long circulation times are sought to increase delivery efficiency. However, in the case of cancer, for example, the median delivery efficiency of most nanocarriers remains critically low at 0.7 %.³ Some possible reasons could be limited tissue penetration of large nanoparticles (≥ 50 nm) to reach deeper targets,^{4–7} particle instability and off-target release of the drug payloads, and their passive accumulation in other organs such as the liver, lung, and spleen.⁸ Although the toxicity profiles of nanoparticle formulations have improved compared to their free drug counterparts, their therapeutic index remains a limiting factor.³⁹ Therefore, to overcome the existing limitations of prolonged systemic exposure and associated off-target or even on-target/off-site accumulation, alternative delivery strategies for immunotherapy have emerged: i.e. from designs emphasizing maximum tolerable concentrations with long circulation times for effective accumulation to those characterized by rapid, cell-type or tissue-specific uptake with limited systemic exposure.¹⁰ For example, receptors on the surface of hepatic stellate

cells or Kupffer cells (liver macrophages) have been targeted to achieve cell-type specific delivery of small molecule drugs for more effective treatment of liver fibrosis.^{11,12}

Thus, attaching ligands to the surface of low nanometer-sized carriers that target receptors present in high density may be attractive to achieve higher immune cell or immunological organ specificity *in vivo* and to enable fast uptake.^{13–18} C-type lectin receptors (CLRs), such as the macrophage mannose receptor (MMR, CD206) or the dendritic cell-specific ICAM-3 grabbing nonintegrin receptor (DC-SIGN, CD209), are ideal candidates.^{19,20} They are a class of pattern recognition receptors (PRRs) highly expressed on the surface of professional antigen-presenting cells (APCs) such as macrophages and dendritic cells, as well as on Kupffer and liver sinusoidal endothelial cells (LSEC).^{21,22} Moreover, they enable the recognition of pathogen-associated molecular patterns (PAMPs) containing saccharide moieties, especially branched mannopyranosides that are found in high mannose glycopeptides.^{23,24} MMR recognizes end-standing single mannose branched structures or di-mannose clusters, like α -(1→2)-mannobiose,²⁵ while DC-SIGN recognizes both internal branched mannose structures with a minimum of three mannoses, like α -(1→3), α -(1→6)-mannotriose^{20,21,26} and end-standing di-mannoses.^{21,27–29} Thus, mannose-based targeting moieties are highly relevant for efficient drug delivery to immune cells.^{30,31} It has been reported that a branched, highly mannosylated structure binds 130 times more strongly to DC-SIGN than single mannose.²⁰ However, progress has been limited because the chemical synthesis of hyperbranched polymannosides is challenging. Therefore, multiple smaller mannose-based oligosaccharide targeting structures attached to a single carrier are more attractive for practical implementation and widespread application. The trimannose (TM) group^{19,32,33} is an excellent candidate as it meets the required binding features³⁴ and the synthesis is easily upscaled.

For widespread application, nanocarriers with exceptional biocompatibility and biodegradability are of particular interest. Proteins have been applied as versatile macromolecular drug delivery systems with smaller dimensions (<50 nm) than many of the conventional nanoparticle formulations. Human serum albumin (HSA), the most abundant human plasma protein, has endogenous transport functions in the bloodstream, is non-immunogenic^{35,36} and has a hydrodynamic diameter of less than 10 nm making it an attractive nanocarrier for drug delivery applications.³⁷ HSA also offers various functional groups on its surface, such as amines, carboxylic acids or a thiol that can be easily modified to accommodate multiple copies of targeting groups or drug molecules.

Inspired by glycogen, in which branches of glucose units surround a core protein, we propose to attach TM in a multivalent manner to the HSA surface (Figure 1A) to enable high cell-type specificity *ex vivo* through active cell targeting and rapid accumulation *in vivo* (Figure 1B, C). The glycogen-inspired design allows the number of TM per HSA to be varied to systematically investigate the influence of ligand density on their selective binding to cell lines expressing MMR/DC-SIGN and on uptake efficiency,^{38,39} which has been limited to date.¹⁹ We also incorporated multiple copies of molecular cargoes, such as toll-like receptors 7/8 agonist (TLR7/8a), into the highly saturated TM₃₈₋₄₁-HSA (henceforth TM-HSA), using a disulfide linker. Cell-type selective activation *ex vivo* was observed in dendritic cells and macrophages through controlled receptor targeting and activation of synergistic immune pathways was achieved using TM-HSA-TLR7/8a. Fast and specific accumulation of the TM-HSA conjugates was observed only in the liver *in vivo*. The TM-HSA nanocarrier, which combines TM and HSA in a core-shell architecture, provides access to a biodegradable, organ-selective, and safe drug delivery system with high drug loading capacity (independent of the drug polarity) and long-term stability to provide new avenues for immunotherapy through cell-type specific or/and liver targeting.

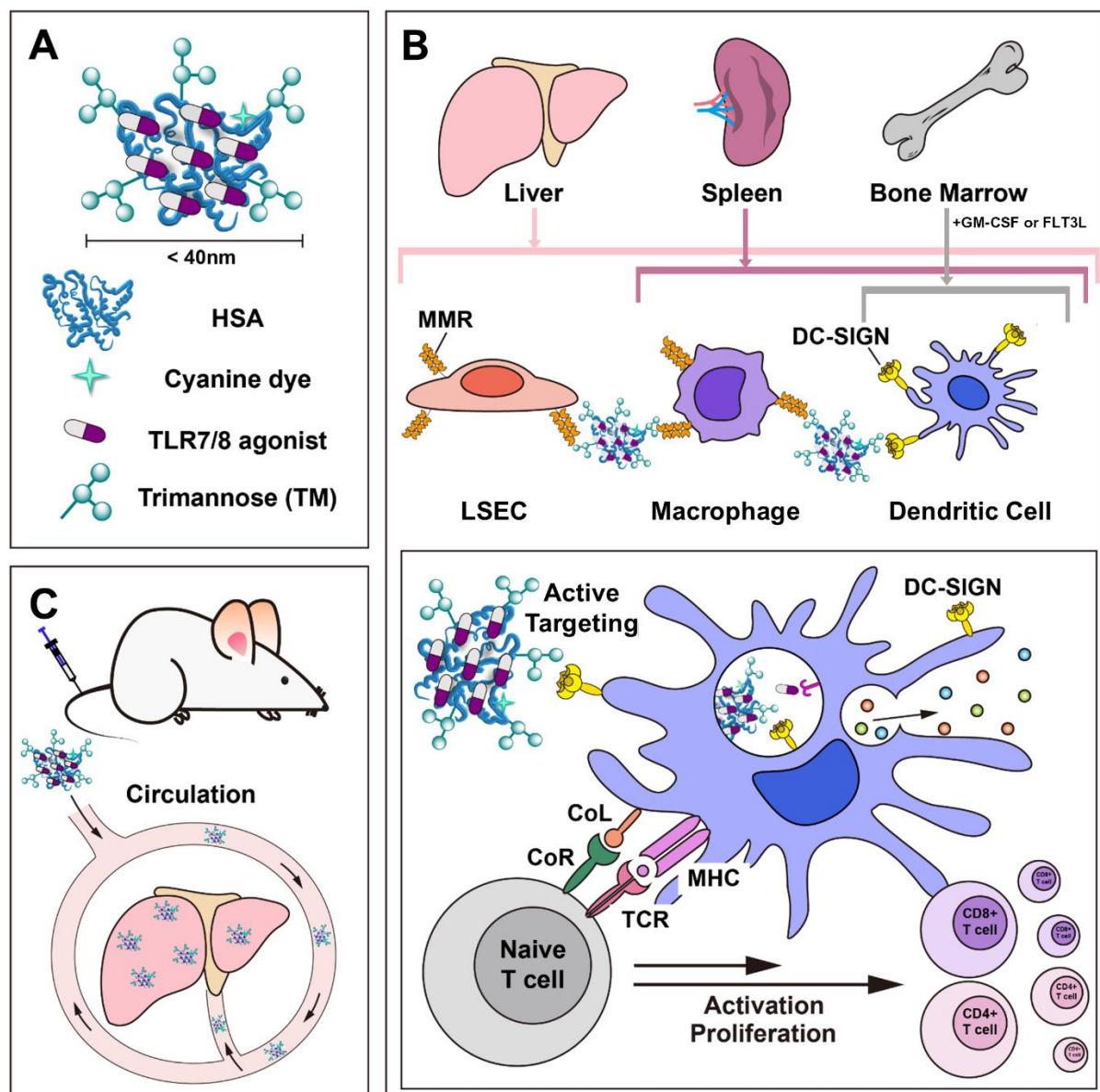


Figure 1 Illustration of A) trimannosylated human serum albumin carrier with TLR 7/8 agonist and B) main target cell populations derived from murine liver (LSECs, macrophages (Kupffer cells), dendritic cells), spleen (macrophages, dendritic cells) and bone marrow (dendritic cells) that were investigated in *ex vivo* experiments regarding carrier uptake (top) and carrier effects on dendritic cells (bottom). C) Biodistribution of trimannosylated HSA nanocarrier *in vivo* in healthy mice. HSA = human serum albumin, CoL = co-stimulatory ligand, CoR = co-stimulatory receptor, TCR = T-cell receptor, MHC = major histocompatibility complex, LSEC = liver sinusoidal endothelial cells.

2. Results and Discussion

2.1. Synthesis and Characterization of $\text{TM}_n\text{-HSA}$

To prepare HSA carriers with varying numbers of TM targeting moieties, a mannotriptide containing an azide-functionalized linker was prepared following an optimized synthesis route based on literature methods.³³ The nine-step synthesis sequence involved two telescoped reaction procedures to reduce the amount of necessary intermediate chromatographic purification steps and to improve the overall yield compared to non-telescoped synthesis procedures.³³ The TM unit was obtained with an overall yield of 42 %, over six linear steps

starting from commercially available D-mannose and triethylene glycol (Scheme S1 in supporting information (SI)). The yield was significantly improved, compared to the 20 % over seven linear steps previously reported.³³ Inclusion of a two-step propargylation and Zemplén deacylation sequence, based on the work of *Krabicová et al.*,⁴⁰ afforded the propargyl mannoside (**1**). A three-step silylation, benzylation, and desilylation sequence, based on the work of *Ramos-Soriano et al.*,⁴¹ efficiently delivered the 2,4-protected mannoside (**2**). Both methods allowed the preparation of the building blocks efficiently in multigram scale reactions for further transformations to yield the trimannose targeting unit (TM, Figure 2A, for abbreviations, see SI). Furthermore, employing the selective silylation strategy, proved to be superior to the formerly used method.³³ The clickable TM and all involved intermediates were characterized by ¹H-, ¹³C-, and 2D-NMR, as well as high-resolution mass spectrometry (see SI). The TM unit was used for subsequent conjugation with dibenzocyclooctyne (DBCO)-modified HSA.

Next, we proceeded to functionalize HSA with varying numbers of TM. To facilitate subsequent characterization, a sulfo-cyanine 5 (Cy5) dye was first incorporated into native HSA via the single unpaired cysteine (Cys 34, Figure 2B) yielding Cy5-labeled HSA. All HSA and chemically modified HSA analogs used in subsequent studies are fluorescently labeled unless otherwise stated. Ring-opening of the succinimide thioether was promoted by incubation in borate buffer at pH 9.2 and 37 °C for 24h⁴² to prevent displacement of the Cy5 via exchange with thiols, which has been reported.⁴³ Next, varying equivalents of NHS-PEG₄-DBCO (5, 10, 20, 2x25, 4x25 equiv.) were added to HSA and different DBCO_m-HSA conjugates were isolated with varying average numbers of DBCO groups (Figure 2B). The increasing amount of DBCO groups bound to HSA was confirmed in agarose gel electrophoresis (Figure S1), in which proteins smaller than 600 kDa are separated predominantly by charge.⁴⁴ By attaching NHS-PEG₄-DBCO, lysine residues in the protein are converted to amides, leading to a more negative overall charge and further migration towards the positive electrode. The average number of DBCO per HSA was calculated to be 3, 6, 15, 30, and 50, respectively, as determined by the average molecular weight of the statistical modification from MALDI-ToF analysis (Table S1). The molecular mass (M) of these DBCO-HSA conjugates was calculated as the *m/z* value of the center of the peak of the singly charged species, with the variation of the respective mass range (Δm) given as the full width at half maximum (FWHM, see SI for calculation method). Thereafter, TM (5–12 equiv. per DBCO) was added to the DBCO_m-HSA conjugates, followed by purification via spin filtration (molecular weight cutoff = 10 kDa) to remove excess reagent. Upon lyophilization,

the TM_n-HSA conjugates, TM₁-HSA, TM₃-HSA, TM₉-HSA, TM₂₀-HSA, and TM₃₈-HSA, were isolated with a 78–95 % recovery rate. Successful click reactions were confirmed by agarose gel electrophoresis (Figure S2). The interaction between TM and agarose leads to a reduced migration of TM-HSA conjugates through the gel with an increasing number of TM attached and when compared to the corresponding DBCO-HSA precursors (Figure S1). The average number of TM groups attached as well as FWHMs (Δm) were determined by MALDI-ToF-MS (Table S2, Figure 2C). Based on the Δm values, we obtained the number variation in the degree of modification for each TM_n-HSA conjugate (Figure 2C). The sizes of the TM_n-HSA bioconjugates were determined using fluorescence correlation spectroscopy (FCS),⁴⁵ through measuring diffusion. The size ranges from 4.7 ± 0.5 to 7.0 ± 0.7 nm, with the greatest increase in size in TM₃₈-HSA (Figure 2C). The trend is consistent with observation in dynamic light scattering (DLS) measurements (Figure S3). The PDI increases with an increasing number of TM (up to 1), presumably due to hydrogen bonding and carrier-carrier interaction increase (Figure S3). Notably, no precipitation for trimannosylated conjugates was observed even after long storage in solution, suggesting that there is no uncontrolled aggregation. Zeta potential measurements (Figure 4B) also indicated that there is not a large difference in charge between the negatively charged HSA (-7.7 ± 1.9 mV) and the TM₃₈₋₄₁-HSA (-9.0 ± 1.9 mV).

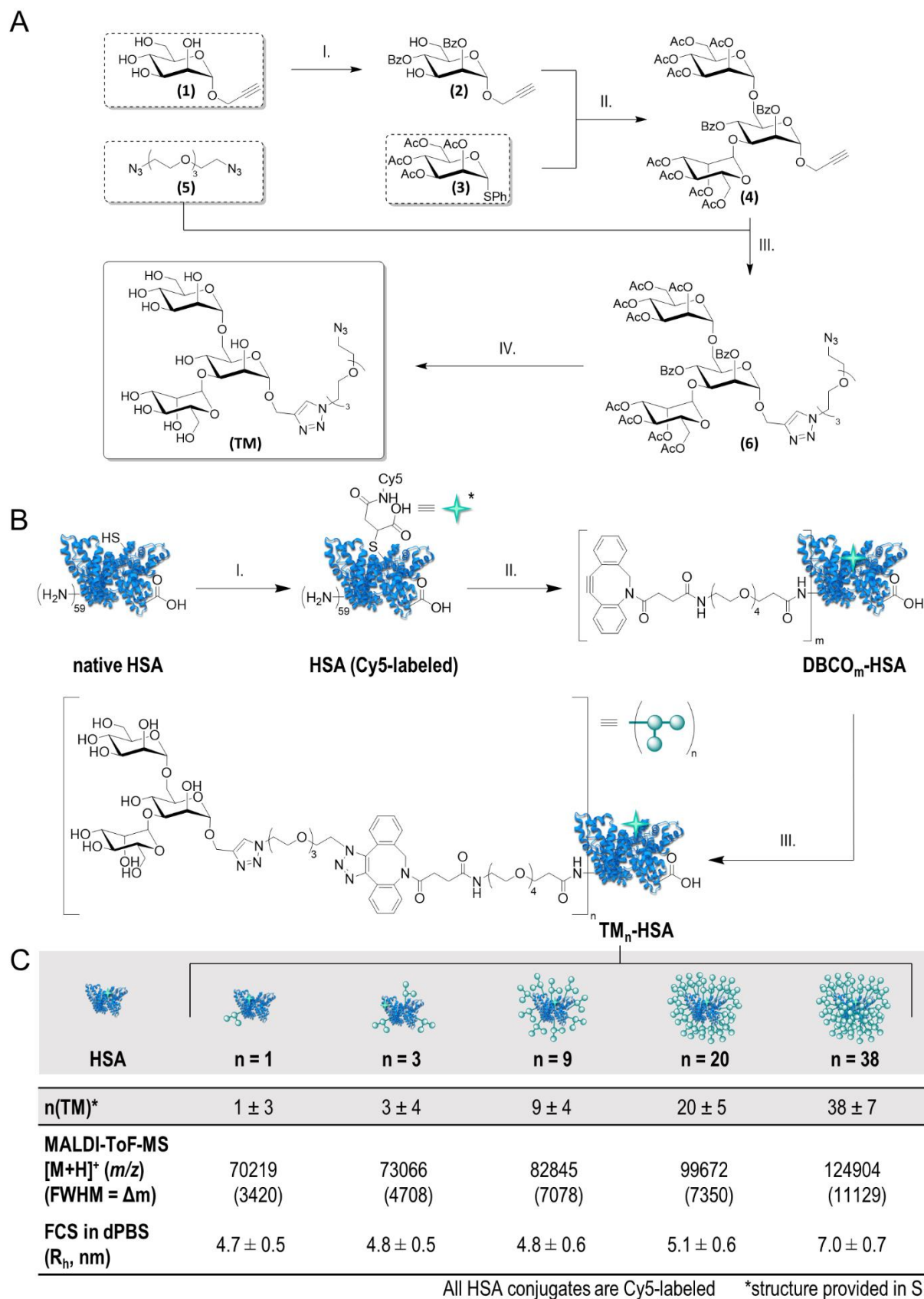


Figure 2 A) Synthesis scheme of TM: I.) i.) TBDMSCl, imidazole, DMF, Ar-atm., MS 3Å, 0 °C, 4 h; ii.) BzCl, Pyr., DMAP, DCM, Ar-atm., MS 3Å, 0 °C to RT, 42 h; iii.) $(\text{HF})_x \times \text{Pyr.}$, Pyr., THF, 0 °C to RT, 24 h, 62 %; II.) NIS, AgOTf, DCM, Ar-atm., -40 °C to -20 °C, 4 h, 70 %; III.) CuBr, PMDETA, DMF, Ar-atm., 45 °C, 3 h, 97 %; IV.) NaOMe, NaOH, MeOH, 45 °C, 4 h, quant. (* telescoped yield, i.e. yield for a sequential one-pot synthesis with reagents added one at a time and without work-up) for further details see Scheme S1). B) Synthesis scheme of trimannosylated HSA conjugate: I.) Sulfo-Cy5-maleimide, 50 mM PB pH 7.4, RT, 4 h; 50 mM Na-borate buffer pH 9.2, 37 °C, o.n., 93 % recovery rate; II.) NHS-PEG₄-DBCO, 50 mM PB pH 7.4, RT, 4–6 h, 95–100 % recovery rate; III.) TM, 50 mM PB (8 M urea, 2 mM EDTA) pH 8, RT, 4–

16 h, 78–95 % recovery rate. C) MALDI-ToF-MS and FCS characterization of HSA conjugates carrying varying numbers of TM. Variation in the degree of modification was calculated from FWHM, see SI for calculation method; see SI Fig. S4 for FCS correlation curves.

Next, we investigated the influence of the number of TM present on the HSA surface on the uptake of cells expressing MMR/DC-SIGN. Chinese hamster ovary (CHO) cells or CHO transductants expressing murine MMR (CHO-MMR⁺) were used and 100 nM of the TM_n-HSA carriers were applied and incubated for 24 h before fluorescence-activated cell sorting (FACS) analysis (Figure 3C). HSA (n(TM) = 0) was used as negative control. We already observed significant differences in cellular uptake of TM₁-HSA carrying an average of one TM group. The uptake increased even further with higher number of TM attached in the MMR-positive CHO cells. Confocal studies of TM₃₈-HSA *versus* HSA further corroborated the TM-mediated internalization into CHO-MMR⁺ cells and low non-specific uptake of the HSA control (Figure 3A).

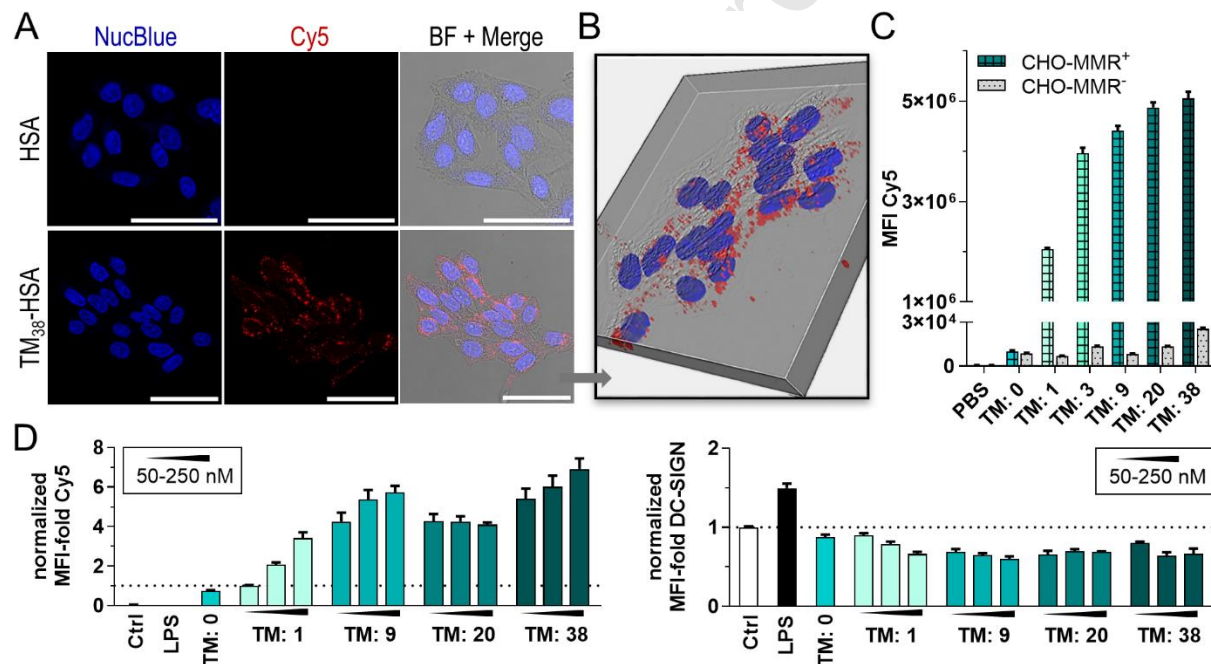


Figure 3 A) Confocal microscopy of MMR-positive CHO cells incubated with trimannosylated TM₃₈-HSA or HSA (1 μ M, 20 h) (Scale bar: 50 μ m, Channels: Nuc Blue (Nuclei), Cy5 (HSA conjugates)) and B) corresponding z-stack of internalized TM₃₈-HSA conjugate. C) FACS analysis of Cy5-labeled HSA conjugates (HSA or TM_n-HSA, 100 nM) with varying numbers of TM groups into MMR-positive and MMR-negative CHO cells after 24 h incubation. D) FACS analysis of concentration-dependent cellular uptake (left) of Cy5-labeled TM_n-HSA conjugates (50, 100, 250 nM) with varying numbers of TM into GM-CSF differentiated BMDC after overnight incubation normalized to 50 nM TM₁-HSA and the corresponding DC-SIGN expression (right) upon treatment normalized to the untreated control. LPS = Lipopolysaccharides (100 ng/mL). Data are presented as mean $\pm$ SEM, n=9 (C), n=5 (D). All HSA and HSA conjugates are Cy5-labeled.

As dendritic cells play a crucial role in shaping adaptive immunity and naturally express the target receptor DC-SIGN, we studied the uptake using a commonly used culture of murine bone marrow (BM) cells treated with granulocyte-macrophage colony-stimulating factor (GM-CSF) to generate BM-derived dendritic cells (BMDCs). Similarly, we observed a significant uptake of the TM_n-HSA carriers into the GM-CSF differentiated BMDC after

overnight incubation (Figure 3D). The corresponding DC-SIGN expression upon treatment was determined, proving consistent receptor availability and showing no increase in expression levels regardless of the amount of TM attached to the carrier in contrast to lipopolysaccharides (LPS) treatment, which serves as a positive PAMP control. The results demonstrate that TM-HSA conjugates exhibit selectivity towards cell lines expressing MMR in first *in vitro* studies. For $n(\text{TM}) = 38$, the highest cellular uptake was obtained indicating that this bioconjugate could potentially serve as nanocarrier for cell-type selective delivery into MMR/DC-SIGN positive cells. For all further studies, we prepared carriers that are saturated with TM ($n(\text{TM}) = 38\text{--}41 \pm 12$) to minimize the influence of smaller variations on our experiments, e.g. changes in receptor densities of the different cells used in this study.

2.2. Synthesis and Characterization of TM-HSA Drug Conjugates

Having determined the optimum range of TM groups for the HSA carrier, we proceeded to prepare various $\text{TM}_{38\text{--}41}$ -HSA conjugates (with average $n(\text{TM}) = 38\text{--}41 \pm 12$, henceforth referred always as TM-HSA) consisting of different cargoes for further studies. All HSA and HSA conjugates are fluorescently labeled with Cy5 or Cy7.5 unless otherwise stated. For cargo loading, we adopted and modified a HSA denaturation and refolding method previously reported by our group.⁴⁶ Specifically, TM-HSA was first denatured and reduced to allow the conjugation of multiple cargoes to the 34 cysteine groups originating from the 17 disulfide groups of HSA (Figure 4A). 1-(4-(Aminomethyl)benzyl)-2-butyl-1*H*-imidazo[4,5-*c*]quinolin-4-amine, an imiquimod derivative as representative TLR 7/8 agonist was selected as a weakly basic cargo after functionalization, while benzylamine was chosen as a neutral, non-active model cargo that has no immunostimulatory effects. At the same time, it serves as a negative control for subsequent investigations, to consider effects of loaded cargoes on the cellular uptake properties of the nanocarrier. Both cargoes were modified with a pyridinyldisulfanyl-ethyl-carbonate linker to form a carbamate bond, which can undergo thiol exchange and thereby attach to the thiol groups of the reduced cysteines of the denatured TM-HSA backbone.^{47–51} In addition, *N*-ethylmaleimide was added to cap unreacted thiols to enhance the stability of the bioconjugate as reported previously.^{52,53} Afterwards, excess reactants were removed *via* spin filtration (molecular weight cutoff = 10 kDa) with the denaturing buffer. By rapid dilution with MilliQ water (MQ), the conjugates were backfolded based on hydrophobic and hydrophilic interactions as reported before.^{46,54} After purification *via* spin filtration with MQ and lyophilization, the TM-HSA drug conjugates, TM-HSA-TLR7/8a and TM-HSA-Benzyl, were isolated with a 34 % and 46 % recovery rate, respectively.

SDS-PAGE analysis showed differences between the migration behavior of the final protein-drug conjugates (TM-HSA-Benzyl and TM-HSA-TLR7/8a) under reducing and non-reducing conditions in comparison to the precursor conjugates, TM-HSA (Figure S6), suggesting successful loading of the cargoes. For further confirmation, the cargo-to-carrier loading was determined, using MALDI-ToF-MS, to consist of an average number of 25 (benzylamine) or 20 (TLR 7/8 agonist). Agarose gel electrophoresis of the final TM-HSA-cargo conjugates indicates absence of large aggregates and no major charge differences to the precursor TM-HSA (Figure S5), which is corroborated by Zeta Potential (ZP) measurements (Figure 4B). Circular dichroism (CD) measurements were performed for TM-HSA-Benzyl, TM-HSA-TLR7/8a, and native HSA, showing slight changes in intensity in HSA's signature peaks at 208 and 222 nm but an overall retained curve indicating that the α -helix-rich secondary structures are preserved even after denaturation and modifications (Figure S7).⁵⁵

Sizes and size distribution profiles of TM-HSA-cargo conjugates were determined using FCS (Figure 4C and Figure S9B) and DLS (Figure 4D and Figure S8), respectively. From FCS, the hydrodynamic radii in Dulbecco's Phosphate-Buffered Saline (dPBS) were determined as follows: HSA (4.1 ± 0.4 nm), TM-HSA (7.1 ± 0.7 nm), TM-HSA-Benzyl (11.0 ± 2.8 nm) and TM-HSA-TLR7/8a (12.0 ± 1.2 nm). The determined hydrodynamic radius of HSA is consistent with previously reported literature³⁷ and the increase in size after denaturing and cargo loading is consistent with previous reports.⁵⁶ DLS shows similarly an increase in size in the TM-HSA drug conjugates compared to TM-HSA after cargo loading. Furthermore, the conjugate sizes were determined after long-term storage (after 1 year) in solution (Figure S9-11), showing that they are stable and no uncontrolled aggregation of the TM-HSA conjugates was observed. As many nanocarriers are known to degrade, aggregate or interact with components in biological media, e.g. to form a protein corona, the hydrodynamic radii of the TM-HSA conjugates in human plasma were also determined using FCS. Here, a higher value of the plasma viscosity (1.5 times higher than that of water) was used according to earlier studies.^{45,57} There was no significant size increase observed for TM-HSA (8.3 ± 0.8 nm) and only a slight increase for both, TM-HSA-Benzyl (15.0 ± 1.5 nm) and TM-HSA-TLR7/8a (16.5 ± 1.7 nm) suggesting no protein corona formation. Additionally, preincubation of TM-HSA-Benzyl and TM-HSA-TLR7/8a with native or heat-inactivated mouse plasma did not reduce or prevent cellular uptake into BMDC (Figure S15). Next, we tested the release profile of the TLR7/8a cargo with the disulfide linker. As the endolysosomal compartments in immune cells are reductive and denaturing,⁵⁸ we have performed the investigation under (1) reductive (+ GSH) and (2) denaturing + reductive (8 M urea + GSH) conditions (Figure S12–

14). These show that under reductive and denaturing conditions we have fast drug release from the carrier. In comparison, under reductive and non-denaturing conditions, the nanocarrier shows a slower drug release, indicating that the drug is folded into the inner parts of the HSA conjugate.

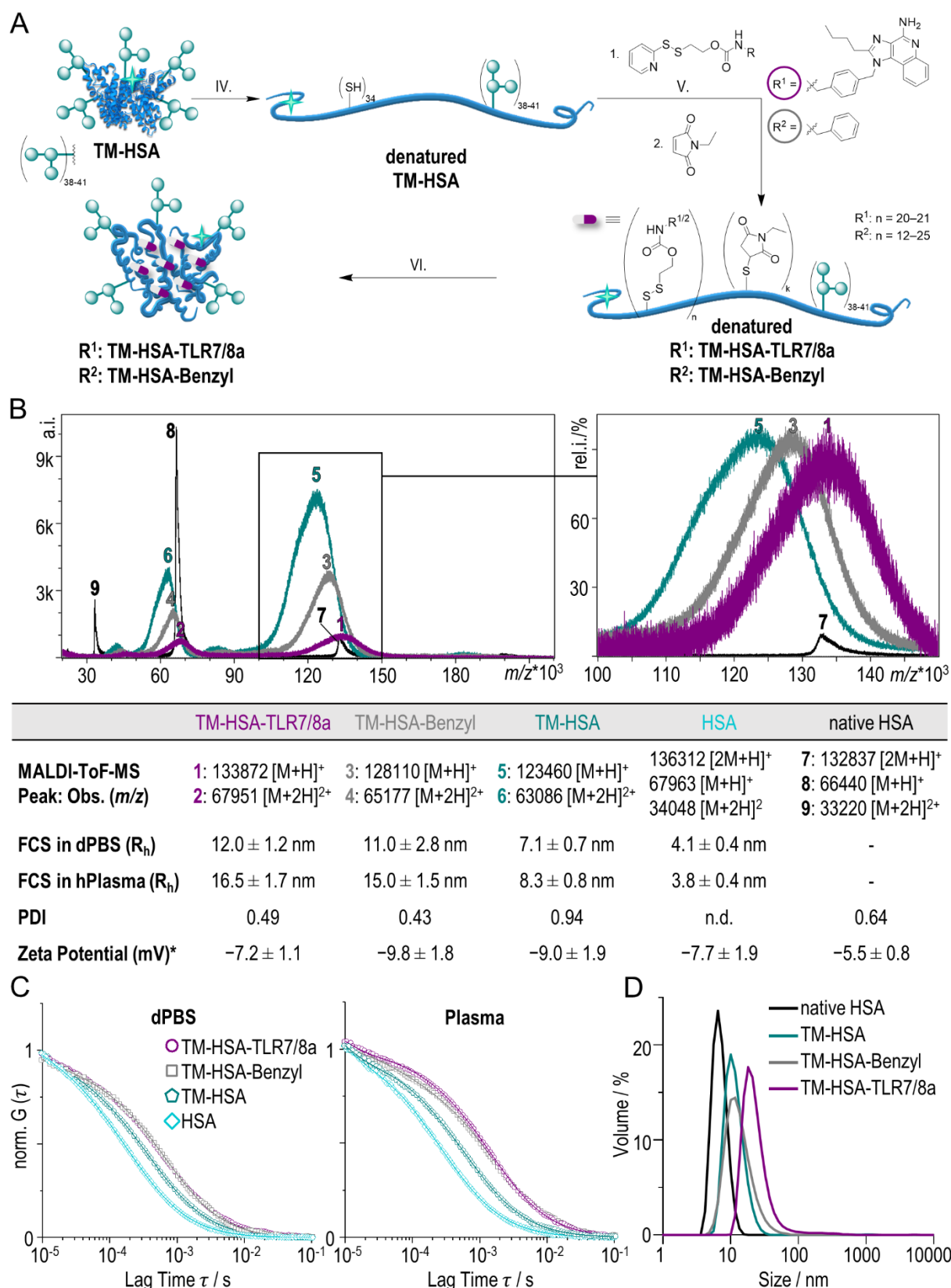


Figure 4 A) Synthesis scheme of drug loading procedure onto trimannosylated HSA to obtain final nanocarrier conjugates; IV.) TCEP, 50 mM PB (8 M Urea, 2 mM EDTA) pH 8 V.) 1. Pyr-S-S-R, 2. N-ethylmaleimide, 50 mM PB (8 M Urea, 2 mM EDTA) pH 8 (if R=TLR7/8 agonist, readjust buffer to pH 6.3 for washing steps) VI.) rapid dilution 1:10, MQ, recovery rate 34–46 % B) MALDI-ToF-spectra of TLR7/8a- / benzyl-loaded nanocarriers as well as precursors, FWHM values are given in the SI (top) and summary of nanocarrier characterization (below). C) Normalized FCS autocorrelation curves measured for TLR7/8a- / benzyl-loaded nanocarriers as well as precursors in dPBS and human plasma. The solid lines correspond to fits with eq. S1. D) Size distribution of final, loaded nanocarriers compared to native HSA and trimannosylated precursor conjugate (1 mg/mL in dPBS) showing sizes of d.nm. = 7.02 ± 1.77 for native HSA, d.nm = 11.81 ± 0.67 for TM-HSA, d.nm = 11.0 ± 2.8 for TM-HSA-Benzyl, and d.nm = 12.0 ± 1.2 for TM-HSA-TLR7/8a.

= 14.28 ± 1.34 for TM-HSA-Benzyl and $d.nm = 25.89 \pm 0.84$ for TM-HSA-TLR7/8a determined by DLS. Measurements were performed in quadruplicates, errors are given as standard deviation. *ZP measurements were performed with Cy7.5-labeled conjugates (see SI, section 6.4).

2.3. *Ex Vivo* Evaluation

Next, we studied the uptake and activation of immune cells of the TM-HSA drug conjugates *ex vivo* (Figure 5 and S17). As APCs are key targets for cancer immunotherapy due to their role in boosting pathogen-specific adaptive immune responses^{59,60} and the liver is an immunological organ, we first investigated the uptake of TM-HSA-Benzyl and TM-HSA-TLR7/8a into BMDC, splenic and liver cells (Figure 5A). Cells that were not treated with HSA derivatives or only treated with HSA were used as controls for comparison. While HSA showed no significant uptake in all cells tested, we observed an exceptional uptake for TM-HSA drug conjugates in differentiated BMDC, in splenic macrophages and DC, and in LSEC, Kupffer cells, and liver DC both after 3 h incubation and overnight incubation time and at low dosage (50 nM Figure S17). This further corroborates the *in vitro* results of TM-mediated and enhanced uptake in cell lines expressing MMR/DC-SIGN. We also observed that TM-HSA-Benzyl has a higher uptake than TM-HSA-TLR7/8a in some of the tested cell types, which could be due to the influence of hydrophobicity of the cargoes.^{61,62} To confirm that the uptake is enhanced by the fast association of the TM to the cell surface, we incubated BMDC with TM-HSA-Benzyl and HSA at 0 °C to slow down energy-dependent endocytosis and analyzed the percentage of Cy5-positive cells after 30, 60 and 180 min (Figure S18). TM-HSA-Benzyl reaches a plateau of Cy5 positive cells already after 30 min, which further supports the high avidity and fast association of the protein conjugate.⁶³ TLR7/8 agonists are applied in cancer immunotherapy for activation of DC and induce a change in receptor expression levels and cytokine production such as TNF α and interleukins (ILs).⁶⁴ Additionally, TLR7 activation has been proposed for the treatment of alcoholic hepatitis, which is a form of alcohol-associated liver disease,⁶⁵ and autoimmune diseases such as systemic lupus erythematoses.⁶⁶ Analysis of the cytokine levels in BMDC, spleen, and liver NPC cultures show that the TM-HSA-TLR7/8a conjugate offers superior performance in cytokine production, in comparison to the untreated or decoy-loaded carrier TM-HSA-Benzyl controls, even at low concentration of 50 nM (Figure 5E and S20). The TM-HSA-TLR7/8a conjugate even outperforms small molecule TLR7/8 agonist at the same applied concentration in inducing interleukin production, e.g. in GM-CSF differentiated BMDC IL-10, -12 and -1 β concentrations were found 3–4-times and IL-6 concentrations 34-times higher. These results are in accordance with previous findings demonstrating a higher immunostimulatory activity of adjuvants when applied at equimolar amounts in a particulate versus soluble form, which may be due to higher cellular

uptake.⁶⁷ FACS analysis shows that CD86 and MHC II expression levels in GM-CSF- and FLT3L-differentiated BMDC after overnight incubation with TM-HSA-TLR7/8a (50 nM) were comparable to incubation with small molecule TLR7/8 agonist at the corresponding equimolar drug concentration (1.025 μ M, 0.371 μ g/mL) (Figure 5B and C), whereas TM-HSA-Benzyl induced no upregulation of these activation markers, nor did unloaded TM-HSA (Figure S16). In addition, we tested the ability of TM-HSA-TLR7/8a to induce T-cell responses by preincubating GM-CSF BMDC with Ovalbumin (OVA) and subsequently with the different HSA conjugates before co-culturing with CD8⁺ (OT-I) or CD4⁺ (OT-II) T cells. Out of all the TM-HSA-TLR7/8a concentrations (1–50 nM) tested (Figure S19), 10 nM was optimal in inducing OVA peptide-specific OT-I and OT-II T cell proliferation and exhibited similar effects as five-times higher concentrations of the free drug (Figure 5D). This observation corresponds to the stronger state of activation evoked by the delivery of particulate adjuvant. It is noteworthy that HSA or TM-HSA-Benzyl did not lead to an increase in the activation markers (CD86, MHC II, OT-I/-II proliferation) compared to untreated controls, which could support that the nanocarrier is immunocompatible. Our results suggest that TM-HSA serves as an efficient and receptor-selective macromolecular nanocarrier for the delivery of TLR 7/8 agonist *ex vivo*.

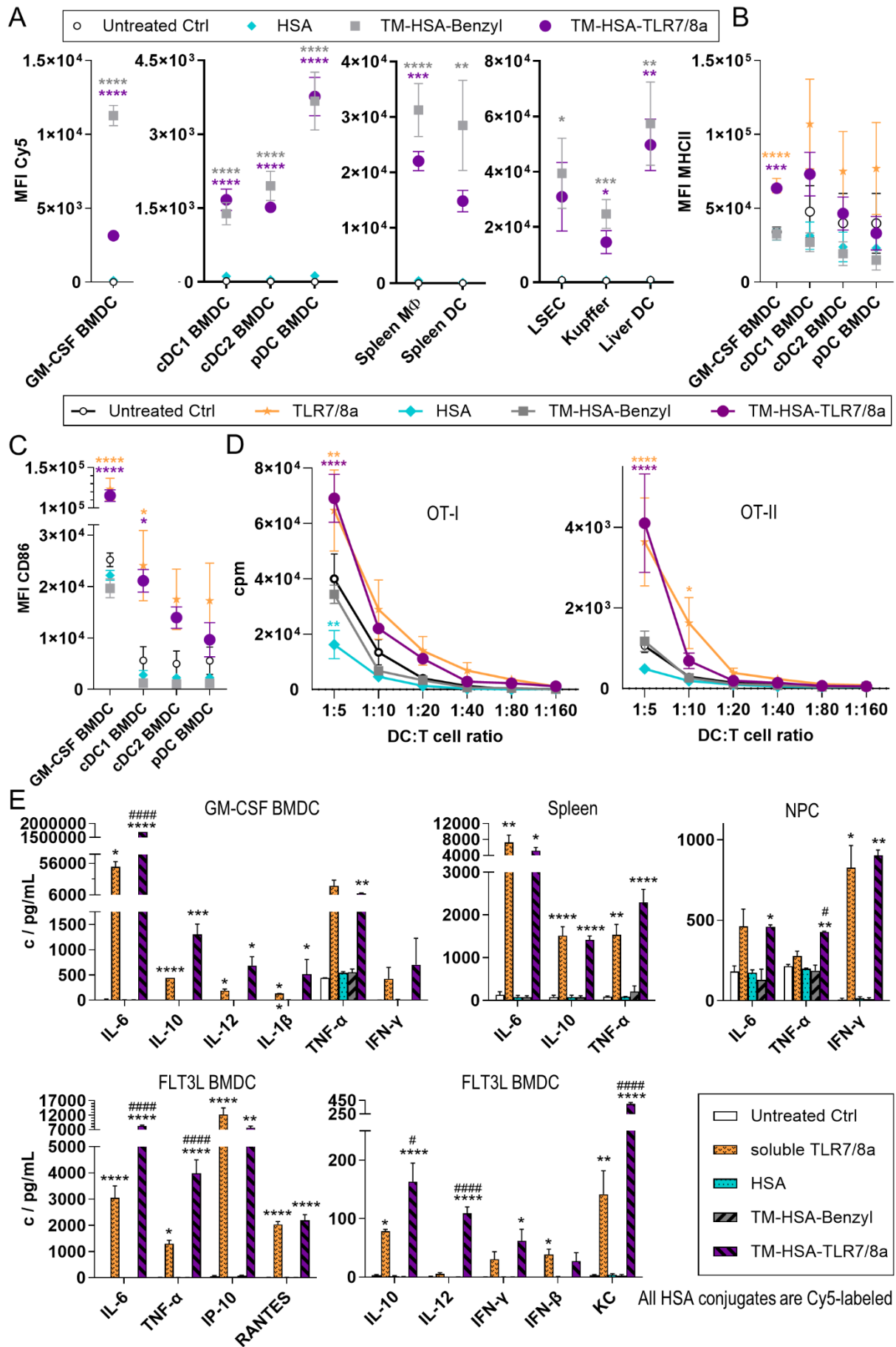


Figure 5 A) FACS analysis of uptake in GM-CSF differentiated BMDC, in FLT3L differentiated BMDC, in splenic macrophages and DC, and in LSEC, Kupffer cells, and liver DC after 3 h incubation with different HSA conjugates (50 nM).

FACS analysis of B) MHC II expression levels and C) CD86 expression levels in GM-CSF- and FLT3L-differentiated BMDC after overnight incubation with different HSA conjugates (50 nM) or small molecule TLR7/8 agonist at the corresponding concentration (1.025 μ M, 0.371 μ g/mL). D) T-cell proliferation (cpm) of OVA peptide-specific CD8⁺ (OT-I) or CD4⁺ (OT-II) after co-culture with GM-CSF-differentiated BMDC which were preincubated with OVA (5 μ g/mL) and subsequently with different HSA conjugates (10 nM, corresponds to 205 nM TLR7/8-cargo) or small molecule TLR7/8 agonist (1.025 μ M, 0.371 μ g/mL). E) Production of cytokines in response to overnight incubation with different HSA conjugates (50 nM) or small molecule TLR7/8 agonist (1.025 μ M, 0.371 μ g/mL) of GM-CSF differentiated BMDC, in FLT3L differentiated BMDC, in splenic macrophages and DC, and in LSEC, Kupffer cells and liver DC. Data are presented as mean $\pm$ SEM, n=4–6 (A–D), n=2–3 (E); Statistical differences are indicated: vs. # soluble TLR7/8 agonist and * Untreated Ctrl (one-way ANOVA, Tukey test or t-test for samples where n=2). *,# p < 0.05, **,## p < 0.01, ***,### p < 0.001, ****,#### p < 0.0001. All HSA and HSA conjugates are Cy5-labeled.

2.4. Rapid Liver Selective Uptake of TM-HSA Conjugates *In Vivo*

To evaluate the uptake and targeting specificity of TM-HSA derivatives *in vivo*, we performed a biodistribution experiment in B6 albino mice. These experiments aimed to identify potential organ-specific accumulation and immunogenic effects of the TM-HSA bioconjugates compared to no TM (HSA) as well as the influence of the drug cargo on carrier biodistribution. For *in vivo* imaging, all HSA and modified HSA used are labeled with Cy7.5 dye (see SI, section 6.4), which is compatible with the instrument settings. We used HSA as control, as well as different TM-HSA conjugates, either with TLR7/8a (TM-HSA-TLR7/8a), benzylamine (TM-HSA-Benzyl), or without cargo (TM-HSA). Next, 100 μ L of a 2.94 μ M solution of these conjugates were injected intravenously (i.v.) into healthy mice (mouse body weight dosage in case of TM-HSA-TLR7/8a: 2 mg/kg mouse corresponding to 0.14 mg/kg TLR7/8 cargo). Biodistribution was assessed through surface fluorescence imaging *via* an *in vivo* imaging system (IVIS), blood sampling followed by plasma analysis, and *ex vivo* analysis of harvested organs. Mice that received complete intravenous tail vein injection were used for quantification and statistical analysis, mice that did not receive the full dose *via* tail vein injection intravenously (Figure S22) were excluded.

In order to monitor the TM-HSA conjugates in the bloodstream, small blood samples (50 μ L) were obtained from the studied mice shortly after injection (0.1 h) and 24 h later, and the generated plasma was analyzed. As a control, plasma samples of mouse blood incubated with TM-HSA-TLR7/8a at a concentration (140 nM) similar to the injected ones (estimated initial blood concentration = 230 nM)⁶⁸ were also studied. The size and the concentration of the TM-HSA conjugates in plasma obtained from all blood samples were measured by FCS. Slight size increases in hydrodynamic radii of 9.1 ± 1.7 nm, 6.2 ± 1.8 nm and 4.9 ± 3.8 nm were observed for trimannosylated conjugates TM-HSA, TM-HSA-Benzyl and TM-HSA-TLR7/8a, respectively, as compared to the measurements in PBS (Figure S9B). Furthermore, the results showed that immediately after injection, HSA exhibited high concentrations of 238 ± 8 nM in the bloodstream, with detectable levels (40 ± 6 nM) persisting up to 24 h post injection (Figure 6B, all correlation curves: Figure S26). In contrast, TM-HSA conjugates showed rapid

clearance from the bloodstream, independent of any cargo. For TM-HSA, TM-HSA-Benzyl and TM-HSA-TLR7/8a, we observed concentrations ranging from 1 ± 1 nM to 4 ± 2 nM shortly after injection to non-detectable levels at 24 h post injection of these conjugates. The plasma control sample, TM-HSA-TLR7/8a incubated *ex vivo* in whole blood, showed no such decrease in concentration, ruling out a reduction in sample concentration due to adsorptive interaction with whole blood components during plasma generation. This was verified by IVIS imaging, showing that HSA distributed throughout the body, with a trend for higher accumulation in the liver, which is a known phenomenon for i.v. administered fluorescently labeled albumin (Figure 6A).^{69,70} Trimannosylation, however, leads to direct accumulation mainly in the liver post injection, which has been shown for some glycosylated systems.^{71,72} *Ex vivo* fluorescence analysis of organs highlighted significant differences in organ enrichment of HSA and the TM-HSA conjugates (Figure 6D and E). Notably, HSA showed enrichment in the heart 24 hours post injection, confirming its prolonged presence in the bloodstream. It also demonstrated higher accumulation in the lungs compared to TM-HSA conjugates as well as in the kidneys, indicating renal clearance of the HSA.⁷³ Conversely, TM-HSA conjugates did not or only to a much lower extent accumulate in these organs, again verifying enhanced selective liver targeting *in vivo*. TM-HSA conjugates were also observed in the spleen, however, the difference to HSA uptake in the spleen was not statistically significant. The ratio of liver fluorescence intensity to total body intensity revealed that liver-specific targeting was significantly enhanced for TM-HSA bioconjugates, also indicating enhanced organ specificity when using TM as targeting units (Figure 6C and S24). Taken together, the results show that the TM moieties enabled fast organ-specific uptake and the type of cargo did not affect the biodistribution profiles. *Ex vivo* FACS analysis of isolated liver NPC and spleen cells revealed that the TM-HSA conjugates were uptaken by target cells (LSEC, Kupffer cells and liver DC) but there is no statistical significance due to variability (Figure S25). Low uptake observed in spleen immune cells is in agreement with the *ex vivo* organ analysis (Figure 6E).

In addition to imaging analysis, we evaluated the potential immunogenic responses towards our protein nanocarriers by measuring plasma cytokine levels 24 hours post injection. A significant increase in IP-10 (CXCL10) and RANTES (CCL5) was observed in the plasma when TLR7/8 ligands were coupled to TM-HSA (TM-HSA-TLR7/8a) indicating immune cell activation possibly mediated by T-cellular or T cell-induced immune activation (Figure S27). No statistically significant increase in plasma MCP-1 and IFN- α levels was observed, which is not unexpected due to the low dosage of TLR 7/8 used for the *in vivo* biodistribution study.

Furthermore, no other cytokines were detectable in the sera, in particular IL1 β , TNF α , IFN α and IFN γ (Figure S27). In conjunction with the fact that the rate of apoptotic leukocytes was not increased in ex vivo samples from treated animals (data not shown), these observations suggest that the TM-HSA-TLR7/8a did not induce systemic inflammation.

In summary, we could demonstrate that trimannosylation of HSA significantly enhanced liver-specific targeting *in vivo* as well as activation, which was shown for the TLR7/8a loaded bioconjugate TM-HSA-TLR7/8a. The TM moieties enabled fast organ-specific uptake and cargo loading and the type of cargo did not affect the biodistribution profiles.

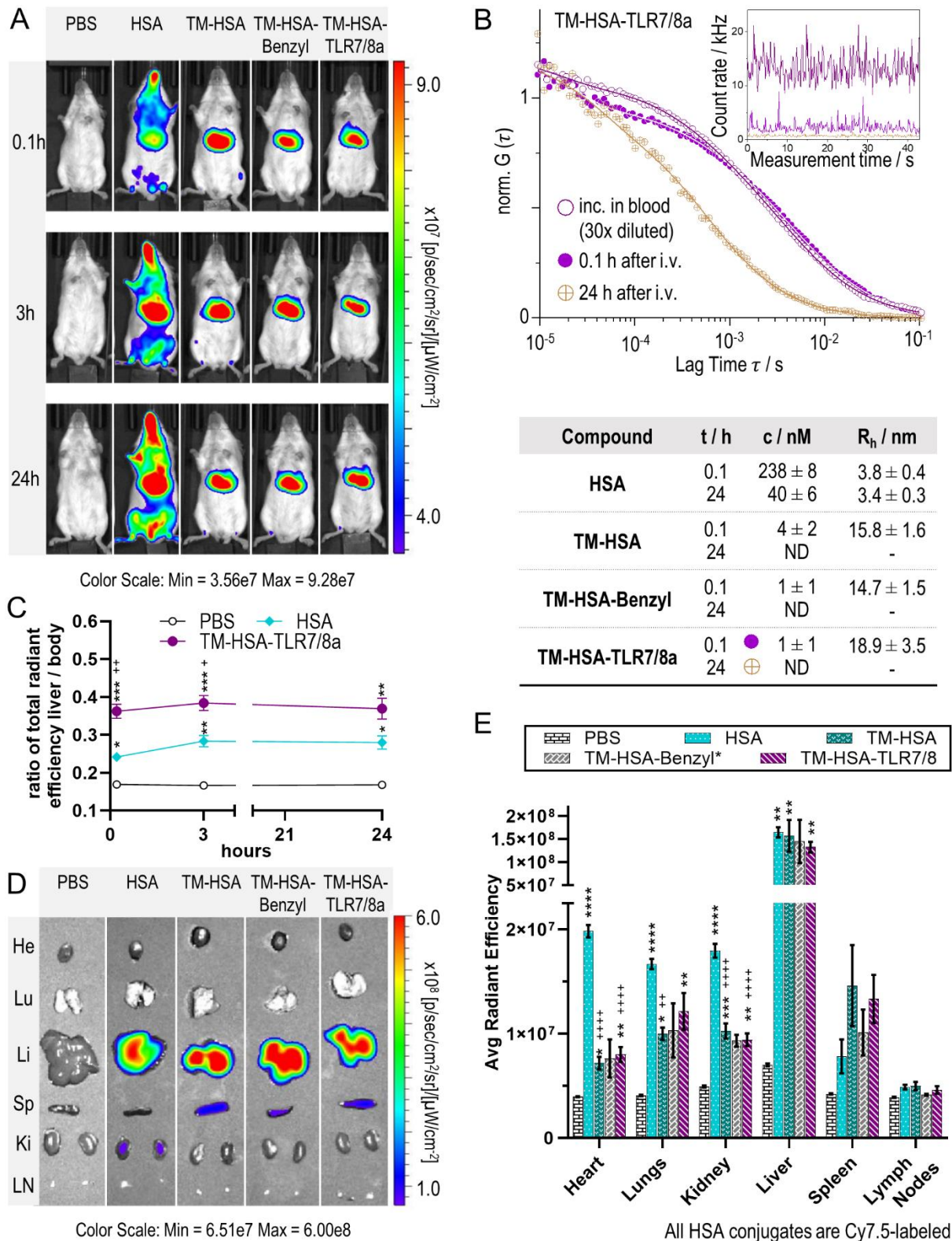


Figure 6 Evaluation of *in vivo* biodistribution experiment. A) *In vivo* imaging of Cy7.5 dye-labeled HSA and TM-HSA conjugates using IVIS after 0.1, 3, and 24 h post intravenous injection. B) (top) Exemplary normalized FCS correlation curves of TM-HSA-TLR7/8a during biodistribution and incubated in whole blood *ex vivo*. The inset shows the corresponding intensity time traces (note that the curves for the sample taken 24 h after i.v. injection originated from a very small amount of initially non-covalently bound Cy7.5 attaching to endogenous mouse blood proteins); (bottom) FCS results for the concentrations (c) and the hydrodynamic radii (R_h) of HSA conjugates in mouse plasma 0.1 h and 24 h after injection. Data are presented as mean ± SD, n=3 (PBS), n=2 (TM-HSA-Benzyl), n=4 (HSA, TM-HSA) and n=5 (TM-HSA-TLR7/8a). C) Ratio of total radiant efficiency of liver to whole body 0.1, 3, 24 h post injection based on IVIS images. D) IVIS images of Cy7.5 dye on organ level 24 h post injection. E) Quantification of organ-specific fluorescence 24 h post injection. Data denote average radiant efficiency ± SEM, n=3 (PBS), n=2 (TM-HSA-Benzyl), n=4 (HSA, TM-HSA) and n=5 (TM-HSA-TLR7/8a) B6 albino mice. C) + E) Statistical differences are indicated: vs. + HSA and * PBS Ctrl ((C) two-way, (E) one-way

ANOVA, Tukey test). *,+ $p < 0.05$, **,++ $p < 0.01$, ***,+++ $p < 0.001$, ****,++++ $p < 0.0001$; *TM-HSA-Benzyl group was excluded from statistical analysis (n=2).

Conclusion

Inspired by the polysaccharide-core-shell protein glycogen, we have reported TM-HSA bioconjugates consisting of an HSA core with a dense trimannose shell. The small-sized (< 40 nm) protein nanocarriers were rapidly and highly selectively uptaken into immune cells as well as the liver. The number of TM groups present on the surface of HSA was varied to tune the uptake efficiency of the nanocarriers. Furthermore, TM-HSA was denatured to incorporate multiple copies of a TLR 7/8 agonist with a redox-sensitive linker for the first time, which allows controlled drug delivery and traceless release under denaturing and reducing conditions, often found in immune cells.

In comparison to HSA, TM-HSA serves as an efficient transporter of cargo molecules as exemplified by the delivery of TLR 7/8 agonist *ex vivo*. Moreover, specific and selective uptake into cell lines expressing MMR/DC-SIGN, such as MMR transductants, BMDC, splenic and liver cells was demonstrated and the resulting TM-HSA-TLR7/8a conjugate even outperformed TLR7/8 in stimulating interleukin production at similar concentrations. The resultant TM-HSA conjugates, through tailored surface modifications, not only revealed enhanced specificity for and binding affinity to immune cells *ex vivo* but also selective uptake into the liver over other organs, *in vivo* in healthy mice.

TM-HSA nanocarriers were rapidly cleared from the bloodstream, independent of the cargo attached (none, Benzyl and TLR7/8a tested), which could serve as a first indication of the potential of TM-HSA conjugates as versatile nanocarriers for targeting liver diseases. The application of TM-HSA nanocarriers providing rapid clearance *via* uptake by the target organ offers the potential to reach immune cells in diseased liver tissue, to minimize off-target as well as on-target/off-site effects, to avoid prolonged organ deposition, and could in the future be employed as a carrier to maximize therapeutic efficacy in immunotherapy of liver cancer or liver diseases. Therefore, the TM-HSA nanocarriers reported herein could offer new possibilities for the treatment of liver fibrosis or cancer. Potentially, this could be further extended in the future to look into diseases that are related to the immune system such as infections, immunodeficiencies or autoimmune disorders.

Methods

Nuclear magnetic resonance spectroscopy (NMR): NMR spectra were measured on Avance-III 700 or 400 Neo (Bruker Corporation, Ettlingen, BW, Germany) NMR spectrometers. For

carbohydrate structures NMR-spectra were recorded utilizing an AC300 (*Bruker Corporation*, Ettlingen, BW, Germany) with 5 mm Dual- ^{13}C -head and B-ACS 60 sample changer for 300 MHz ^1H -NMR-, 75.5 MHz ^{13}C -NMR- as well as 2D-NMR-experiments, an Avance-II 400 (*Bruker Corporation*, Ettlingen, BW, Germany) with 5 mm BBFO-head with z-gradient and ATM as well as SampleXPress 60 sample changer for 400 MHz ^1H -NMR-, 100.6 MHz ^{13}C -NMR- and 2D-NMR experiments and an Avance-III 600 (*Bruker Corporation*, Ettlingen, BW, Germany) with 5 mm TCI-Cryoprobe with z-Gradient and ATM as well as SampleXPress Lite 16 sample changer for 600 MHz ^1H -NMR-, 151.0 MHz ^{13}C -NMR- and 2D-NMR experiments. Samples were dissolved in an appropriate deuterated solvent (CDCl_3 , CD_3OD , D_2O). Spectra were processed using the MestReNova software (*Mestrelab Research*, Santiago de Compostela, Spain) for baseline correction (Whittaker smoother) and automated phase correction. Spectra were referenced to the signal of the respective deuterated solvent (CDCl_3 : ^1H : $\delta = 7.26$ ppm, CD_3OD : ^1H : $\delta = 3.31$ ppm, D_2O : ^1H : $\delta = 4.79$ ppm) and using the absolute reference function of the analysis software. Observed signals are described using their chemical shifts (δ) in ppm, multiplicity (s-singlet, d-doublet, t-triplet, dd-double of doublet, etc. and m-multiplet; app-apparent and br-broad), integral and locator of ^1H - and ^{13}C -position in the molecule. Signal assignment was supported utilizing 2D-NMR spectra: COSY, HSQC, HSQC-NoDec, HMBC, TOCSY, NOESY (data not shown).

Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-ToF-MS): Mass spectra for nanocarrier structures were acquired using sinapinic acid matrix on a ToF MS rapifleX (*Bruker Corporation*, Ettlingen, BW, Germany).

Dynamic light scattering (DLS): DLS measurements of the HSA based compounds (1 mg/mL in dPBS or MQ unless otherwise noted) were performed at 25 °C using a Zetasizer Nano S (*Malvern Instruments*, Malvern, England) equipped with a He/Ne Laser ($\lambda = 633$ nm) and a narrow band filter at a fixed scattering angle of 173 °. Volume distribution of DLS measurements of nanocarriers in dPBS was used to determine their size range.

Fluorescence correlation spectroscopy (FCS): FCS experiments were performed using a commercial confocal LSM 880 microscope (*Carl Zeiss*, Jena, TH, Germany) equipped with a C-Apochromat 40 $\times$ /1.2 W (*Carl Zeiss*, Jena, TH, Germany) water immersion objective. A HeNe laser ($\lambda = 633$ nm) fiber coupled to the LSM 880 was used for the excitation of the Sulfo-Cyanine5 dye. The emission light in the spectral range 655 – 699 nm (Cy5) was

detected using a Quasar spectral detection unit (*Carl Zeiss*, Jena, TH, Germany) that comprises a diffraction grating and a 32 channel GaAsP photomultiplier array operating in the photon counting mode. Sulfo-Cyanine7.5 dye was excited at 780 nm by a strongly attenuated Mai Tai Ti:Sa laser (*Newport Spectra Physics*, Milpitas, CA, USA) coupled to the LSM 880. The emission light passed through a band-pass filter EM835/70 (*Chroma Technology*, Bellows Falls, Vermont, USA) and was directed to a PDM SPAD detector (*Micro Photon Devices*, Bolzano, Italy) connected to a TimeHarp 260 TCSPC board (*PicoQuant*, Berlin, B, Germany). For each sample, 30 μL of solution (PBS, human plasma or mouse blood serum) was added to a 15 wells chamber (*ibidi*, Munich, BY, Germany). The confocal detection volume was placed $\sim 30 \mu\text{m}$ above the glass coverslip and series of 20 measurements, 10 s each, were performed at room temperature (23 $^{\circ}\text{C}$). For experiments with human plasma, the nanocarriers were incubated with plasma for 1 h at 23 $^{\circ}\text{C}$ before the measurement. The obtained experimental autocorrelation curves were fitted with the following analytical model function:

$$G(\tau) = 1 + \left[1 + \frac{f_T}{1-f_T} e^{-\tau/\tau_T} \right] \frac{1}{N} \sum_{i=1}^m \frac{f_i}{\left(1 + \frac{\tau}{\tau_{D,i}} \right) \sqrt{1 + \frac{\tau}{S^2 \tau_{D,i}}}} \quad (\text{eq. S1})$$

whereby N is the average number of diffusing fluorescence species in the observation volume, f_T and τ_T are the fraction and the decay time of the triplet state, $\tau_{D,i}$ is the diffusion time of the i -th type of species, f_i is the fraction of the component i ($1 \leq i \leq m$), and S is the structure parameter, $S = z_0/r_0$, where z_0 and r_0 represent the axial and radial dimensions of the observation volume. The fit yielded the values of $\tau_{D,i}$ and N . The diffusion coefficients of the species D_i are related to the respective diffusion times $\tau_{D,i}$ and the radial dimension r_0 of V_{obs} by $D_i = r_0^2/(4\tau_{D,i})$. By inserting D_i into the Stokes-Einstein equation, the hydrodynamic radius can be calculated as $R_h = \frac{k_B \cdot T}{6 \cdot \pi \cdot \eta \cdot D}$, here, k_B is the Boltzmann constant, T is the temperature, and η is the viscosity of the solvent. For the viscosity of water and plasma at 23 $^{\circ}\text{C}$ values of 0.93 mPas and 1.4 mPas, respectively were used. Errors are given as standard deviation but rounded up to 10 % of absolute values of hydrodynamic radii (R_h) if $\text{SD} < 10 \%$ of R_h to account for systematic errors based on temperature, viscosity, diffusion coefficient value of reference sample and later individual variations in plasma samples. Furthermore, the concentration of the fluorescent species was calculated as $c = N/V_{\text{obs}}$. For calibration of the confocal volume V_{obs} , dyes with known diffusion coefficients in water, i.e. Alexa 647 and IRDye[®] 800CW were used.

Fluorescence activated cell sorting: For FACS analysis the CHO^{MMR+/-} cells (200000 cells/well, 24-well plates, 24 h seeding, 24 h treatment, 0.1 μ M of TM_n-HSA, HSA, or PBS) were washed with dPBS and harvested using cell dissociation buffer for 15 min at 37 °C. After centrifugation for 10 min at 350 x g and 5 °C cell pellets were resuspended in dPBS. All samples were kept on ice prior to flow cytometric analysis by a BD Accuri C6 (BD Biosciences, Franklin Lakes, NJ, USA). Data were processed with FlowJo software. For *ex vivo* uptake experiments, cells were harvested, washed with staining buffer (PBS, 1 % FBS, 0.5 mM EDTA) and incubated with rat anti-mouse CD16/CD32 antibody (clone 2.4G2; 15 min, 4 °C) to prevent antibody binding to Fc γ receptors. Following this, samples were incubated with fluorescence-labeled antibodies (20 min, 4 °C). Then, samples were washed with PBS and incubated with FVD to identify live/dead cells. Measurements were carried out using an Attune NxT flow cytometer and data were analyzed using Attune NxT software (both Thermo Fisher, Waltham, MA, USA).

Cytokine detection: Cytokines were detected using the BDTM Cytometric Bead Array (CBA) Mouse Flex Set (BD Biosciences, Franklin Lakes, NJ, USA), which was performed according to the manufacturer's specifications.

Animals: *In vivo* experiments followed an approval by the local ethics committee on animal care (number 23 177-07/G 20-1-123, Government of Rhineland Palatinate, Germany). B6N-Tyr^{c-Brd}/BrdCrCrl were purchased from Charles River. Female mice were 8 weeks old (body weight ~20 g) and kept according to the guidelines of the institute and the local government. To explore the biodistribution of the nanoparticles 100 μ L of a solution of the respective Cy7.5 labeled nanoparticles (2.94 μ M in dPBS, corresponding to mouse body weight dosages of TM-HSA-TLR7/8a: 2.0 mg/kg, TM-HSA-Benzyl: 1.9 mg/kg, TM-HSA: 1.9 mg/kg, HSA: 1.0 mg/kg) were intravenously administered to B6 albino mice (n = 5, three mice receiving dPBS as controls). Directly after injection and 3 hours, the mice were once again anesthetized with isoflurane gas and placed into an IVIS (*in vivo* imaging spectrum) system (Caliper Life Sciences, Hopkinton, MA, USA) for *in vivo* NIR fluorescence imaging. The picture integration time of the fluorescence source was set to 10 s. Recording parameters were adjusted for excitation at 745 nm and emission at 820 nm to visualize the Cy7.5 labeled nanoparticles. Additionally, after injection blood was retrieved from *vena facialis*. After 24 hours, mice were imaged *in vivo* and afterwards anesthetized with Ketamine/Rompun. After heart puncture, the mice were euthanized, the organs harvested and returned to the

imaging chamber for *ex vivo* signal quantification by Live Image software (Perkin Elmer, Waltham, MA, USA). Organs were analyzed by region-of-interests (ROIs), and the fluorescence was quantified as the average of area-normalized radiant efficiency. Mice that received complete intravenous tail vein injection were used for quantification and statistical analysis.

Trimannosylation and Nanocarrier Loading: Detailed experimental including all precursor molecule synthesis can be found in the supporting information. For the optimized procedure for the SPAAC reaction to attach TM to HSA, DBCO-HSA (15.0 mg, 166 nmol, 1.00 eq.) was dissolved in 50 mM phosphate buffer with 8 M urea and 2 mM EDTA (pH = 8) to a final protein concentration of 1 mg/mL. The azide functionalized TM (14.1 mg, 17.9 μ mol, 115 eq. or 2.5 eq/DBCO) dissolved in MQ (10 mg/mL) was added and the reaction solution was shaken at room temperature overnight. Afterwards, the reaction solution was purified (5 x MQ) and concentrated *via* ultracentrifugation spin filter (Vivaspin, PES, 10 kDa). The product was lyophilized and obtained in form of a blue lyophilizate. For drug loading, e.g. TLR7/8a, TM-HSA (1.13 mg, 9.19 nmol, 1 eq.) was dissolved in denaturing buffer (50 mM phosphate buffer + 8 M urea + 2 mM EDTA, 1.00 mL) and inverted for 20 min before TCEP (263 μ g, 919 nmol, 100 eq.) dissolved in MQ (23 μ L) was added. The reaction solution was inverted for 1–2 hours and the residual TCEP was removed *via* ultracentrifugation spin filter (2 x denaturing buffer, Vivaspin, PES, 10 kDa). To the protein solution (1 mg/mL) Pyr-S-S-TLR7/8a (1.11 mg, 1.38 μ mol, 150 eq.) dissolved in DMF (105 μ L) was added. The reaction solution was inverted for 1 h and the buffer exchanged *via* ultracentrifugation (denaturing buffer) before reaction control *via* MALDI-ToF-MS to determine drug modification rate. *N*-Ethylmaleimide (173 μ g, 1.38 μ mol, 150 eq) was added to cap residual free thiols on the HSA for 14 h. The product was washed (5 x denaturing buffer) and backfolded *via* rapid dilution in MQ (1:10). If aggregation during washing steps occurred, the washing solutions were adjusted to pH = 6.3. The product was washed again (5 x MQ) and concentrated *via* ultracentrifugation spin filter (Vivaspin, PES, 10 kDa). The product was lyophilized and obtained in form of a blue lyophilizate with a modification rate of 20.5 TLR7/8a units per HSA.

Declaration of competing interests

BL, YZ, RF, SLK, MB, TO and TW are coinventors of a pending patent that claims to use human serum albumin based nanocarriers for targeted immunotherapy.

Supporting Information

Supplementary data to this article can be found online at [https://doi.org/\(\(will be filled in by the editorial staff\)\)](https://doi.org/((will be filled in by the editorial staff))).

Acknowledgements

The authors would like to thank the Max Planck Society and the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) project number 213555243 – SFB 1066 (Q05, B16, B05) for financial support, Prof. Jo A. Van Ginderachter for the MMR-positive CHO cells, Ingrid Tubbe and Nadine Röhrig for technical assistance in the biodistribution experiments and the MPI-P mass spectrometry department for measurements.

Received: ((will be filled in by the editorial staff))

Revised: ((will be filled in by the editorial staff))

Published online: ((will be filled in by the editorial staff))

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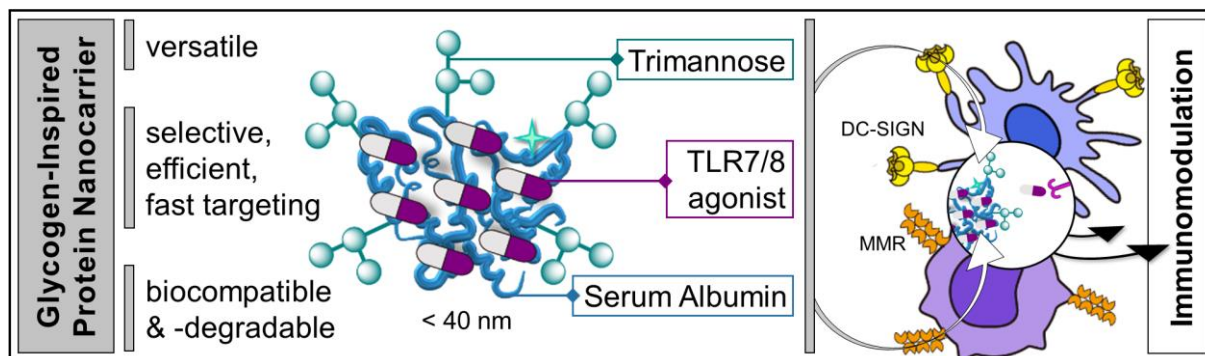
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Graphical abstract



Highlights

- A trimannose-HSA nanocarrier is designed for active targeting through DC-SIGN/MMR
- Redox-sensitive, traceless linkers enable precise control over drug release
- Cell-type specific delivery of TLR7/8 agonist is achieved *ex vivo*
- The bioconjugates demonstrate fast, efficient, and selective liver targeting *in vivo*
- The nanocarrier holds immense promise for targeted immunotherapy of liver diseases

6.5. Publication 5

“Transcriptional Targeting of Dendritic Cells Using an Optimized Human *Fascin1* Gene Promoter”

Published in: *International Journal of Molecular Sciences*. **2023** Nov 29;24(23):16938.

DOI: 10.3390/ijms242316938

Transcriptional targeting of dendritic cells (DCs) was used to enhance DNA vaccine efficacy. A plasmid (pFscnLuc) was constructed using an optimized *fascin1* promoter that includes a DC-specific enhancer region to ensure selective activity in DCs. Enhanced DC-focused activity of pFscnLuc was observed *in vitro* compared to a standard plasmid containing a cytomegalovirus promoter (pCMVLuc). *In vivo*, pFscnLuc showed favorable expression in the spleen, specifically in splenic DCs, indicating improved targeting of DCs. These findings suggest that using the *fascin* promoter for transcriptional targeting could be applied to develop more effective and selective DNA vaccines for cancer immunotherapy.

Contribution:

As first author of this paper, I performed most *in vitro* experiments and conducted the processing of the *in vivo* experiment. I also contributed to data analysis, visualization, manuscript draft preparation, as well as review and editing.

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Zeyn Y, Hobernik D, Wilk U, Pöhmerer J, Hieber C, Medina-Montano C, Röhrig N, Strähle CF, Thoma-Kress AK, Wagner E, Bros M, Berger S. Transcriptional Targeting of Dendritic Cells Using an Optimized Human *Fascin1* Gene Promoter. *Int J Mol Sci*. 2023 Nov 29;24(23):16938. doi: 10.3390/ijms242316938.



Communication

Transcriptional Targeting of Dendritic Cells Using an Optimized Human *Fascin1* Gene Promoter

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Abstract: Deeper knowledge about the role of the tumor microenvironment (TME) in cancer development and progression has resulted in new strategies such as gene-based cancer immunotherapy. Whereas some approaches focus on the expression of tumoricidal genes within the TME, DNA-based vaccines are intended to be expressed in antigen-presenting cells (e.g., dendritic cells, DCs) in secondary lymphoid organs, which in turn induce anti-tumor T cell responses. Besides effective delivery systems and the requirement of appropriate adjuvants, DNA vaccines themselves need to be optimized regarding efficacy and selectivity. In this work, the concept of DC-focused transcriptional targeting was tested by applying a plasmid encoding for the luciferase reporter gene under the control of a derivative of the human *fascin1* gene promoter (pFscnLuc), comprising the proximal core promoter fused to the normally more distantly located DC enhancer region. DC-focused activity of this reporter construct was confirmed in cell culture in comparison to a standard reporter vector encoding for luciferase under the control of the strong ubiquitously active cytomegalovirus promoter and enhancer (pCMVLuc). Both plasmids were also compared upon intravenous administration in mice. The organ- and cell type-specific expression profile of pFscnLuc versus pCMVLuc demonstrated favorable activity especially in the spleen as a central immune organ and within the spleen in DCs.

Keywords: *fascin1*; cytomegalovirus; promotor activity; plasmid DNA; gene delivery; polyplex; polyethylenimine; dendritic cell; tumor cell



Citation: Zeyn, Y.; Hobernik, D.; Wilk, U.; Pöhmerer, J.; Hieber, C.; Medina-Montano, C.; Röhrig, N.; Strähle, C.F.; Thoma-Kress, A.K.; Wagner, E.; et al. Transcriptional Targeting of Dendritic Cells Using an Optimized Human *Fascin1* Gene Promoter. *Int. J. Mol. Sci.* **2023**, *24*, 16938. <https://doi.org/10.3390/ijms242316938>

Academic Editor: Nikolai Petrovsky

Received: 27 October 2023

Revised: 21 November 2023

Accepted: 23 November 2023

Published: 29 November 2023



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1. Introduction

Based on a deeper understanding of the pathophysiology and the important role of immune dysfunction in cancer development and progression, nucleic acid-based immunotherapeutic approaches have attracted growing interest in recent years [1–15]. Hereby, the aims are (i) to modulate the immune system in such a way that it effectively fights the tumor and (ii) to create an immunological memory for long-term protection. DNA-based vaccination of dendritic cells (DCs) and other antigen-presenting cells (APCs) with transgenes that encode tumor-associated or -specific antigens and nucleic acid-based or encoded adjuvants aims to induce adaptive anti-tumor T cell immune responses [1]. So far, the overall efficacy of such DNA vaccines in clinical trials has been limited [1,16,17].

Their combination with other immunotherapies (e.g., check-point inhibitors, chimeric antigen receptor (CAR) T cells, or oncolytic viruses) or with conventional cancer treatments (e.g., chemotherapy, irradiation) may improve the clinical outcome [1]. Moreover, DNA vaccines can be optimized by (i) the careful choice of suitable antigens, (ii) the selection of appropriate adjuvants to stimulate APCs [18–24], (iii) the use of efficient delivery systems (e.g., nanoparticles like polyplexes [25–30]), and (iv) the structural optimization of the DNA itself. The latter can be achieved by using a strong APC-specific promoter that confines tumor antigen expression to APC. By this, unwanted gene expression in tumor-induced immune-regulatory cell types (e.g., tumor-associated macrophages or myeloid-derived suppressor cells), which in turn can induce tumor immune tolerance, may be prevented [1]. As DCs are the most potent T cell stimulatory APC population [31], they represent an ideal target for such an approach. Previously, the promoter of the murine *fascin1* (*Fscn1*) gene was identified to be suitable for transcriptional targeting of activated DCs [32]. *Fscn1*, a cytoskeletal actin-bundling protein, is evolutionarily highly conserved, expressed upon activation of DCs, and is relevant for their motility, migration, and T cell stimulatory activity, as recently reviewed [33]. *Fscn1* is also required by tumor cells for metastasis [34]. Therefore, *Fscn1* inhibitors have been developed and clinically tested for tumor therapy [35]. Interestingly, the pharmacological inhibition of *Fscn1* activity in tumor-bearing mice has been reported to promote anti-tumor activity also due to direct effects on intratumoral DCs [36]. Wang and co-worker demonstrated an inhibitor-dependent accumulation of DCs within the tumor that internalized more tumor antigen and upon co-treatment with the PD-1 checkpoint inhibition stimulated cytotoxic T lymphocytes (CTLs).

In the current work, we aimed to assess the suitability of the human *Fscn1* gene promoter [37] to achieve transcriptional targeting of *Fscn1*-expressing cell types with regard to the translational perspective of DNA-based vaccination in the future. The concept of DC-focused transcriptional targeting was tested in vitro and in vivo by using a plasmid encoding for the firefly luciferase reporter gene under the control of an optimized derivative of the human *Fscn1* gene promoter (pFscnLuc), in comparison to a standard firefly luciferase encoding plasmid under the control of the strong ubiquitously active cytomegalovirus promoter and enhancer (pCMVLuc). As effective non-viral plasmid DNA (pDNA) carriers in vitro and in vivo, linear polyethylenimine 22 kDa (LPEI) [38,39] as well as succinylated branched polyethylenimine 25 kDa (succPEI, 10% succinylation degree) [40] were used. Both cationic polymers are able to efficiently compact pDNA into nanoparticulate complexes (so-called polyplexes [25]) via electrostatic interactions. Altogether, our study demonstrated preferable DC-focused activity of pFscnLuc both in vitro and in vivo compared to the standard CMV promoter.

2. Results and Discussion

2.1. Firefly Luciferase Reporter Gene Construct under the Control of an Optimized Human *Fscn1* Gene Promoter

We have previously reported that both the murine [32,41] and human [37] *Fscn1* gene promoters allowed for transcriptional targeting of DCs. Subsequent studies demonstrated that plasmids containing the murine *Fscn1* promoter to drive antigen expression favorably stimulated a Th1-biased immune response [41–43]. With regard to subsequent in vivo studies in mice, we first assessed the activity of human *Fscn1* gene promoter reporter constructs in the murine DC-like cell line DC2.4 (Figure 1A). DC2.4 cells are known to be of mature phenotype [32,44] and thus are considered to express *Fscn1*, which was confirmed by us via intracellular *Fscn1* staining (Figure 2A and Figure S1). We have previously shown that a 1.6 kb fragment of the human *Fscn1* gene promoter (phF1.6) (Figure 1A) evoked stronger activity in human DCs than the 3.1 kb encompassing parental vector [37]. In murine DC2.4 cells, phF1.6 showed cross-species activity and exerted similar activity as the positive control (pGL3-Ctrl) (Figure 1B, left). Further, we have reported that DC-specific activity was mediated by an enhancer region located within a 170 bp sequence stretch at the 5'-end of phF1.6 (Figure 1A). When fusing this enhancer sequence with the core

promoter of the human *Fscn1* gene promoter as apparent in pHF0.21, the resulting construct pFscnLuc (previously described as pHF-TRR in [45]) exerted stronger transcriptional activity in DC2.4 cells than the parental constructs (2.5-fold higher compared to pHF1.6; 5-fold higher compared to pHF0.21). This enhancer/core promoter hybrid retained DC-focused transcriptional activity as observed in *Fscn1*-negative cell lines (i.e., macrophage-like P388 cells (Figure 1B, middle) and B cell-like A20 cells (Figure 1B, right)), yielding only about double activity as the negative control (pGL3-Basic) in either case.

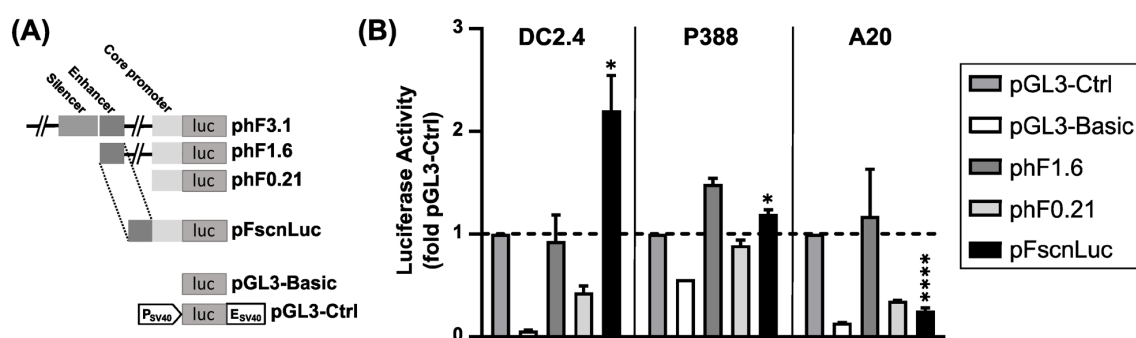


Figure 1. Activity of human *Fscn1* promoter reporter constructs. (A) Scheme of human *Fscn1* gene promoter luciferase reporter constructs (pHF) derived from parental pHF3.1 in comparison to control vectors pGL3-Basic (promoter-less) and pGL3-Ctrl (hybrid SV40 promoter/enhancer). (B) DC-like DC2.4 cells, macrophage-like P388 cells, and B cell-like A20 cells (all of murine origin; each 20,000 cells/well) were transfected with *Fscn1* promoter firefly luciferase reporter constructs as indicated and control vectors (pGL3-Basic, pGL3-Ctrl) at equimolar amounts normalized to 950 ng of the largest plasmid, respectively, and a constant amount of 50 ng of a renilla luciferase encoding control vector (pRL-EF1 α [37]) using the transfection reagent JetPEI according to the manufacturer's protocol (Polyplus, Illkirch, France). On the next day, firefly luciferase activities were determined. Data denote the relative luciferase activity (fold pGL3-Ctrl; $n = 4$; mean + SEM). Significant differences versus pGL3-Ctrl: * $p \leq 0.05$; **** $p \leq 0.0001$ (unpaired Student's two-tailed t -test with Welch's correction; GraphPad PrismTM 9.5.1).

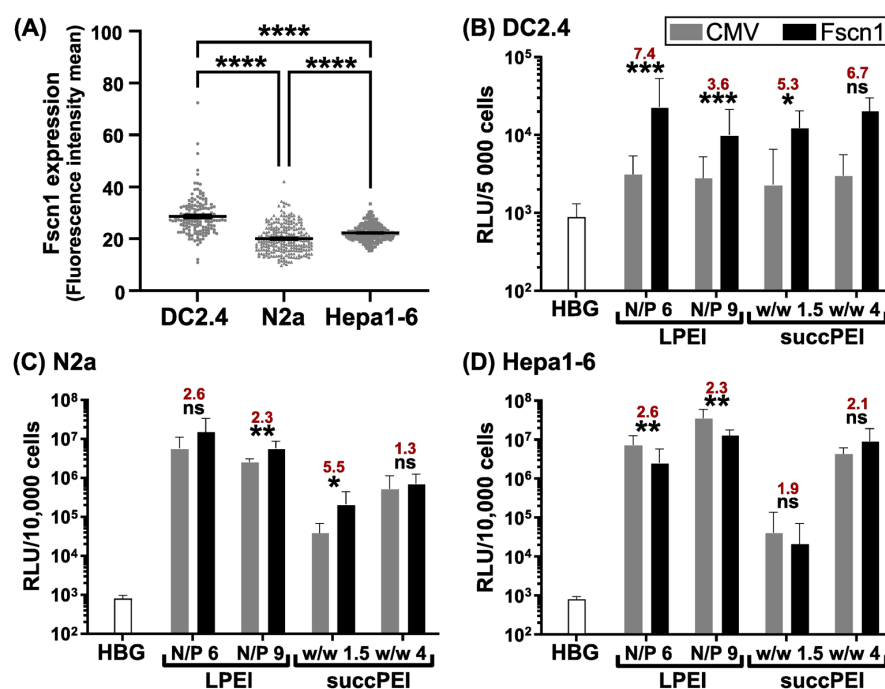


Figure 2. Quantification of *Fscn1* levels and promoter-dependent reporter activities in DC-like (DC2.4) and tumor (N2a, Hepa1-6) cell lines (all of murine origin). (A) Data denote the corresponding

fluorescence intensities per cell and the mean $\pm$ SEM of 154–301 cells per group after CLSM imaging. Corresponding cytospin images are displayed in Figure S1. (B–D) Luciferase gene expression ($n = 3$; mean $\pm$ SD) was assayed in comparison to HBG buffer-treated control cells at 24 h after transfection with LPEI (N/P 6 and 9, resp.) and succPEI (w/w 1.5 and 4, resp.) polyplexes containing pFscnLuc or pCMVLuc, resp., in the DC-like cell line DC2.4 (B) at a pDNA concentration of 500 ng/well, as well as in the neuroblastoma cell line N2a (C) and the hepatoma cell line Hepa1-6 (D) at a pDNA concentration of 200 ng/well. Red numbers indicate the fold difference in RLU values between pFscnLuc and pCMVLuc. Note: For succPEI, w/w ratios of 1.5 and 4 represent N/P ratios of ~ 11.25 and ~ 30 of an unsubstituted PEI. (A–D) Significance levels are indicated as follows: (A) **** $p \leq 0.0001$ (one-way ANOVA, Tukey test, GraphPad PrismTM 9.5.1); (B–D) significant differences between both plasmids: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; ns, not significant (unpaired Student's two-tailed t -test with Welch's correction; GraphPad PrismTM 9.5.1).

In the following, the optimized human *Fscn1* gene promoter was evaluated in comparison to the standard CMV promoter in terms of its transcriptional activity in vitro and in vivo.

2.2. Tumor Cell Lines Express *Fscn1* at Lower Levels Than DCs

Under homeostatic conditions, *Fscn1* expression is mainly restricted to neuronal/glia cells and activated DCs, mediating membrane protrusions and the formation of filopodial extensions [46–48]. As mentioned above, the de novo expression of *Fscn1* is also often found in tumor cells and correlates with cancer aggressiveness by promoting cancer progression, migration, and metastasis [34,46].

To prove that the murine neuroblastoma cell line N2a and the murine hepatoma cell line Hepa1-6 express *Fscn1* and hence are suitable for pFscnLuc transfections, intracellular *Fscn1* detection was conducted on cytospun cells. CLSM imaging revealed that both tumor cell lines indeed express *Fscn1* at a certain yet lower extent compared to DC2.4 cells (Figure 2A and Figure S1), and thus can be used for further evaluation of the human *Fscn1* promoter.

2.3. PEI Derivatives Are Suitable Carriers for pDNA Delivery

For the comparison of the optimized human *Fscn1* promoter with the standard CMV promoter in vitro and in vivo, LPEI [38,39,49] as the established gold standard for pDNA transfections as well as succPEI [40] were selected as pDNA carriers. Both cationic polymers were able to compact either plasmid at in vitro and in vivo concentrations into well-defined, homogenous nanoparticles by electrostatic interactions with hydrodynamic diameters (z-average) between 55 and 140 nm, polydispersity indices (PDI) below 0.3, and a positive surface charge (zeta-potential ranging from +10 to +30 mV) (Table S1).

Despite the potent transfection capability of PEI, its multi-faceted cytotoxicity can be an issue [49]. Furthermore, PEI-mediated effects on the immune system such as complement activation [50] or interactions with Toll-like receptors (TLRs) [51–53] have been reported, mediating either immune toxicity or immune stimulation [49]. Lowering the N/P (nitrogen-to-phosphate) ratio of LPEI pDNA complexes from highly effective N/P 9–10 [38,54–56] to N/P 6 [39] may be a compromise between efficacy and toxicity. Another option to reduce toxicity could be the surface modification of PEI [49] as for example demonstrated by the succinylation of branched PEI (succPEI) [40].

In the following, LPEI and succPEI were tested regarding their possible toxicity. The viability of DC-like DC2.4 cells treated overnight with LPEI and succPEI complexed with pFscnLuc at different N/P (LPEI; N/P 6 and 9, resp.) or w/w (weight-to-weight) ratios (succPEI; w/w 1.5 and 4, resp.) was moderately ($\sim 35\%$) reduced for LPEI but not for succPEI compared to untreated control cells (Figure S2A). In agreement, DC2.4 cells showed reduced metabolic activity in response to overnight incubation with either polyplex formulation in a plasmid-independent manner, particularly at higher carrier amounts (LPEI N/P 9, succPEI

w/w 4) (Figure S2B). In contrast, we observed no carrier- or plasmid-dependent effects on the metabolic activity of the two *Fscn1*-expressing tumor cell lines (Figure S2C,D).

2.4. The Optimized Human *Fscn1* Promoter Confers Enhanced Reporter Gene Expression in *Fscn1*-Expressing Cell Lines

The cell-type-specific activity of pFscnLuc in comparison to standard plasmid pCMVLuc [57] was evaluated in the different *Fscn1*-expressing cell lines (see Figure 2A and Figure S1). In parallel assays, LPEI [49,54] as well as succPEI [40] were used as potent transfection agents at the indicated N/P (LPEI; N/P 6 and 9, resp.) or *w/w* ratios (succPEI; *w/w* 1.5 and 4, resp.). As depicted in Figure 2B, the DC-focused activity of pFscnLuc was confirmed for all four formulations since corresponding polyplexes mediated up to 7.4-fold higher RLU values in DC2.4 cells than polyplexes formed with the standard plasmid pCMVLuc. In contrast to DC2.4 cells, the preference for the *Fscn1* promoter plasmid was less pronounced in the neuroblastoma cell line N2a (Figure 2C) and in the hepatoma cell line Hepa1-6 (Figure 2D). This is in line with the lower *Fscn1* expression level compared to DC2.4 cells (Figure 2A and Figure S1). Yet, pFscnLuc was expressed in both tumor cell lines at almost similar luciferase expression levels as the standard plasmid pCMVLuc (Figure 2C,D). Altogether, these findings suggest on the one hand that the optimized *Fscn1* promoter was suitable to drive stronger transgene expression in DCs as mediated by the standard CMV promoter/enhancer, allowing for DC-focused DNA vaccination. On the other hand, the observation that the *Fscn1* promoter was also highly active in tumor cells may pave the way to employ DNA therapeutics that (co-)target tumor cells. For example, transgenes under the control of the *Fscn1* promoter encoding for anti-tumor cytokines (e.g., tumor necrosis factor (TNF)- α) can be used to transcriptionally address tumor cells, leading to apoptosis [1,58–61]. Hence, one could envision a DNA vaccine which encodes for a tumor antigen and at the same time for TNF- α , driving the activation of transfected DCs and at the same time exerting tumoricidal effects in the case of transfected tumor cells.

2.5. The Optimized Human *Fscn1* Promoter Mediates Preferential Reporter Expression in DCs also In Vivo

2.5.1. LPEI at Higher N/P Ratio Exerts Moderate Toxicity and Activation of DCs at the Same Time

In light of the potential cytotoxicity of LPEI polyplexes on DC2.4 cells, we tested for the corresponding effects of LPEI and succPEI also on bone marrow-derived (BM) DCs. Besides viability, also the expression of activation markers comprising MHCII (major histocompatibility complex) for antigen-presentation and CD80 as well as CD86 as costimulatory receptors for antigen-specific T cell activation [62–64] was analyzed. Despite the far higher carrier amounts (*w/w* ratios of 1.5 and 4 represent N/P ratios of ~11.25 and ~30), succPEI polyplexes were well tolerated by BMDCs, whereas LPEI especially at an N/P ratio of 9 moderately reduced their viability by around 30% (Figure S3A). Nevertheless, in contrast to succPEI, LPEI also enhanced the expression of CD86 by means of the viable BMDC (at N/P 9, 1.5-fold increase compared to untreated control cells) (Figure S3D), whereas the MHCII and CD80 expression levels remained largely unaffected (Figure S3B,C). This effect may be due to an intrinsic activating potential of the LPEI polyplexes. In this regard, it has been reported that PEI stimulated DCs via TLR5 [52] and macrophages via TLR4 [51,53]. Further, it cannot be ruled out that necrotic cells within the culture released endogenous danger factors like HMGB1 (high mobility group box-1 protein), which binds to TLR4 as well [65], thereby triggering DC activation.

Due to these findings, the next isolated murine primary splenic immune cells were evaluated in a pre-experiment for subsequent in vivo studies. Splenic immune cell types were distinguished based on the expression of the corresponding lineage surface markers. In accordance with moderate cytotoxicity of LPEI polyplexes at N/P 9 in DC2.4 cells (Figure S2A,B) and BMDCs (Figure S3A), we observed a decrease in the viability of primary DCs by around 30% as compared to untreated control cells (Figure 3A). On the contrary, LPEI polyplexes at lower N/P ratio and succPEI polyplexes in general had no significant

effect on DC viability. Opposed to DCs, the viability of other splenic immune cell types was not significantly affected by either polyplex as assessed for macrophages (MAC), polymorphonuclear neutrophils (PMN), B cells and natural killer cells (NK) (Figure 3A).

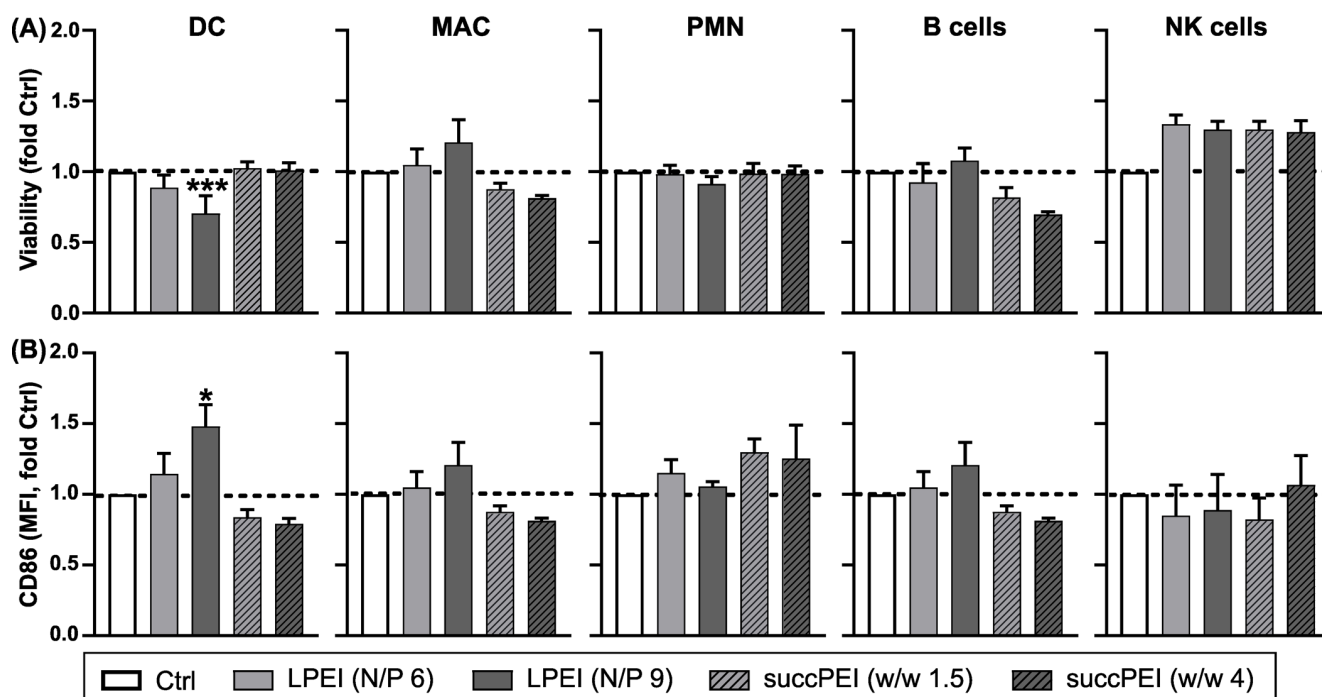


Figure 3. Effect of LPEI and succPEI polyplexes on the viability and activation of splenic immune cells. Splenic immune cells (2×10^6 cells per well) were incubated with LPEI and succPEI polyplexes at the indicated ratios using $1 \mu\text{g}$ pFscnLuc/well. On the next day, the viability (A) and CD86 expression (B) of leukocyte populations was assessed by flow cytometry. Splenic immune cell populations were delineated by sequential gating, as described in Figure S5. Graphs denote (A) the frequencies of FVD (fixable viability dye)-negative viable cells normalized to the value of untreated cells (Ctrl) ($n = 4$; mean + SEM) and (B) the MFI (mean fluorescence intensity) of CD86 expression in relation to the according expression level of untreated cells (Ctrl) (each $n = 4$; mean + SEM). (A,B) Significant differences versus Ctrl: * $p \leq 0.05$; *** $p \leq 0.001$ (one-way ANOVA, Tukey test; GraphPad PrismTM 9.5.1). DC, dendritic cells; MAC, macrophages; PMN, polymorphonuclear cells. NK, natural killer cells. Note: For succPEI, w/w ratios of 1.5 and 4 represent N/P ratios of ~11.25 and ~30 of an unsubstituted PEI.

In accordance with our observations on BMDCs, LPEI polyplexes at an N/P ratio of 9 conferred a significant increase (~1.5-fold compared to untreated control cells) in CD86 activation marker expression on splenic DCs (Figure 3B). Since only DCs were activated, it is more likely that LPEI at N/P 9 exerted intrinsic stimulatory activity since a release of danger signals by necrotic cells may have broadly activated most of the monitored cell types.

2.5.2. The Optimized Human Fscn1 Gene Promoter Evokes Reporter Gene Expression Preferably in Spleens

The performance of the *Fscn1* and the CMV promoter was compared also in vivo by the intravenous administration of corresponding LPEI and succPEI pDNA polyplexes in BALB/c mice. Despite showing moderate toxicity in DCs (see Sections 2.3 and 2.5.1), LPEI polyplexes at an N/P ratio of 9 were considered as suitable for subsequent in vivo evaluation also with regard to their presumably intrinsic immunostimulatory potential. It is known from the literature that LPEI at N/P ratios of 9–10 is highly active in vivo

and mediates only minor, if any, toxic effects [38,55,56]. For reasons of comparability and based on preliminary in vivo results (unpublished data), a *w/w* ratio of 1.5 was chosen for succPEI polyplexes, which corresponds to an N/P ratio of ~11.25 for unsubstituted PEI. The two carriers promoted different luciferase expression profiles (Figure 4A, Table S2) with lower overall luciferase expression levels for the succPEI group (esp. in the lung; 210-fold for CMV, 22-fold for *Fscn1*). LPEI showed a strong preference for confer luciferase reporter expression in the lungs. In the LPEI group, the CMV promoter was ubiquitously active at a high level. In contrast, the *Fscn1* promoter mediated an overall lower luciferase expression, especially in the lungs and liver (10-fold less RLU), but conferred luciferase expression in the spleen as high as that observed for the CMV promoter. This indicates a clear shift towards expression in the spleen for the *Fscn1* promoter construct, directing expression

containing high numbers of APCs. In the case of succPEI polyplexes, the *Fscn1* promoter favored reporter gene expression (12.6-fold higher RLU compared to CMV promoter) in the spleen. RLU levels in the lungs and liver remained constant, suggesting a preferable shift towards spleen expression mediated by the *Fscn1* promoter. Spleen/lung and spleen/liver luciferase expression ratios are expected to be higher in the LPEI group since in both the LPEI and succPEI group (Table S3).

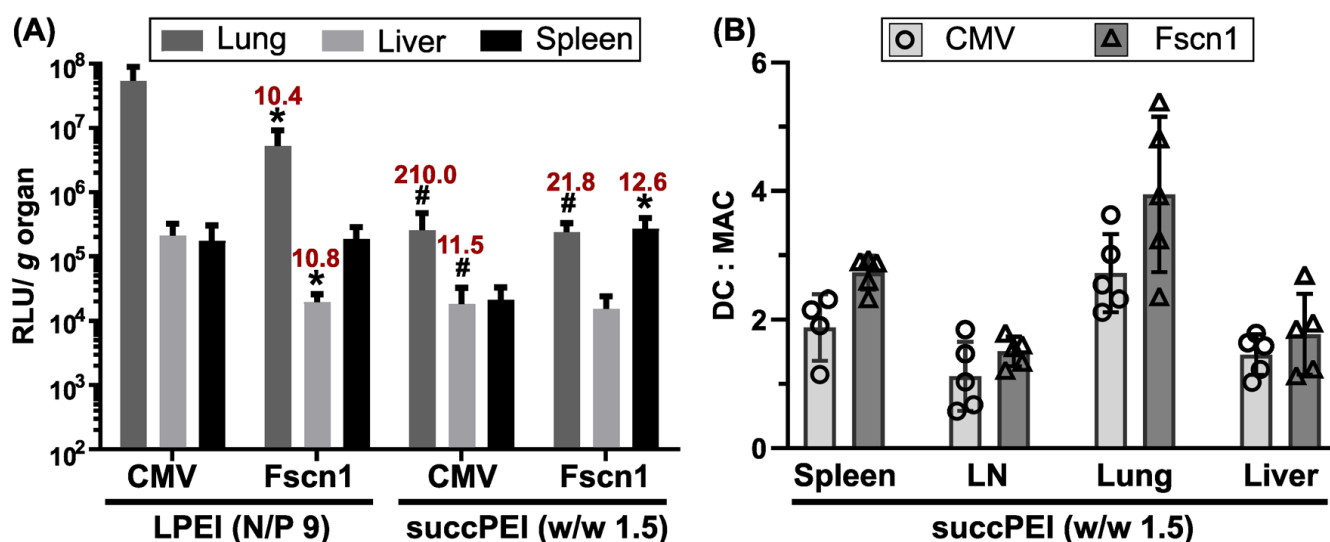


Figure 4. In vivo comparison of *Fscn1* and CMV promoter activities in BALB/c mice. **(A)** Luciferase expression in organs ($n = 5$; mean $\pm$ SD) assessed via an ex vivo luciferase assay at 24 h after intravenous injection of LPEI (N/P 9) and succPEI (*w/w* 1.5) polyplexes, resp., formed at a pDNA dose of 60 μ g/animal in 200 μ L of HBG buffer. Significant differences between pCMV and p*Fscn1* within the LPEI and succPEI groups, resp., $p < 0.05$ (unpaired Student's *t*-test with Welch's correction; GraphPad PrismTM 9.5.1). Red numbers indicate the fold difference in RLU values between the compared groups of the significance tests. For clarity, all significance levels and fold changes are displayed in Table S2. **(B)** Comparison of *Fscn1* and CMV promoter activities on the single cell level. Ex vivo analysis of single cell suspensions retrieved from different organs at 24 h after the intravenous injection of 200 μ L of succPEI polyplexes (*w/w* 1.5), containing 60 μ g pDNA. Cells were isolated and subjected to flow cytometric analyses. Gating strategies for the cell suspensions retrieved from the distinct organs are pictured in Figures S5 and S6. Ratios of luciferase-expressing DCs (dendritic cells) to MACs (macrophages) of $n = 5$ are displayed. The ratio calculation is based on the data shown in Figure S4. LN, lymph nodes.

2.5.3. The Optimized Human *Fscn1* Gene Promoter Shows DC-Focused Activity In Vivo

In addition, promoter-dependent reporter expression activity was examined in single-cell suspensions retrieved from the spleen, lymph nodes, liver, and lungs of accordingly treated BALB/c mice via intracellular immunostaining with a luciferase-specific

2.5.3. The Optimized Human *Fscn1* Gene Promoter Shows DC-Focused Activity In Vivo

In addition, promoter-dependent reporter expression activity was examined in single-cell suspensions retrieved from the spleen, lymph nodes, liver, and lungs of accordingly treated BALB/c mice via intracellular immunostaining with a luciferase-specific antibody (Figure 4B and Figure S4). succPEI was used as carrier for this evaluation, since it showed a more selective spleen activity compared to LPEI in the ex vivo luciferase assay (Figure 4A, Tables S2 and S3). Moreover, we observed that succPEI-complexed pFscnLuc conferred a significantly stronger splenic luciferase expression than succPEI/pCMVLuc transfection complexes (12.6-fold increase, p value = 0.0102; Table S2). pFscnLuc expression was determined in DCs alongside macrophages, both representing APC-like cell lines, and detected in DC populations of all tested organs (Figure S4). Standard plasmid pCMVLuc was also highly expressed due to the strong and ubiquitously active CMV promoter, not in favor of a real benefit of pFscnLuc at first glance. However, a comparison of calculated ratios of luciferase expressing DC/MAC in the single organs revealed a slightly better performance of pFscnLuc with preferential luciferase expression in DCs, particularly in the spleen and lymph nodes (Figure 4B). For example, the DC:MAC ratio in the spleen was approx. 2.7 for pFscnLuc compared to approx. 1.9 for pCMVLuc.

3. Materials and Methods

3.1. Materials

3.1.1. Plasmids

Plasmids pCMVLuc [57] (encoding *Photinus pyralis* firefly luciferase under control of cytomegalovirus promotor and enhancer) and pFscnLuc (described as pHF-TRR in [45]; encoding *Photinus pyralis* firefly luciferase under the control of a derivative of the human *Fscn1* gene promotor) were obtained from Plasmid Factory GmbH (Bielefeld, Germany). Promoter-less expression vector pGL3-Basic (promoter-less expression vector encoding for *Photinus pyralis* firefly luciferase), and pGL3-Control (encoding *Photinus pyralis* firefly luciferase under the control of the hybrid SV40 promotor/enhancer) (all from Promega, Mannheim, Germany) were obtained from Plasmid Factory GmbH (Bielefeld, Germany) as endotoxin-free preparations. The *Renilla reniformis* luciferase encoding vector pRL-EF1 α [37] was amplified using the Endofree Plasmid Maxi Kit (Qiagen, Hilden, Germany).

3.1.2. Chemicals

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was obtained from Biomol (Hamburg, Germany), disodium ethylenediaminetetraacetic acid (EDTA) as well as glucose from Merck (Darmstadt, Germany). Cell culture consumables were obtained from Faust Lab Science (Klettgau, Germany) or Greiner Bio-One (Frickenhausen, Germany). Cell culture media, bovine serum albumin (BSA), fetal bovine serum (FBS), antibiotics, and trypsin/EDTA were purchased from Sigma Aldrich (Munich, Germany) and PAN-Biotech (Aidenbach, Germany); β -mercaptoethanol was obtained from Roth (Karlsruhe, Germany). Cell culture 5 $\times$ lysis buffer, CellTiter-Glo Reagent, and D-luciferin sodium salt were obtained from Promega (Mannheim, Germany); dithiothreitol (DTT), adenosine 5'-triphosphate (ATP) disodium salt trihydrate, coenzyme A trilithium salt, and protease and phosphatase inhibitor cocktail were from Sigma-Aldrich (Munich, Germany). In the case of co-transfection using firefly luciferase and renilla luciferase encoding vectors, luciferase activities were determined using the Dual-luciferase reporter assay kit (Promega, Mannheim, Germany).

3.1.3. Transfection Agents

Succinylated branched PEI 25 kDa (succPEI; succinylation degree of 10%) and unmodified linear PEI 22 kDa (LPEI) were synthesized and analyzed as described previously [40,66]. The starting products branched PEI (brPEI) 25 kDa as well as poly(2-ethyl-2-oxazoline) were purchased from Sigma Aldrich (Munich, Germany). The transfection reagent JetPEI (linear PEI) was obtained from Polyplus (Illkirch, France).

3.1.4. Cell Lines

The murine neuroblastoma cell line Neuro2a (N2a; Cellosaurus database entry: https://www.cellosaurus.org/CVCL_0470; accessed on 30 October 2023), the murine macrophage-like cell line P388 (Cellosaurus database entry: https://www.cellosaurus.org/CVCL_7222; accessed on 30 October 2023), and the murine B cell-like cell line A20 (Cellosaurus database entry: https://www.cellosaurus.org/CVCL_1940; accessed on 30 October 2023) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The murine hepatoma cell line Hepa1-6 (Cellosaurus database entry: https://www.cellosaurus.org/CVCL_0327; accessed on 30 October 2023) was obtained from ATCC (Gaithersburg, MD, USA), and the murine DC-like cell line DC2.4 (Cellosaurus database entry: https://www.cellosaurus.org/CVCL_J409; accessed on 30 October 2023) was from Merck Millipore (Darmstadt, Germany).

3.1.5. Antibodies

PE-/APC- and PE-Cy7-labeled CD11c-specific (clone N418), FITC-CD86 (GL-1), SB600-CD11b (M1/70), PE-NK1.1 (PK136), PE-eFl610-Ly6G (1A8-L6g), V500-B220 (RA3-6B2), SB702-CD45 (30-F11), eFl506- or FITC-CD3 (17-A2), AF647-Luciferase (EPR17789), eFl450-F4/80 (BM8), AF488-CD19 (6D5), AF647-XCR1 (ZET), PE-Cy7-CD172a (P84), and PE-CD32b (AT130-2) antibodies were used for flow cytometric analysis (FACS). FACS antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA), BioLegend (San Diego, CA, USA), or ThermoFisher (Waltham, MA, USA). eFl780-FVD (fixable viability dye) used to identify dead cells was obtained from ThermoFisher. Unlabeled mouse anti-human Fcsc1 antibody (55K2; Sigma-Aldrich, Munich, Germany), a corresponding isotype control antibody (mouse IgG1, clone MOPC-21, BioLegend), and secondary AF488-labeled IgG goat anti-mouse antibody (948492; ThermoFisher) were used for confocal laser scanning analysis (CLSM).

3.2. pDNA Polyplex Formation

pDNA and calculated amounts of LPEI at indicated N/P (nitrogen/phosphate) ratios or of succPEI at indicated *w/w* (weight/weight) ratios, respectively, were diluted with HBG (HEPES buffer with glucose; 20 mM of HEPES, 5% (*w/v*) glucose; pH 7.4) in separate vials at equal volumes. pDNA and LPEI or succPEI solutions were mixed by rapid pipetting and incubated for 30 min at room temperature (RT) in a closed vial.

Note: In the case of succPEI, the N/P ratio was considered to be not suitable for calculation of the amounts of succPEI to pDNA since some amino groups are substituted with succinic acid and thus not involved in nucleic acid binding. Therefore, the *w/w* ratio was used instead of the commonly used N/P ratio [40]. A *w/w* ratio of 1 corresponds to an N/P ratio of ~7.5 for unsubstituted PEI.

3.3. Physico-Chemical Characterization of pDNA Polyplexes

Polyplexes were formulated in HBG buffer as described above (see Section 3.2.) at pCMVLuc concentrations used for the in vitro experiments (i.e., 10 µg mL⁻¹ in the case of transfections in tumor cell lines N2a and Hepa1-6; 25 µg mL⁻¹ in the case of transfections in the DC-like cell line DC2.4) and for the in vivo studies (i.e., 300 µg mL⁻¹). Measurements of size and zeta-potential were performed by dynamic and electrophoretic laser-light scattering (DLS, ELS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, Worcestershire, UK) in a folded capillary cell (DTS1070). Size and polydispersity index were measured in 100 µL polyplex solution using the following instrument settings: equilibration time 30 s, temperature 25 °C, refractive index 1.330, and viscosity 0.8872 mPa·s. Samples were measured three times with six sub runs per measurement. For measurement of the zeta-potential, all samples were diluted to 800 µL with HBG directly before measurement. Parameters were identical to the size measurement apart from an equilibration time of 60 s. Three measurements with 15 sub runs lasting 10 s each were performed, and zeta-potentials were calculated with the Smoluchowski equation.

3.4. Cell Culture

Immortalized cell lines. N2a and Hepa1-6 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (1 g L^{-1} glucose) supplemented with 10% (*v/v*) FBS, 4 mM of stable glutamine, 100 U mL^{-1} of penicillin, and $100 \text{ } \mu\text{g mL}^{-1}$ of streptomycin. The DC2.4 cell line was grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% (*v/v*) FBS, 4 mM of stable L-glutamine, 100 U mL^{-1} of penicillin, $100 \text{ } \mu\text{g mL}^{-1}$ of streptomycin, and $50 \text{ } \mu\text{M}$ of β -mercaptoethanol. Bone marrow-derived cells (BMDCs, $2 \times 10^5 \text{ mL}^{-1}$) were seeded in 12-well plates (Greiner Bio-One, Frickenhausen, Germany) in an IMDM-based culture medium (see above) including recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng mL^{-1} ; Miltenyi Biotec, Bergisch Gladbach, Germany) to differentiate DCs. DC culture media were replenished on days three and six of culture. Cells were cultured at 37°C and either 5% (cell lines) or 10% (BMDC) CO_2 in an incubator with a relative humidity of 95%.

3.5. Confocal Laser Scanning Microscopy (CLSM)

Cytospins were generated, and *Fscn1* expression was detected by CLSM, as described previously [46]. In brief, cells were re-suspended ($5 \times 10^5 \text{ mL}^{-1}$) in PBS (phosphate-buffered saline; 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , and 1.5 mM KH_2PO_4). Sets of 50,000 cells were cytopun (500 rpm, 5 min, RT) onto microscope slides (Superfrost Plus; VWR, Darmstadt, Germany) using a Cytospin 3 (ThermoFisher, Waltham, MA, USA), air-dried overnight, and stored at -20°C . For staining, cytopins were incubated first with pre-cooled methanol (Roth, Karlsruhe, Germany) for 10 min to permeabilize the cell membrane and washed twice with PBS. During all subsequent incubation steps, samples were kept in a humidified chamber. Cytospins were incubated with a Fc receptor blocking antibody (clone 2.4-G2; 1:50-diluted in PBS/2% (*v/v*) BSA) for 10 min at RT. Then, an *Fscn1*-specific or isotype control antibody (each 1:50-diluted in PBS/2% (*v/v*) FBS) was added, and samples were incubated for 20 min at RT. Afterwards, samples were washed and incubated with AF488-labeled secondary anti-mouse antibody (1:400-diluted in PBS/2% (*v/v*) FBS) for 20 min at RT. After two washing steps with PBS, HCS Cell Mask Orange ($2 \text{ } \mu\text{g mL}^{-1}$, ThermoFisher, Waltham, MA, USA) was applied and samples were incubated for 30 min and finally washed with water. As a control, samples were left untreated or incubated with one agent only, respectively. All samples were covered with fluorescence mounting medium (DAKO; Agilent, Santa Clara, CA, USA) and a coverslip. For CLSM, samples were imaged using a Leica SP8 confocal microscope (Mannheim, Germany) equipped with a 20/0.75 NA air objective using a 405 nm laser for transmission images, a 488 nm laser for AF488 (*Fscn1*) excitation (emission and detection within a spectral window of 499–581 nm), and 552 nm laser exposure for AF555 (HCS Cell Mask Orange) excitation (emission and detection within a spectral window of 562–632 nm). Cellular *Fscn1* intensities were calculated using Imaris software version 9.3.1 (Bitplane, Zurich, Switzerland).

3.6. In Vitro Transfection and Luciferase Detection

One day prior to transfection, 10,000 N2a, 10,000 Hepa1-6, or 5000 DC2.4 cells were seeded per well into 96-well plates. For transfection, 200 ng (N2a, Hepa1-6) or 500 ng (DC2.4) pDNA per well were applied, respectively. HBG served as a negative control. All transfection experiments were performed in biological triplicates. The medium was replaced with 80 μL of fresh medium containing 10% (*v/v*) FBS, and polyplexes formed at indicated N/P (LPEI) or *w/w* (succPEI) ratios in 20 μL HBG, as described above (see Section 3.2.), were added to each well. The plates were incubated at 37°C and 5% CO_2 without a change in medium. After 24 h of incubation, the medium was removed, cells were lysed with 100 μL of cell culture $0.5\times$ lysis buffer (Promega, Mannheim, Germany), and the samples were frozen at -80°C at least overnight. Prior to measurement of the luciferase activity, the plates were equilibrated for 1 h at RT. Luciferase activity in 35 μL of the cell lysate was measured for 10 s by using a Centro LB 960 plate reader luminometer (Berthold Technologies, Bad Wildbad, Germany) after the addition of a 100 μL LAR buffer

(20 mM glycylglycine; 1 mM MgCl_2 ; 0.1 mM EDTA; 3.3 mM DTT; 0.55 mM ATP; 0.27 mM coenzyme A, pH 8–8.5), supplemented with 5% (*v/v*) of a mixture of 10 mM luciferin and 29 mM glycylglycine. Transfection efficiency was calculated for the seeded number of cells and presented as relative light units (RLU) per well.

In some experiments (results displayed in Figure 1), cells (DC2.4, P388, A20; each 20,000 per well in a 24-well plate) were co-transfected with firefly (950 ng of largest vector and equimolar amounts of smaller vector) and renilla luciferase (50 ng) encoding vectors using JetPEI (Polyplus, Illkirch, France), as recommended by the manufacturer. On the next day, plates were centrifuged, media were removed, and 100 μL of Passive Lysis Buffer (Promega, Mannheim, Germany) was applied to each well. In total, 10 μL of a cell lysate were assayed for luciferase activities by subsequently applying firefly and renilla substrate as recommended by the manufacturer (Promega, Mannheim, Germany) and measuring luciferase activities every 5 s. Firefly luciferase activities were divided by renilla luciferase activities to account for differences in transfection efficacy.

3.7. Metabolic Activity of Transfected Cells

Cell transfections were performed as described in Section 3.6. The supernatant was removed at 24 h after transfection, and 25 μL of medium as well as 25 μL of CellTiter-Glo Reagent were added to each well. After incubation on an orbital shaker for 30 min at RT, luminescence was recorded using a Centro LB 960 plate reader luminometer (Berthold Technologies, Bad Wildbad, Germany). The luminescent signals (in RLU) of the samples were set in relation to the luminescent signal of the negative control (HBG buffer-treated control cells). Results are presented as relative metabolic activity related to the negative control. Experiments were performed in biological triplicate.

3.8. In Vivo Application of pDNA Polyplexes

In vivo experiments were performed according to the guidelines of the German Animal Welfare Act and were approved by the animal experiments ethical committee of the Government of Upper Bavaria (accreditation number Gz. ROB-55.2-2532.Vet_02-19-19). Six-week-old female BALB/c mice (Janvier Labs, Le Genest-Saint-Isle, France) were randomly divided into groups of five. The mice were housed in isolated ventilated cages under specific pathogen-free conditions with a 12 h day/night interval, and food and water ad libitum. Weight and general well-being were monitored continuously. The experiments were performed by intravenous tail vein injection of polyplexes formed at indicated N/P (LPEI) or *w/w* (succPEI) ratios, as described above (see Section 3.2), respectively, each containing 60 μg of pCMVLuc or pFscnLuc in 200 μL HBG. Mice were euthanized at 24 h after injection. The organs were dissected and washed carefully with PBS, followed by analysis via an ex vivo luciferase gene expression assay of the lungs, liver, and spleen (see Section 3.9) as well as an immunostaining of single cell suspensions retrieved from dissected organs (lungs, liver, spleen, and lymph nodes; see Sections 3.10 and 3.11).

3.9. Ex vivo Luciferase Gene Expression Assay after In Vivo Transfection

For the detection of luciferase activity, organs were homogenized in Luciferase Cell Culture Lysis Reagent 1 $\times$ (Promega, Mannheim, Germany), supplemented with 1% (*v/v*) protease and phosphatase inhibitor cocktail (Sigma-Aldrich, Munich, Germany) using a tissue and cell homogenizer (FastPrep[®]-24, MP Biomedicals, Santa Ana, CA, USA). Then, the samples were frozen overnight at $-80\text{ }^{\circ}\text{C}$ to ensure full lysis. The samples were thawed and centrifuged for 10 min at maximum speed ($\sim 13,000\text{ rpm}$) at $4\text{ }^{\circ}\text{C}$. Luciferase activity in 25 μL supernatant was measured for 10 s, as described above (see Section 3.6). The luciferase expression is presented as RLU per gram (g) of organ after background subtraction (lysis buffer).

3.10. Single Cell Preparation after In Vivo Transfection

Spleens and lymph nodes were meshed using a pestle and a 40 μ M cell strainer (Greiner Bio-One, Frickenhausen, Germany) to yield a single-cell suspension. Spleen cells (2×10^6 in 500 μ L) were kept in FACS tubes overnight in a medium (IMDM, containing 5% (v/v) FBS, 2 mM of L-glutamine, 100 U mL⁻¹ of penicillin, 100 μ g mL⁻¹ of streptomycin, and 50 μ M β -mercaptoethanol). Murine liver non-parenchymal cells (NPCs) were enriched by liver dissociation, as described previously [67]. In brief, an enzymatic dissociation mixture (Liver Dissociation Kit; Miltenyi Biotec, Bergisch-Gladbach, Germany) was pre-incubated for 15 min in a water bath at 37 °C. Then, the liver tissue was dissected in little pieces in the dissociation mixture, subsequently transferred into prepared C tubes (Miltenyi Biotec). The C tubes were placed into a gentleMACS Dissociator to be minced (program m_liver_03; Miltenyi Biotec). The derived cell suspension was incubated under continuous shaking for 30 min at 37 °C, followed by another round of gentleMACS-mediated dissociation (m_liver_04). After erythrocyte lysis, liver NPCs were enriched by density centrifugation employing 30% Histodenz-HBSS (both from Sigma-Aldrich, Munich, Germany). Lung cell suspensions were obtained using the Lung Dissociation Kit (Miltenyi Biotec) according to the manufacturer's protocol.

3.11. Flow Cytometry

To assess living cells via flow cytometry, cells were washed in staining buffer (PBS, 1% (v/v) FBS, 0.5 mM EDTA) and were incubated with Fc receptor blocking antibody (2.4-G2) for 15 min at RT. Then, samples were incubated with fluorescence-labeled antibodies for 20 min at 4 °C. Afterwards, samples were washed with PBS and subsequently incubated with eFl780-FVD to identify dead cells. For intracellular staining, samples were fixed with 0.5 mL Fluorofix Fixation Buffer for 20 min and washed twice with 0.5 mL Perm Wash Buffer (both from BioLegend, San Diego, CA, USA). Then, the antibody for intracellular luciferase detection was added, and the samples were incubated for 20 min at RT in the dark. Finally, samples were washed two times with every 1 mL of Perm Wash Buffer. Gating was performed according to the strategies described in Figures S5 and S6. Samples were assayed using an Attune NxT flow cytometer, and data were analyzed using Attune NxT software v3.2.1 (both ThermoFisher, Waltham, MA, USA).

3.12. Statistics

Results are presented as arithmetic mean and standard deviation (SD) or standard error of the mean (SEM), respectively, of at least three experiments. Unpaired Student's two-tailed *t*-test with Welch's correction, as well as ordinary one-way ANOVA (multiple comparison, Tukey test), respectively, were performed using GraphPad PrismTM 9.5.1 in order to analyze statistical significances between groups. Significance levels are indicated with symbols: ns, not significant; $p > 0.05$; * or # $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$.

4. Conclusions

The efficacy of DNA vaccines in clinical trials is still low [1,16,17] but may be improved by transcriptional targeting of DCs using a DC-specific promoter. In the current study, we evaluated a hybrid enhancer/core human *Fscn1* gene promoter in this regard. This derivative showed preferable DC-focused activity in vitro compared to the standard CMV promoter. Upon systemic administration in mice, the expression profile of pFscnLuc was encouraging, with favorable activity in the spleen as a central immune organ, and in DCs at a somewhat higher level than in macrophages. This suggests that the human *Fscn1* gene promoter may be suitable for DC-focused transcriptional targeting. Moreover, pFscnLuc mediated high reporter gene expression in *Fscn1*-expressing tumor cells. Further, we observed carrier-dependent differences in the immunostimulatory potential, toxicity, as well as the in vivo luciferase expression profile, which are to be analyzed in more detail in follow-up studies.

Dual transcriptional targeting of DNA to both DCs and tumor cells may be an option to enhance the efficiency of DNA vaccines. This could be realized by employing DNA vaccines that encode for a tumor antigen to induce anti-tumor T cell responses in transfected DCs [1]. In transfected tumor cells, this may enhance antigen presentation via MHCI, thereby supporting recognition by tumor antigen-specific CTLs. Further, a DNA vaccine needs to co-deliver an adjuvant to ensure activation of the APC to prevent antigen-specific tolerance induction [68]. In the case of a DNA vaccine that may co-transfect APC and tumor cells, this adjuvant could be, for example, a TNF- α encoding gene, promoting DC activation by engaging TNF receptor 2 [69,70]. In tumor cells, TNF- α may induce apoptosis due to TNF receptor 1 signaling [60,61]. Alternatively, the vaccine may encompass the sequence for a short hairpin (sh)RNA that inhibits inhibitory signaling, e.g., of the transcription factor signal transducer and activator of transcription (STAT3). STAT3 knockdown in DCs has been reported to promote their anti-tumor activity [71]. Further, in tumor cells, STAT3 inhibition counteracted tumor growth and tumor resistance towards various therapeutic approaches [72]. Besides the design of nanovaccines that may transcriptionally co-address DCs and tumor cells, either cell type may be specifically targeted by conjugation of the nanocarrier system with corresponding targeting moieties [73–75]. In this regard, the attachment of antibodies that bind, e.g., CD205 [76–78], or of mannose or trimannose units, which address mannose recognizing receptors CD206 (macrophage mannose receptor) and CD209 (DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) [79–86], has been shown by us and others to yield the DC-focused internalization of a nanoformulation. In a complementary manner, we and others demonstrated preferential targeting of tumor cells by nanoformulations, e.g., by means of the conjugation of folate [75].

As a perspective, the specificity and efficacy of nanotherapeutics may be considerably improved by using receptor-addressing nanoformulations that deliver transcriptional targeting pDNA. This approach would aim to combine both levels of cell targeting to circumvent the side effects resulting from biological activity of the cargo in unwanted cell types.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/ijms242316938/s1>.

Author Contributions: Conceptualization, E.W., M.B., and S.B.; methodology, Y.Z., D.H., C.F.S., A.K.T.-K., E.W., M.B., and S.B.; software, Y.Z., D.H., M.B., and S.B.; validation, Y.Z., D.H., E.W., M.B., and S.B.; formal analysis, Y.Z., D.H., C.F.S., A.K.T.-K., M.B., and S.B.; investigation, Y.Z., D.H., U.W., J.P., C.H., C.M.-M., N.R., C.F.S., and S.B.; resources, Y.Z., D.H., U.W., J.P., C.H., C.M.-M., N.R., C.F.S., A.K.T.-K., and S.B.; data curation, Y.Z., D.H., M.B., and S.B.; writing—original draft preparation, Y.Z., E.W., M.B., and S.B.; writing—review and editing, Y.Z., E.W., M.B., and S.B.; visualization, Y.Z., M.B., and S.B.; supervision, E.W., M.B., and S.B.; project administration, E.W., M.B., and S.B.; funding acquisition, E.W. and M.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Deutsche Forschungsgemeinschaft, grant numbers B5 (E.W., M.B.) and B15 (M.B.), each SFB1066, and B4 (E.W.) of SFB1032 (project-ID 201269156).

Institutional Review Board Statement: The animal study was performed according to the guidelines of the German Animal Welfare Act and were approved by the animal experiments ethical committee of the Government of Upper Bavaria (accreditation number Gz. ROB-55.2-2532.Vet_02-19-19). Mice were sacrificed for organ retrieval according to § 4(3) TierSchG.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available from the corresponding authors on request.

Acknowledgments: We thank Gregory Harms (Imaging Core Facility of the Research Center for Immunotherapy (FZI), University Medical Center Mainz, Germany) for assistance in CLSM. We thank Wolfgang Rödl (Pharmaceutical Biotechnology, LMU Munich, Germany) for providing LPEI,

and Janin Germer (Pharmaceutical Biotechnology, LMU Munich, Germany) for providing succPEI. The Graphical Abstract was created with BioRender.com (Toronto, ON, Canada).

Conflicts of Interest: The authors declare no conflict of interest.

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6.6. Usage of KI Tools

I hereby declare that I used “DeepL Write” and “Perplexity” to assist in structuring or formulating sentences in the main text and appendix summaries, in order to improve readability and clarification of context. I further declare that I independently reviewed and finalized all modified sentences myself.

7. Curriculum Vitae

8. Publications

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