

Characterization of the Role of Lymphatics in Nucleoside-modified mRNA Vaccine Induced Immune Response



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ABBREVIATIONS

5'-5'-triphosphate bridge – ppp

7-methylguanosine – m7G

Anti-reverse cap analogs – ARCA

Antigen presenting cells – APCs

Clustered regularly interspaced short palindromic repeats-CRISPR-associated protein – CRISPR-Cas

Dendritic cells – DCs

Dil-labelled LNPs – Dil-LNPs

Diphtheria toxin – DT

Diphtheria toxin receptor– DTR

enhanced Green Fluorescent Protein – eGFP

Five-prime end cap – 5' cap

High Endothelial Venules – HEVs

In vitro transcribed – IVT

Initiation elongation factor 4 – eIF4

Lipid-nanoparticles – LNPs

Lymphatic endothelial cells – LECs

P-body-associated deadenylases – PABDs

Polyadenosyl binding proteins – PABPs

Polyethylene glycol – PEG

Puerto Rico/8 Hemagglutinin – PR8 HA

Untranslated regions – UTRs

Vascular endothelial growth factor receptor-3 – VEGFR-3

INTRODUCTION

The Lymphatic System

The lymphatic system is a part of the cardiovascular system. Lymphatics extend into every major region of the body, and it is a blind-ended open vasculature. It consists of the lymphatic vessels, collecting ducts, lymph nodes, lymphoid organs and tissues, and central lymphatic vessels transporting lymph to the venous system [1].

Structure of the Lymphatic System

Lymphatic capillaries are blind-ended structures, composed of a single layer of lymphatic endothelial cells (LECs). LECs have discontinuous button-like junctions, which allows fluid and leukocytes to enter the vessel lumen [2] [3, 4] [5]. Lymphatic capillaries usually lack basement membrane and are noncontractile. The initial lymphatics rely on anchoring filaments connected to LECs, and these filaments regulate the valve-like openings into the lymphatic vessel lumen [6] [7] [8].

Lymphatic capillaries are drained into collecting lymphatics [9] [10]. These vessels are surrounded by a smooth muscle layer, a basement membrane, and the LECs form zipper-like junctions and bileaflet valves [3] [11].

The structure of the large lymphatic vessels is similar to the blood vessels; these transport the lymph into the venous circulation. There are two lymphatic ducts: the ductus lymphaticus dexter which drains from the right side of the head and neck, the thorax, and the upper right extremity, and the ductus thoracicus that drains the rest of the body [12] [13].

The collecting vessels pass through lymph nodes, which are secondary lymphoid organs showing a follicular structure. These consist of lymphoid lobules surrounded by sinuses, which are filled with lymph and are enclosed by capsules. The walls of the sinuses are made up of LECs. The nodes are compartmentalized with narrow fluid crevices, and a vascular compartment opposes each for fluid exchange and cell transport [9] [14] [15].

Classical Functions of the Lymphatic System

Fluid Balance Maintenance

The lymphatic system drains fluid and proteins from the interstitial space and returns it to the blood circulation. The lymphatics play an important role in the regulation of the fluid balance in the interstitial space [2] [3] [4]. From the blood circulation water and smaller molecules are

released into the interstitial space [1]. The excessive fluid is diverted into the initial lymphatics, that are easily accessible for macromolecules, thus serving a primary role in the maintenance of fluid balance in the extracellular space [16].

Dietary Fat Absorption

Intestinal lymphatic vessels absorb and transport dietary lipids. These are transported as chylomicron lipoproteins, which are larger lipoprotein particles created by enterocytes of the intestine, which cells also carry out the absorption and secrete the lipoproteins into the central lymphatic vessel [17] [18] [19].

Immune Functions

The lymphatics play a crucial role in the transportation of immune cells to local lymph nodes for immune response elicitation [20]. Immune cells can enter the lymphatic system through the initial lymphatics. Leukocytes, located within the lymphatic vessels are dendritic cells (DCs), T – and B lymphocytes, monocytes, macrophages, neutrophils, and eosinophils. Upon the immune system's response, the antibody-secreting plasma cells are also present [21]. Memory T-cells and conventional CD11c^{hi} MHC II⁺ DCs, and in a fewer amount macrophages and monocytes also enter through the initial lymphatics [22] [23] [24].

The first step in an immune response is the entry of the antigen. The initial lymphatics openings create a free entry, which allows the fluid, containing antigens, to flow into the lymphatic vessel's lumen [24]. The lymphatic system's vessels are the main conduits for soluble antigens and antigen presenting cells (APCs) from peripheral tissues to the lymph nodes for mounting of immune responses. The afferent lymphatic vessel's LECs attract activated DCs; T- and B-cells [25] [26]. After activation, the DCs enter the lymphatic capillaries and interact with the LECs, meanwhile migrating towards the lymph node [27].

The lymph nodes provide a suitable microenvironment for naive lymphocytes to interact with free antigens and for the DCs for immune surveillance and therefore, lymphocyte activation can occur [28] [29] [30].

Smaller, free antigens can migrate quickly through the lymph nodes' conduit system, which provides a fast-transporting pathway from the sinus throughout the lymph nodes' parenchyma. Lymph node resident DCs are in direct contact with this pathway, thus are able to take up and process said small antigens [31] [32] [33]. The larger antigens, such as viruses, are excluded

from this route, these flow into the sinus, where lymph resident macrophages - located at the LECs area of the nodes - sample them. These macrophages are directly exposed to the antigens, and have a key role in capturing and delivering pathogen derived antigens to the lymphocytes, initiating adaptive immune response [34] [35] [36] [37] [38].

Once these pathogens enter the lymph nodes' subcapsular sinus, a complex multi-step process begins to induce the adaptive immune response [39] [40] [41].

Recent studies suggest that the LECs can express peripheral tissue antigens, therefore suggesting their direct role in immune regulation [42] [43]. LECs express MHC class I and II molecules and due to their positions in the lymph node sinus, LECs are directly exposed to the lymph, containing antigens, this equip LECs to function as APCs [44] [45] [46] [47] [48].

Novel, Organ-specific Functions of the Lymphatics

In addition to the classical functions, novel functions of the lymphatics have been recently described.

The lung's lymphatic vessels have been reported to play an important role in the fluid balance maintenance within the lung tissues, keeping the alveoli free for the optimal gas exchanges. [49]. In a recent study, they have suggested that the lymphatics clearance of fluid occurs prenatally and that the pulmonary lymphatic function is present prior to birth [50].

In a recent study, a research group has shown that high-salt diets in rat model leads to interstitial hypertonic sodium ion accumulation in skin, therefore resulting in increased density in hyperplasia of the lymphatic network. This study shows that in mononuclear phagocyte system's cells, the tonicity-responsive enhancer binding protein - Vascular endothelial growth factor C signaling pathway plays a major role in the extracellular volume and blood pressure homeostasis. This suggests that the lymphatic system also plays a role in regulating blood pressure [51].

The existence of lymphatic circuits in the central nervous system has only been more widely accepted in the recent years, due to the observation of lymphatic vessels in the dura mater of dog, mouse, and human brain [52] [53] [54]. The newly found lymphatics are known to absorb the cerebrospinal fluid, which is primarily formed in the choroid plexus in the later, third and

fourth ventricles. Their functions are still not fully understood, but recent studies suggest that they may also play an important role in the brain's immune defense [55].

As mentioned in the previous chapters, the lymphatic system is a very extensive network, its intact functioning is essential for the physiological functioning of almost all the organs and organs systems. The lymphatic's role is still unclear in many of the physiological processes.

The mRNA-LNP Technology

The novel, genome-based vaccine design offers great potential over conventional vaccine approaches. Nucleic acid-based vaccines paved the way for safe and efficacious vaccines. The mRNA-LNP vaccines are non-infectious; are free from contamination originating from proteins or virus-delivery, thus these are well-tolerated for therapeutic applications [56].

First Attempts

The first application of *in vitro* transcribed (IVT) mRNAs *in vivo* happened in 1990, where IVTs without any delivery system were injected into the skeletal muscle of mice. Results showed that the injection of IVTs can be translated *in vivo* [57]. However, these early attempts presented severe limitations such as the mRNA's instability, its stimulation of high innate immunogenicity, and the inefficient *in vivo* delivery. [58].

Modifications

Several methods have been developed to increase the intracellular stability and the translation of mRNAs, like nucleoside modifications [59], modifications in the untranslated regions (UTRs) [60], in the five-prime end cap (5' cap) analog [61] and the poly(A) tailing [62].

To extend the biological half-life of the mRNAs, the encapsulation of the naked mRNAs into lipid-nanoparticles (LNPs) is one of the most used approach, this protects the mRNAs from the degradation by ubiquitous RNAses [63] [64].

Nucleoside and UTRs Modifications

To improve the stability and translational efficiency, the modification of mRNAs with alternative nucleotides can be done [59]. By replacing uridine and cytidine with pseudouridine, 1-methylpseudouridine, and 5-methylcytidine, they have found that it can reduce intracellular mRNA decay and reduce innate immune activation and can increase the translational capacity of mRNAs *in vivo* [59] [65] [66]. Another option for the improvement of the mRNA translation and concealment from the innate immune response is the post-transcriptional epigenomic RNA

modifications. It has been reported that the post-transcriptional RNA modification with N4-acetylcytidine enhances the mRNA translation both *in vivo* and *in vitro* [67].

It has also been shown that mRNAs' 5' UTRs can be optimized to enhance its stability and accuracy of translation [60].

5' Cap Modifications

5' cap is not encoded by the DNA; therefore, a synthetic cap analogue can be added to the IVT mRNAs. Naturally, the eukaryotic mRNA has a 7-methylguanosine (m7G) cap, which has several functions at the 5' end of the mRNA cap, with a 5'-5'-triphosphate bridge (ppp) [68]. The m7GpppN structure protects the mRNA from degradation by exonucleases, plays a key role in the translation, and prevents the innate immune systems from recognizing the mRNA [69]. Thus, capping of mRNAs is necessary for its proper function and the prevention of its degradation. The incorporation of the 5' cap analogue to the 3' hydroxide of the pentose carrying the non-methylated guanine, which is used for the phosphodiester bridge to the second residue of the mRNA, leads to a proper, functional mRNA, which is a result of the initiation elongation factor 4 (eIF4) protein's recognition of the methylated 5' end [70].

5' capping can be achieved by enzymatic and chemical methods for mRNAs. In practice, the most widely spread method used for *in vitro* post-translational capping, is the enzymatic, Vaccinia capping system, consisting of two subunits: the D1 provides the m7Gppp addition to the mRNA, and the D12 plays a key role in the activation of D1 subunit [61] [71]. The chemical capping methods can add cap analogs co-transcriptionally, however a regular cap analog, which is added during IVT can be reversely incorporated into the mRNA sequences. This results in the not proper methylation of the one third of the mRNA molecules, which leads to the low efficiency of the downstream mRNA translation; to prevent this, anti-reverse cap analogs (ARCA) have been developed. ARCA ensures the addition of nucleotide only at the non-methylated guanines; therefore, ARCAs can increase and prolong the protein expression *in vitro*. This method provides a lower capping efficiency, around 60-80% [72] [73].

Poly(A) Tailing

The poly(A) tail plays a key role in the mRNA translation and in the enzymatic stability of the mRNA by binding to several polyadenosyl binding proteins (PABPs) while working synergistically with the 5' m7G cap sequences through translation initiation factors, to regulate the translational efficiency [74]. Poly(A) sequences can also slow down the degradation of mRNA and has a key role in the prevention of de-capping [75]. Thus, it is an important addition to IVT mRNAs, in order for their proper functions. eIF4E binds to the 5' m7G cap, which

creates complexes with eIF4G and eIF4A. P-body-associated deadenylases (PABDs) interact with eIF4G, resulting in the formation of messenger ribonucleoprotein or a polysome complex [76]. For the circularization of the mRNA, a suitable long poly(A) tail is required [77].

With the increase of the poly(A) tail lengths to 120 bases, in IVT mRNAs, the protein level expressions also increase, but increasing the number of bases beyond 120 does not further enhance translation [78]. Poly(A) tail addition can be achieved by two methods. The first method is encoding the poly(A) tail in the DNA template, the second one is the extension of the IVT RNA after transcription using a recombinant poly(A) polymerase. However, the second method results in variable poly(A) tail length, thus resulting in polyadenylated mRNAs with varying lengths. Therefore, the widely used and preferred method for the generation of poly(A) tails is the first one, generating well-defined length poly(A) tails from DNA templates [62].

Lipid Nanoparticles

The encapsulation of mRNAs serves as physical protection from degradation, and can aid the intracellular uptake and endosomal escape of the mRNAs [79]. Cationic lipid and polymer-based delivery have been widely used for mRNA delivery in the past few years. Recent studies showed that LNPs are effective tools for the *in vivo* delivery of mRNAs [80] [81]. The mechanism underlying the LNP-mediated delivery of the mRNAs is still not clear, but the LNP particles are suggested to be internalized by endocytosis and attached electrostatically and fused with cell membranes by inverted non-bilayer phases [58].

Applications

The improvements of the mRNA platform, the increasement of the stability and the translation efficiency has led to various clinical and pre-clinical trials with this technique against infectious diseases, cancer, and are also used for protein replacement therapies and genetical engineering [82] [83] [84] [85] [86] [87] [88] [89].

mRNA vaccines have been developed to yield specific immune responses against infectious diseases and cancer. These vaccines can mediate a rapid, transient expression of the coded protein, and its production-time is also well defined, based on the mRNA platform. Its functional mechanism is not dependent on active cell division, whereas mRNA exerts its functions in the cytoplasm, and it is a generally safe vector [82]. The Food and Drug Administration approved first, mRNA-based vaccine was the mRNA vaccine against SARS-CoV-2, in 2020 [83].

Because of its mentioned advantages, mRNA vaccines have become a promising platform for cancer immunotherapy too. mRNA vaccinations against cancer can be designed to target tumor-associated antigens, therefore initiating innate and/or adaptive immune response [90].

The abnormal protein expression is a common cause of many diseases. The mRNA technique can also be used for the therapeutic applications of proteins, which includes the replacement of proteins. mRNAs used in protein replacement therapies are designed to display lower immunogenicity, a prolonged stability, and potent translation. The mRNAs intracellular delivery enables the expression of the desired protein *in vivo*, in addition it allows the preservation of post-translational modifications of the proteins [84].

Genetic engineering, based on programmable nucleases with Clustered regularly interspaced short palindromic repeats-CRISPR-associated protein (CRISPR-Cas)-based methods, by the delivery of direct changes in the DNA, have improved personalized medical and basic research [91]. The mRNA technology-based delivery of the modified nucleases has several advantages; Cas9's mRNA is also single-stranded, thus it can be encapsulated in nanoparticles, for both *in vivo* and *in vitro* delivery, a transient expression can be achieved, therefore the off-target rate is also decreased. This approach avoids genomic integration, and it has a high editing efficiency [87].

Inducible Lymphatic Deficient Transgenic Mouse Model

An *in vivo* lymphatic deficient model can be utilized to study the role of the lymphatics in several processes. An inducible, targetable lymphatic deficient transgenic mouse model has been developed recently. The Flt4-CreER^{T2}; iDTR^{fl/fl} transgenic mouse model utilizes the CreER^{T2}-lox system, in which the Cre recombinase enzyme is fused with a mutant estrogen receptor, that can be activated with tamoxifen and the recombinase catalyzes the deletion of a target sequence flanked by two loxP sites [92, 93]. In this mouse model CreER^{T2} recombinase is driven by the Flt4 promoter, mainly expressed by LECs in adults [94] [95]. The human isoform of the heparin binding EGF like growth factor, – which is the vector for the internalization of the diphtheria toxin (DT) – is driven by a ubiquitous promoter, but the first exon includes a nonsense codon flanked by loxP sites. Therefore, the administration of tamoxifen for these mice results in the deletion of the nonsense codon and thus the expression of the DT vector expressed exclusively on the surface of LECs. After tamoxifen treatment, subcutaneous administration of DT induces the apoptosis of LECs and lymphatic dysfunction around the injection site [95] [96] [97].

OBJECTIVES

As described, nucleoside-modified mRNA-LNP vaccines induce molecular and cellular processes, and the lymphatic systems' role in the mRNA-LNP vaccine induced immune responses remains unclear. Our aim is to investigate the role of the lymphatics in the immune response induced by mRNA-LNP vaccines.

MATERIALS AND METHODS

Mouse Models

Young adult C57Bl/6 wild-type mice or Flt4-CreER^{T2}; iDTR^{fl/fl} transgenic mice aged between 56-150 days were used and were maintained in a minimal disease facility [97].

Flt4-CreER^{T2}; iDTR^{fl/fl} were treated with tamoxifen dissolved in corn oil, in a 20 mg/ml concentration, as intraperitoneal injections, with 4.5 mg/40-gram body weight for 5 consecutive days.

Following a week of incubation, mice were treated with subcutaneous 60 ng of DT for 3 consecutive days to induce local lymphatic deletion. Control mice were injected with PBS as control.

Characterization of the Drainage of Lipid Nanoparticles and mRNA-complexes

5 µg of Dil-labelled LNPs (Dil-LNPs) or 1 µg of enhanced Green Fluorescent Protein (eGFP) encoding mRNA-LNPs were injected in 1 µg/µl concentration, subcutaneously into the hind paw of untreated C57Bl/6 mice or Flt4-CreER^{T2}; iDTR^{fl/fl} mice 8 days after the first injection of the described DT treatment. Mice were terminated after different incubation times of mRNA-LNP treatment, fluorescent images were captured before and after the surgical removal of the lymph popliteal lymph nodes. Representative images were taken with standardized background after the surgical removal, as the fluorescent intensity measurements were made with the standardized background. Images of the injection site and the popliteal lymph nodes were captured with a Nikon SMZ25 stereo microscope connected to a Nikon DS-Ri2 camera.

The injection site and popliteal lymph node samples were then fixed in 4% paraformaldehyde (PFA) solution overnight.

Histology and Microscopy

Hind paw samples were decalcified with OSTEOSOFT solution for 40 days. Following excessive PBS washing, the hind paw samples were dehydrated in a series of ethanol solutions of 50%, 70%, 95% and 100% for 24-hours each. After the incubation of samples in xylene for 2×3 hours, samples were embedded into paraffin blocks. 7 µm slides were prepared, using Thermo Scientific microtome (HM340E).

Fluorescent immunohistology staining were performed, using primary antibodies: anti-GFP (Life Technologies A11122), anti-LYVE1 (R&D systems AF2125), anti-GR1 (BD, 550291), anti-CD8a (Invitrogen, 14-0081-86), anti-CD4 (Invitrogen, 14-0041-86) in 1:100 dilution overnight. Samples then were incubated with secondary antibodies: goat anti-rabbit (Invitrogen, A11034), goat anti-rat (Invitrogen, A11077), donkey anti-goat (Invitrogen, A11057)

conjugated with Alexa Fluor 488 or 568 fluorophores. Mounting was performed using mounting medium containing DAPI for nuclei staining (Vector, H-1200-10).

Images were captured with a Nikon ECLIPSE Ni-U upright microscope connected to a Nikon DS-Ri2 camera.

Immunization in Lymphatic Deficient Mice

Flt4-CreER^{T2}; iDTR^{fl/fl} mice, following tamoxifen treatment were treated with DT or PBS in the back skin, as previously described. 8 days after the first DT injection, mice were vaccinated with 5 µg of influenza strain Puerto Rico/8 Hemagglutinin (PR8 HA) mRNA-LNPs or 5 µg of luciferase encoding mRNA-LNPs as a control in a 0.1 µg/µl concentration. Peripheral blood samples were collected prior, and every 2 weeks for 10 weeks after vaccination through retroorbital bleeding.

Monitoring PR8 HA-specific IgG Titers

96-well plates were coated with PR8 HA protein in 2 µg/ml concentration overnight at 4 °C. Wells were blocked with a PBS solution containing 2% goat serum and 0.05% Tween-20 for 1 hour at room temperature. Mouse serum samples were loaded in 1:50 000 dilution in duplicates and were incubated for 1 hour at room temperature along with a serial dilution of mouse anti-influenza PR8 HA antibody (Invitrogen, MA5-29938). The 96-well plates were then incubated with HRP-conjugated goat anti-mouse IgG antibody for 1 hour at room temperature. After washing, 1-Step Ultra TMB-ELISA substrate was added and was incubated for 15-minutes at room temperature, the reaction was stopped with 1 mol/liter sulfuric acid. Absorbance values were measured at 450 nm with ThermoFisher Multiskan FC.

Data Processing and Analysis

Microscopic images were processed via Nikon NIS-Elements Imaging Software (BR4.60.00). Fluorescent intensity was quantified on stereo microscopic images using the Polygonal ROI tool and background intensity were subtracted. Data processing and analysis were performed using Microsoft Excel 2019 and GraphPad Prism 8.

RESULTS

Subcutaneously Injected LNPs are Drained into the Popliteal Lymph Nodes

Fluorescent signal was detected in the popliteal lymph node of wild-type mice as early as 10 minutes after the injection of the Dil-fluorophore labelled LNPs into the hind paw and was still present 14 day after the administration (Figure 1). The kinetics were also investigated by the quantification of the fluorescence signals. A major increase in the fluorescent intensity in the popliteal lymph node of Dil-LNP injected side was observed after 30 minutes. The highest fluorescent intensity was observed 24-hours after the administration, thereafter a constantly decreasing signal was detected (Figure 2). In addition, we could also observe a transient enlargement of the lymph nodes 8 and 24 hours after the LNP injection (Figure 3 and 4).

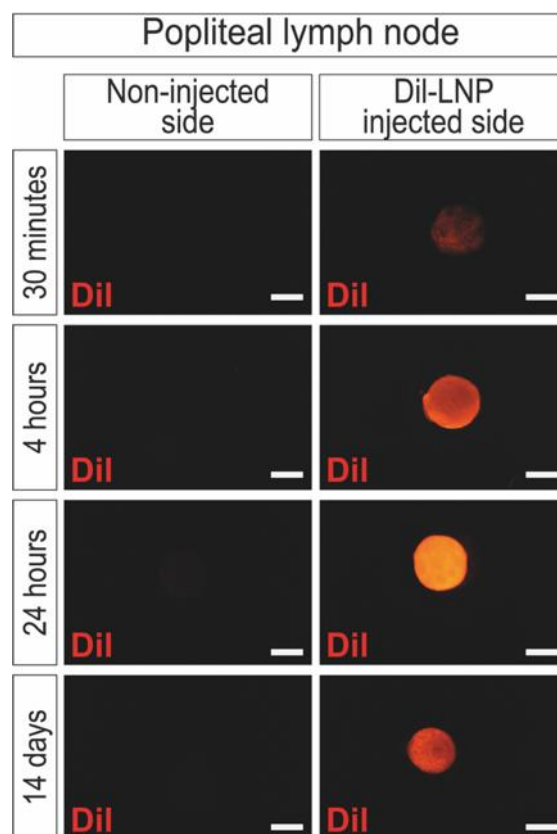


Figure 1: Representative Fluorescent Images of the Popliteal Lymph Nodes After the Injection of Dil-labeled LNPs

Injection of Dil-LNPs into the hind paw of wild-type mice results in fluorescent signal in the popliteal lymph nodes of the ipsilateral side. (n = 5, bars: 1 mm)

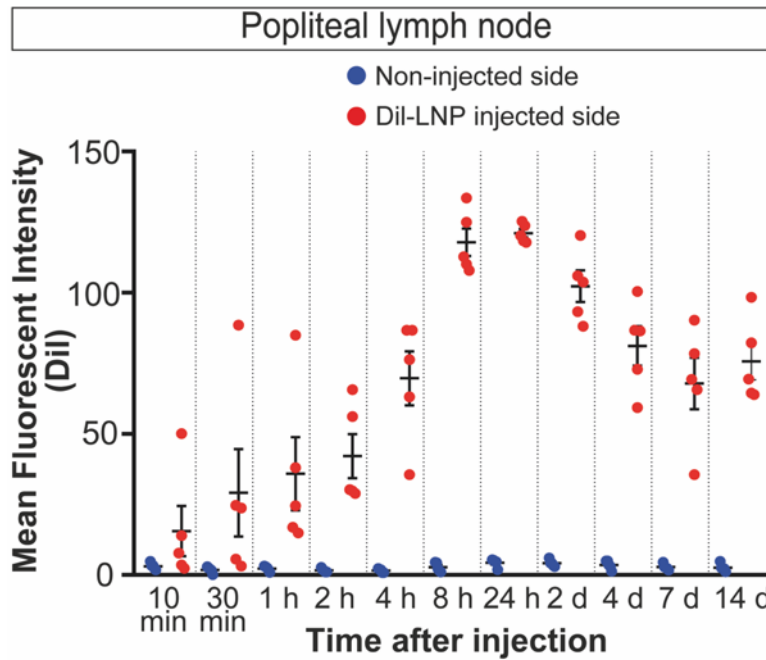


Figure 2: Quantification of Fluorescent Signal in the Popliteal Lymph Nodes after Dil-LNP Injection

Dil fluorophore-labelled LNPs were injected into the hind paw of wild-type mice and the mean fluorescent signal intensity in popliteal lymph nodes was quantified at several time points after injection. (n = 5 at each time point, mean ± SEM.)

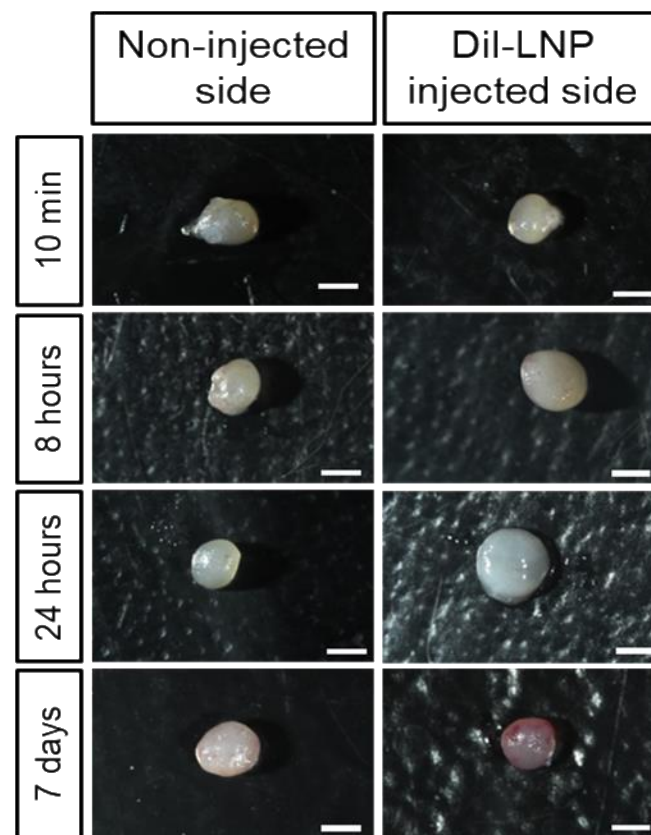


Figure 3: Representative Brightfield Images of the Popliteal Lymph Nodes After Dil-labeled LNP Injection

Following the injection of Dil fluorophore-labelled LNPs into the hind paw of wild-type mice, we could observe a transient enlargement of the lymph nodes 8 and 24 hours after the LNP injection. (n = 5, bars: 1 mm)

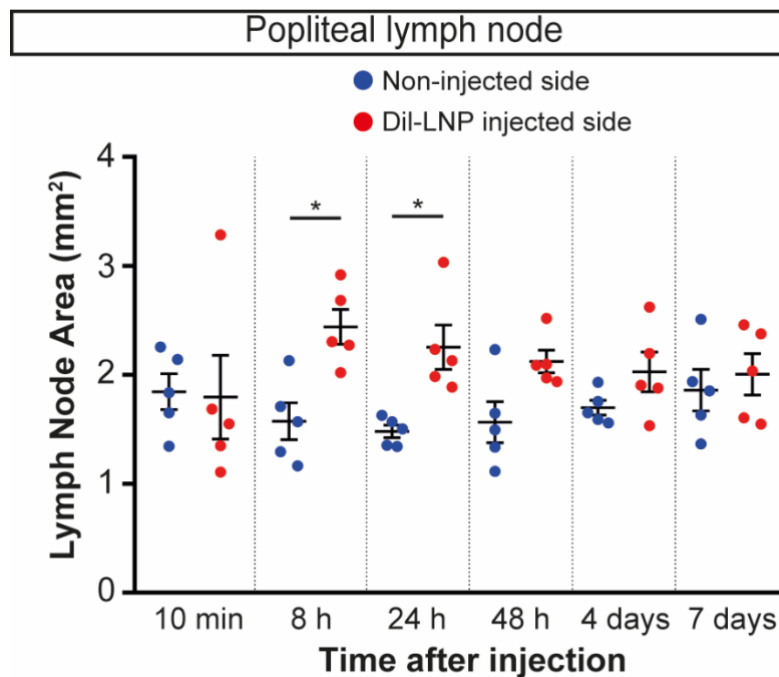


Figure 4: Quantification of Lymph Node Areas After Dil-labeled LNP Injection

Dil-labelled LNPs were injected into the hind paw of wild-type mice, which resulted in a transient enlargement of the lymph nodes, the changes in the size of lymph nodes were quantified at several time points after the administration. (Mean±SEM, $p = 0.0219$ at 8 hours, $p = 0.0352$ at 24 hours; paired t-test)

Subcutaneously Injected mRNA-LNPs are Expressed at the Injection Site and in the Popliteal Lymph Nodes

The mRNA expression was examined at the injection site and in the popliteal lymph node by the administration of eGFP encoding mRNA-LNPs. eGFP expression was detectable after 4 hours at the injection site (Figure 5). The intensity of the eGFP fluorescent signal was quantified, showing that the fluorescence signal was detectable 4 hours after the injection and reached its maximum 48 hours following the administration and was still detectable after 14 days later (Figure 6).

Fluorescent signals were also detectable 4 hours after injection in the popliteal lymph nodes (Figure 7). The intensity of the eGFP fluorescent signal was also quantified, which has shown that the eGFP signal was detectable 4 hours after the administration, reached its maximum 24 hours after the injection and was still detectable after 14 days later (Figure 8).

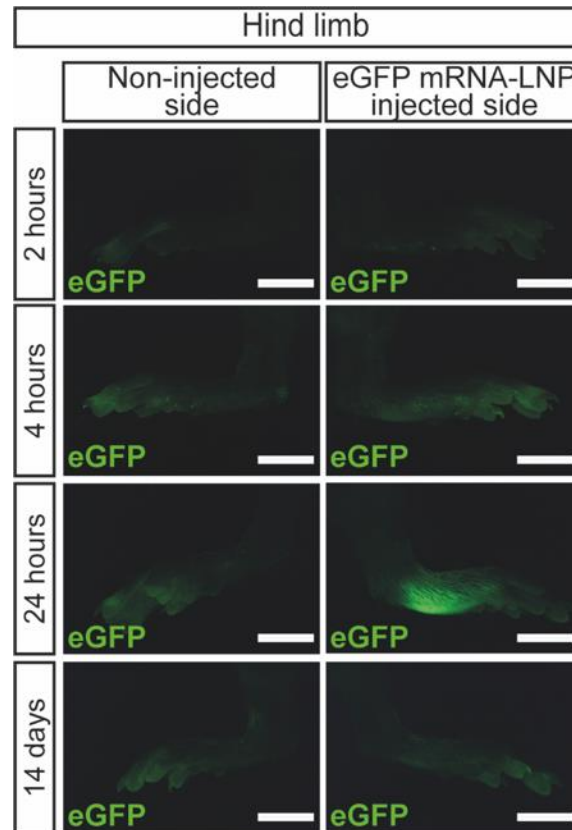


Figure 5: Representative Fluorescent Images of the Injection Site After the Injection of eGFP Encoding mRNA-LNP

eGFP mRNA-LNP was injected into the hind paw of wild-type mice, and eGFP expression was monitored with stereo microscopy at the injection sites. (n = 2-7, bars: 1 mm)

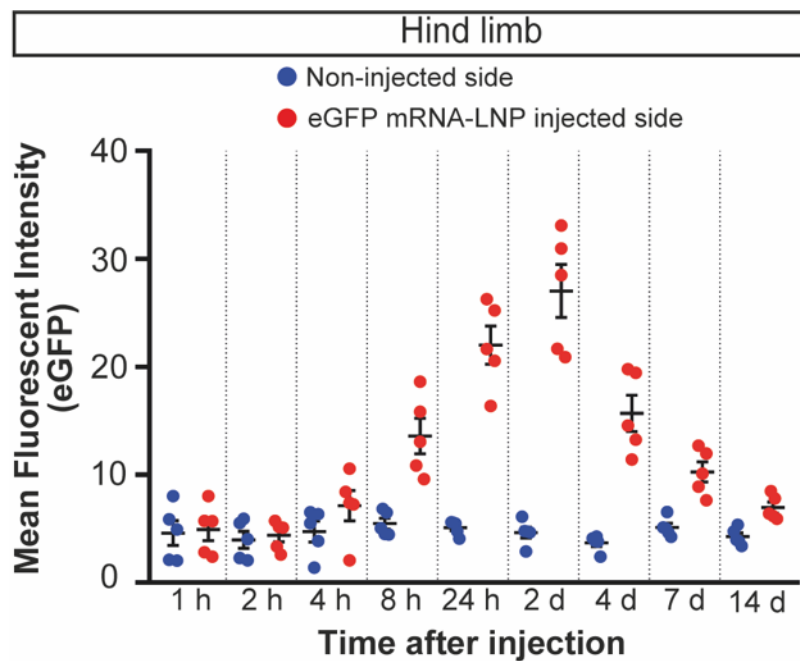


Figure 6: Quantification of eGFP Fluorescent Intensity at the Injection Site

Following the eGFP mRNA-LNP injection, the mean fluorescent signal intensity was quantified at the injection sites. (n = 2-7, mean $\pm$ SEM.)

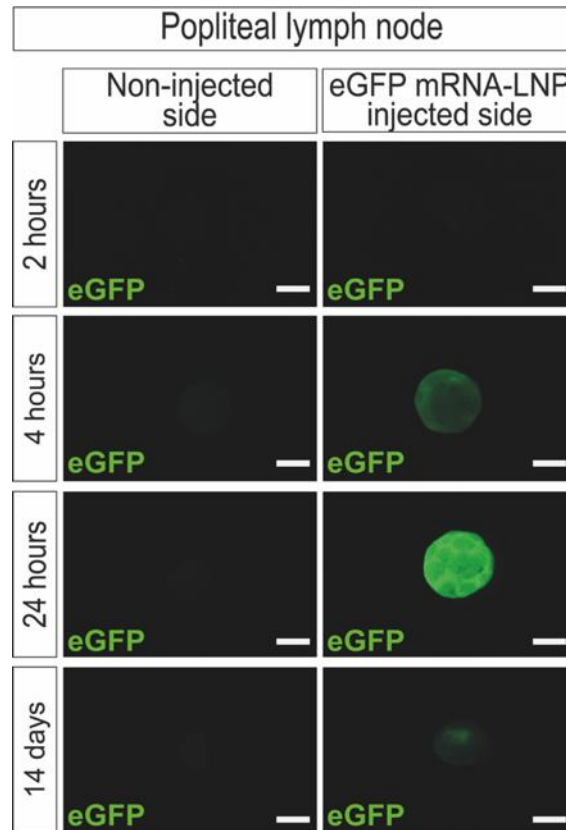


Figure 7: Representative Fluorescent Images of the Popliteal Lymph Nodes After the Injection of eGFP Encoding mRNA-LNPs

eGFP mRNA-LNP was injected into the hind paw of wild-type mice, and eGFP expression was monitored with stereo microscopy in the popliteal lymph nodes. (n = 5, bars: 1 mm)

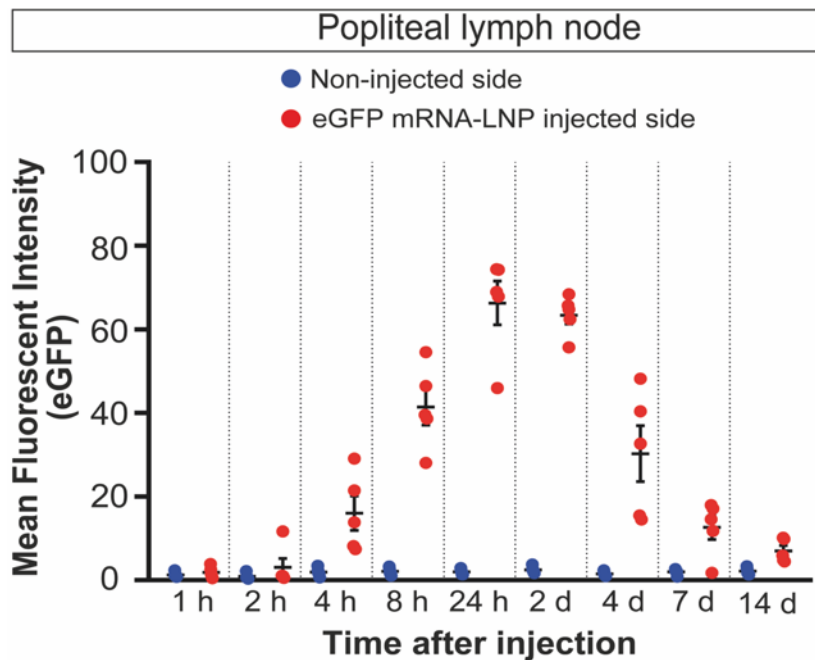


Figure 8: Quantification of eGFP Fluorescent Intensity in the Popliteal Lymph Nodes

eGFP mRNA-LNP was injected into the hind paw of wild-type mice and the mean fluorescent signal intensity was quantified in the popliteal lymph nodes. (n = 5; mean $\pm$ SEM)

Local Lymphatic Deficiency Prevents Subcutaneously Injected eGFP mRNA-LNP Expression in the Popliteal Lymph Nodes

Injection of eGFP mRNA-LNP into lymphatic deficient regions resulted in a greater degree of vascularization, however no significant difference in size could be detected following the administration into regions with intact lymphatics and lymphatic deficient regions (Figure 9). In addition, eGFP mRNA-LNP injected into lymphatic deficient regions did not result in eGFP expression in the popliteal lymph node, even with as high doses as 20 μ g (Figure 10). The intensity of the eGFP fluorescent signal was also quantified, showing that the fluorescent signal constantly increased with the increased amount of eGFP mRNA-LNP injected into the intact lymphatics, injection into the lymphatic deficient regions did not result in fluorescent signal with any of the doses (Figure 11).

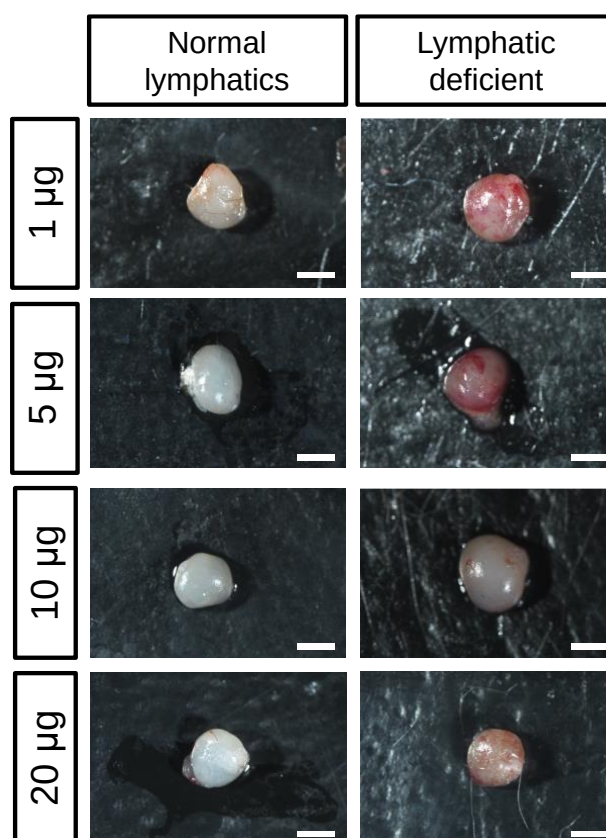


Figure 9: Representative Brightfield Images of the Popliteal Lymph Nodes After eGFP mRNA-LNP Injection into Locally Lymphatic Deficient Mice

The brightfield image of eGFP mRNA-LNP injected into the normal and lymphatic deficient hind paws. (n=2-6, bars: 1 mm)

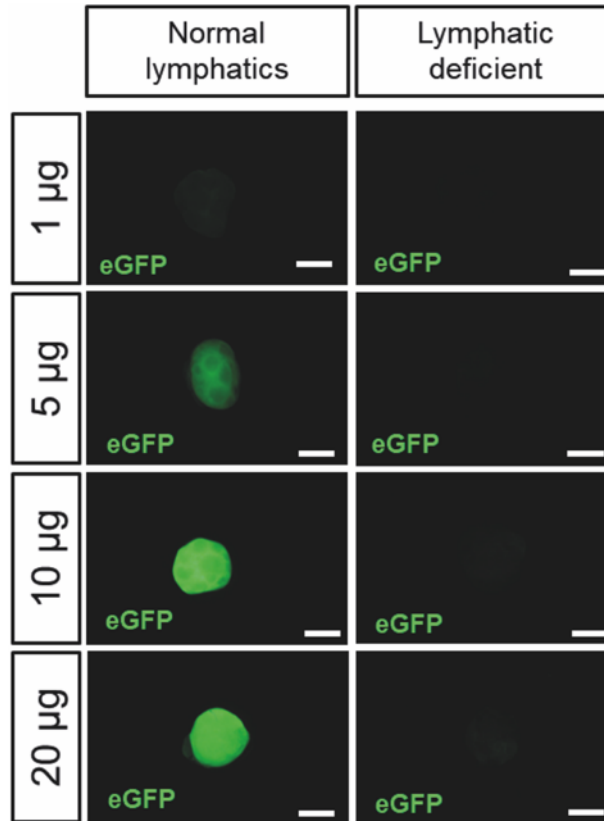


Figure 10: Representative Fluorescent Images of the Popliteal Lymph Nodes After eGFP mRNA-LNP Injection into Locally Lymphatic Deficient Mice

eGFP mRNA-LNP injected into the lymphatic deficient hind paws resulted in no eGFP expression in the popliteal lymph nodes. (n=2-7, bars: 1 mm)

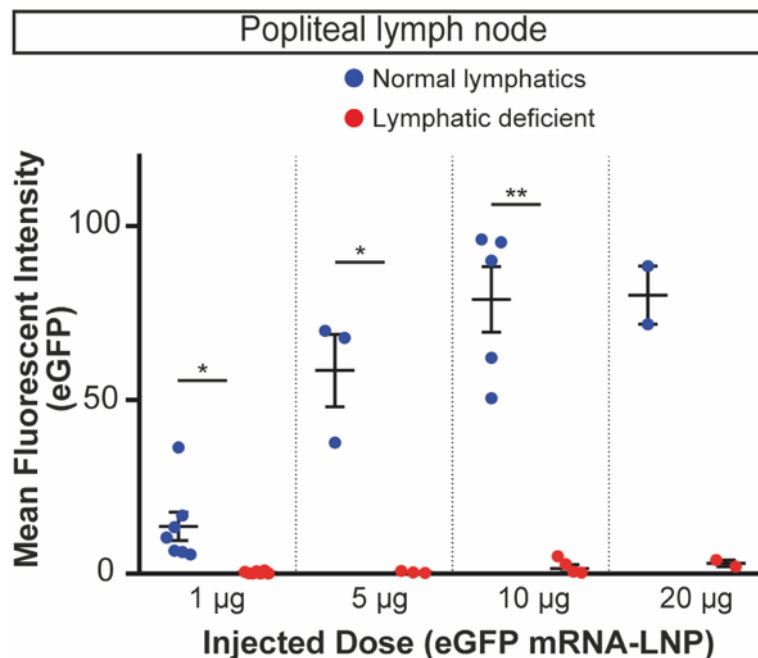


Figure 11: Quantification of eGFP Fluorescent Signal After the Injection of eGFP mRNA-LNP into Lymphatic Deficient Regions

eGFP mRNA-LNP was injected into regions with intact lymphatics and lymphatic deficient regions, the mean fluorescent signal intensity was quantified in the popliteal lymph nodes. No eGFP expression was observed in the popliteal lymph nodes when eGFP mRNA-LNP was injected into lymphatic deficient regions. (n = 2-7, Two-tailed paired t-test, $p=0.0196$ at 1 μ g, $p=0.0317$ at 5 μ g, $p=0.001110$ at 10 μ g, Mean $\pm$ SEM)

Histological Characterization of GFP mRNA-LNP Treated Tissues

Fluorescent immunohistology showed that eGFP expressing cells are present around the injection site. Additionally, our results showed that LYVE1⁺ stained LECs seemed to express mRNA-LNPs only to a small extent (Figure 12).

Immunohistochemical staining of lymph nodes yielded similar kinetics of eGFP expressing cell numbers, compared to the injection site (Figure 13).

Furthermore, a subset of cells in close contact to eGFP expressing cells were successfully characterized by immunostaining with markers for helper T cells (CD4), cytotoxic T cells (CD8) and neutrophil granulocytes (GR1). Although these cells did not show significant eGFP expression, the results suggest that these cells are in close cell-cell contact with the eGFP expressing cells (Figure 14).

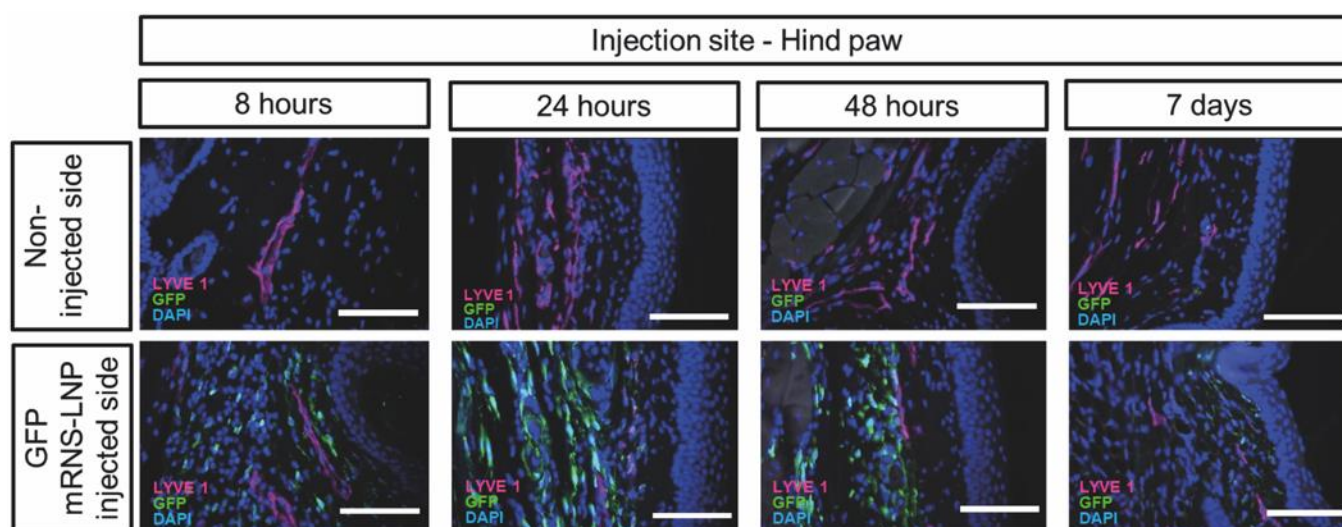


Figure 12: Fluorescent Immunohistology of Injection Site After eGFP mRNA-LNP Injection
eGFP (green) expressing cells could be detected around the injection site 8, 24, 48 hours and 7 days after the administration of eGFP mRNA-LNPs. LYVE1⁺ lymphatic capillaries (magenta) showed little to no eGFP expression. (n = 5, bars: 100µm)

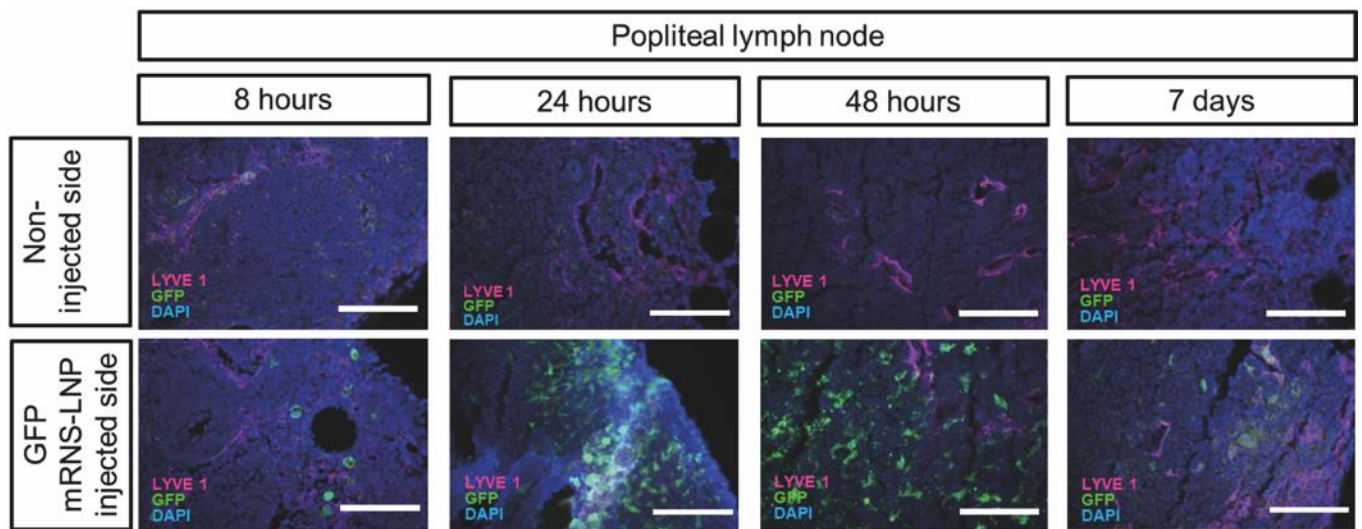


Figure 13: Fluorescent Immunohistology of the Popliteal Lymph Nodes After eGFP mRNA-LNP Injection

eGFP (green) expressing cells could be detected in the popliteal lymph nodes, 8, 24, 48 hours and 7 days after eGFP mRNA-LNP administration. LYVE1+ (magenta) LECs show little to no eGFP expression. (n = 5, bars: 100µm)

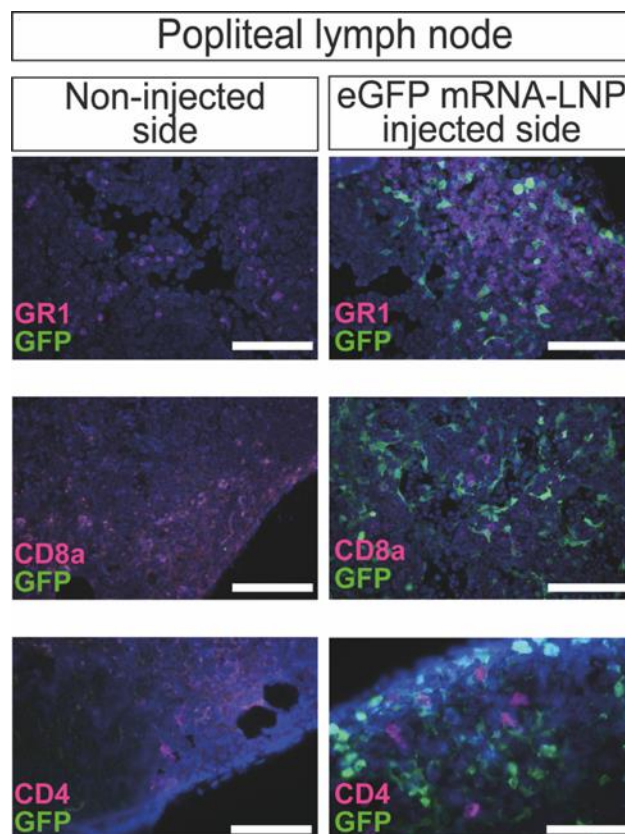


Figure 14: Fluorescent Immunostaining with Different Immune Cell Markers in the Popliteal Lymph Nodes 24 Hours After eGFP mRNA-LNP Injection

After eGFP mRNA-LNP injection eGFP expressing cells can be detected in close proximity of GR1+ neutrophils, CD8a+ cytotoxic T cells and CD4+ helper T cells, yet these populations showed no eGFP expression. (n = 5, bars: 100µm)

Immunization in Lymphatic Deficient Regions Resulted in Undetectable Levels of Specific IgG Production

In the sera of control, luciferase mRNA-LNP injected mice, no PR8 HA specific IgG response was detected. Mice vaccinated with PR8 HA encoding mRNA-LNP into a region with intact lymphatics produced an adequate IgG response, already detectable two weeks after injection. Anti-PR8 HA IgG serum titers reached its maximum at the fourth week, after which a decreasing trend could be observed, but a significant humoral immune response could be observed in the following weeks. In contrast, the vaccination with the PR8 HA mRNA-LNP into lymphatic deficient regions resulted in PR8 HA specific IgG levels mostly below detectable signals (Figure 15).

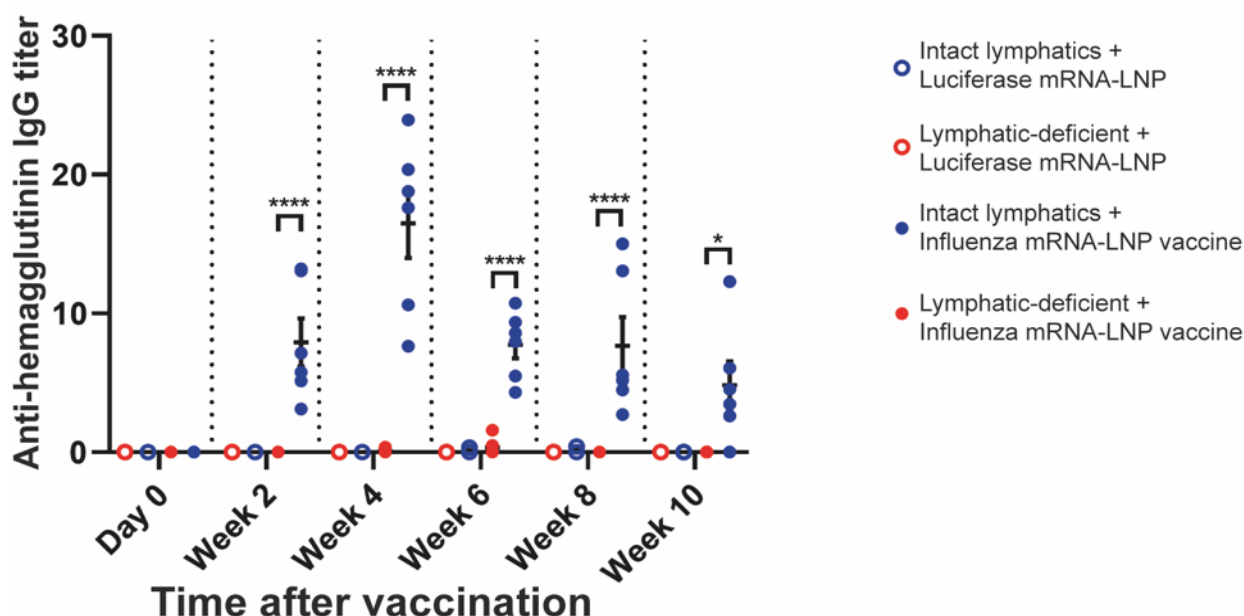


Figure 15: Anti-PR8 HA IgG Serum Titers After Vaccination with PR8 HA Encoding mRNA-LNP in Lymphatic Deficient Mice

PR8 HA coding mRNA-LNP was injected into region with intact lymphatics and into lymphatic deficient regions. (n=5-6 for each group: mean $\pm$ SEM, three-way ANOVA, $p=1.9369 \times 10^{-6}$; (intact lymphatic vaccinated vs. lymphatic deficient vaccinated groups $p=2.9229 \times 10^{-6}$ for 2. week; $p<10^{-15}$ at week 4; $p=1.8101 \times 10^{-5}$ at week 6; $p=6.8146 \times 10^{-6}$ at week 8; $p=0.0434$ at week 10; Tuckey's post-hoc analysis)

CONCLUSIONS

Nucleoside-modified mRNA-LNPs have been successfully used as vaccines against the SARS-CoV-2 [98]. Vaccines based on the mRNA-LNP technology are able to induce significant humoral immune response including high neutralizing antibody titers, however, the underlying molecular and cellular mechanisms in the development of immune response are not fully known. The role of the lymphatic system in immunological processes is well known [99]. In the light of this, the role of the lymphatic system in the immune response during vaccination is also emerging, however the molecular mechanisms of this process are not yet fully understood. Therefore, our aim is to investigate the role of the lymphatics in the immune response induced by mRNA-LNP vaccines using transgenic mouse models.

Our results suggests that the lymphatic vessels take up and deliver the LNPs into the popliteal lymph nodes (Figure 1).

By analyzing the kinetics of the process, we could observe that the LNPs appear in the popliteal lymph nodes after only 30 minutes, suggesting that this is a quite rapid process, presumably LNPs are being directly transported (Figure 2) [100]. Furthermore, LNPs are “stuck” in the lymph nodes, which can be assumed to be due to the LNPs are being taken up by certain cell types, but this process requires further studies.

Investigating the expression of mRNA-LNPs by injecting mRNA-LNPs encoding the fluorescent protein eGFP, we have found that 4 hours after the injection eGFP signals were present in the popliteal lymph nodes, which approximates to the uptake and translation time of mRNAs, suggesting that mRNA-LNPs are taken up and are expressed by lymph node resident cells (Figure 7-8).

Based on our results performed on the inducible lymphatic deficient mouse model, local lymphatic capillaries play a major role in the delivery of mRNA-LNPs to the lymph nodes, in their absence, no protein expression could be observed in the lymph nodes (Figure 10-11). Conversely, in these mice increased eGFP signal was observed at the injection site (data not shown), suggesting that more mRNA-LNPs are taken up by resident cells, thus fewer APCs are reached by mRNA-LNPs.

We have successfully visualized a subset of cells which are in close contact with the eGFP-expressing cells, in this ongoing experiment our final goal is to characterize the lymphatics dependent cellular processes in the expression of mRNA-LNPs [82] (Figure 14).

Investigation of the humoral immune response induced by mRNA-LNP vaccination, showed that mRNA-LNP vaccines administered to a region with impaired lymphatics significantly

reduced the specific IgG response. These suggest that the afore-mention lymphatic-depending mechanism is required to elicit an adequate immune response and that the lymphatics is a potential target to increase the efficacy of mRNA-LNP vaccines (Figure 15).

Overall, our results suggest that the lymphatics are involved in the uptake, transportation, and expression of mRNA-LNPs. These results may increase our scientific understanding of the processes that occur *in vivo* in response to the mRNA-LNP technology-based vaccines and could therefore be used to further develop the mRNA-LNP technology. Improvements based on this knowledge could contribute to increasing the efficiency of vaccination based on mRNA-LNP technology.

SUMMARY AND KEYWORDS

Introduction: Vaccines based on the lipid nanoparticle (LNP)-encapsulated nucleoside-modified messenger RNA (mRNA-LNP) technology recently gained major scientific interest, as two vaccines against SARS-CoV-2 accepted for emergency use utilize this technology. It has been presented that mRNA-LNP vaccines are able to induce potent humoral immune response, however the underlying molecular and cellular mechanisms involved are still not fully understood. The lymphatic system is involved in several immunological processes, but the role of lymphatics in the mRNA-LNP vaccine induced immune response requires further investigation.

Objectives: Our aim is to characterize the role of the lymphatics in the mRNA-LNP vaccine induced immune response in experimental mouse models.

Methods: Fluorescently labelled LNPs were injected into the hind paw of wild-type mice and fluorescent signal intensity was quantified at the popliteal lymph nodes. eGFP encoding mRNA-LNP was injected into the hind paws, and its expression was also monitored. Tissue samples were collected and examined by fluorescent immunostaining. eGFP mRNA-LNP expression was also characterized in a transgenic mouse model in which diphtheria toxin injection induces local lymphatic deficiency. Influenza hemagglutinin-encoding mRNA-LNP vaccine was injected into skin regions with intact lymphatics or in lymphatic deficient regions and then serum samples were collected, and anti-influenza titer was determined multiple time points after vaccination.

Results: Our results showed that fluorescently labelled LNPs were drained into the popliteal lymph nodes. eGFP mRNA-LNP was expressed at the injection site and in the popliteal lymph nodes. Local deletion of the lymphatics inhibited GFP protein expression in regional lymph nodes. Injection of the mRNA-LNP-based vaccine into the lymphatic deficient regions resulted in a significantly lower specific antibody titer compared to the control group vaccinated in skin areas with normal lymphatics.

Conclusions: Our results suggest that lymphatic vessels are involved in the uptake and transportation of mRNA-LNPs. Furthermore, intact lymphatic function is required for the induction of an adequate humoral immune response by mRNA-LNP vaccines. Our results may contribute to the understanding of the lymphatic-dependent mechanisms of action induced by mRNA-LNP-based vaccines, as well as to the optimization of such vaccines.

Keywords: lymphatic system, mRNA-LNP vaccine, immunology

ÖSSZEFOGLALÓ ÉS KULCSSZAVAK

Bevezetés: A lipid nanopartikulumba (LNP) csomagolt nukleozid-módosított, hírvivő RNS (mRNS-LNP) technológián alapuló vakcinákat jelentős tudományos érdeklődés övezi, mivel kettő, a vészhelyzetileg elfogadott SARS-CoV-2 elleni vakcina is ezen a technológián alapul. Irodalmi adatok alapján jelentős humorális immunválaszt képesek kiváltani, azonban az ebben szerepet játszó molekuláris és celluláris mechanizmusok a mai napig sem teljesen ismertek. A nyirokrendszer részt vesz számos immunológiai folyamatban, azonban az mRNS-LNP vakcinák indukálta immunválaszban betöltött szerepe további vizsgálatokat igényel.

Célkitűzések: Célunk, hogy jellemezzük a nyirokrendszer szerepét az mRNS-LNP vakcinák által indukált immunválaszban kísérleti egérmodellek segítségével.

Módszerek: Vad típusú egerek talpába fluoreszcensen jelölt LNP-ket injektáltunk, majd vizsgáltuk a fluoreszcenciát az injektálás területén és regionális nyirokcsomóban. Továbbá eGFP mRNS-LNP-t injektálva térképeztük fel annak expresszióját. Szövetmintákat gyűjtöttünk, amelyeket fluoreszcens immunfestéssel vizsgáltunk. A fentieken túl diftéria toxinnal indukálható, lokálisan nyirokérhiányos transzgenikus egértörzsben is követtük az eGFP mRNS-LNP expresszióját. Ezt követően az influenza hemagglutinint kódoló mRNS-LNP vakcinával kezeltünk ép nyirokérrendszerű, vagy nyirokérhiányos régióban állatokat, majd több időpontban gyűjtött szérummintából meghatároztuk a specifikus IgG titert.

Eredmények: Eredményeink alapján a fluoreszcensen jelölt LNP-k elszállítódtak a regionális nyirokcsomókba. Az eGFP mRNS-LNP az injektálás területén és a nyirokcsomóban is kifejeződött. A nyirokerek lokális hiánya gátolta a GFP fehérje expresszióját a regionális nyirokcsomókban. A nyirokérhiányos régióba való mRNS-LNP alapú vakcina injektálása, szignifikánsan alacsonyabb specifikus antitest titert eredményezett a kontroll, ép nyirokérrel rendelkező területen vakcinált csoporthoz viszonyítva.

Következtetések: Eredményeink arra utalnak, hogy a nyirokerek részt vesznek az mRNS-LNP-k felvételében és szállításában. Továbbá az ép nyirokérfunkció szükséges a megfelelő humorális immunválasz kialakulásához. Eredményeink hozzájárulhatnak az mRNS-LNP alapú vakcinák kiváltotta, nyirokerektől függő hatásmechanizmusok megértéséhez, illetve a vakcinák optimalizálásához és a lehetséges mellékhatások csökkentéséhez.

Kulcsszavak: nyirokérrendszer, mRNS-LNP vakcina, immunológia

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