ENGINEERED BIOMATERIALS FOR DRUG DELIVERY AND THERAPEUTIC MODULATION IN LYMPHATIC TISSUES

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Presented to
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by

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ENGINEERED BIOMATERIALS FOR DRUG DELIVERY AND THERAPEUTIC MODULATION IN LYMPHATIC TISSUES

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To my friends and family – my parents, Krista and Wade, sister Kacey, and husband Jesse – for their constant support.
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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS \hspace{4cm} iv

LIST OF FIGURES \hspace{4cm} viii

LIST OF SYMBOLS AND ABBREVIATIONS \hspace{4cm} x

SUMMARY \hspace{4cm} xiii

**CHAPTER 1.** Introduction \hspace{4cm} 1
1.1 Motivation \hspace{4cm} 1
1.2 Specific Aims \hspace{4cm} 5
   1.2.1 Specific Aim 1: Investigate effects of nitric oxide on lymph node access of biomolecules and particulates. \hspace{4cm} 5
   1.2.2 Specific Aim 2: Develop a nanoparticle system to encapsulate and deliver hydrophobic small molecule drug to lymphatic vessels for the treatment of diseases of lymphatic dysfunction. \hspace{4cm} 6
1.3 Significance \hspace{4cm} 7

**CHAPTER 2.** Background and Literature review \hspace{4cm} 11
2.1 Introduction \hspace{4cm} 11
2.2 Drug delivery to lymphatic vessels \hspace{4cm} 11
   2.2.1 Lymphatic structure and function \hspace{4cm} 11
   2.2.2 Lymphatic dysfunction in disease \hspace{4cm} 14
   2.2.3 Regulation of lymphatic vessel pumping \hspace{4cm} 17
   2.2.4 Lymphatic vessel drug delivery challenges \hspace{4cm} 19
   2.2.5 Biomaterials as tools to improve LV drug delivery \hspace{4cm} 22
2.3 Drug delivery to lymph nodes \hspace{4cm} 23
   2.3.1 Lymph node structure and function \hspace{4cm} 23
   2.3.2 LNs as therapeutic targets in disease \hspace{4cm} 25
   2.3.3 Barriers to LN drug delivery \hspace{4cm} 26
   2.3.4 Nitric oxide in the lymphatics \hspace{4cm} 29
   2.3.5 Biomaterials for NO delivery \hspace{4cm} 31

**CHAPTER 3.** Lymph-directed nitric oxide increases immune cell access to lymph-borne nanoscale species \hspace{4cm} 33
3.1 Introduction \hspace{4cm} 33
3.2 Materials and Methods \hspace{4cm} 36
   3.2.1 Nanoparticle synthesis and characterization \hspace{4cm} 36
   3.2.2 Fluorescent tracers \hspace{4cm} 38
   3.2.3 Biodistribution experiments \hspace{4cm} 39
   3.2.4 Lymph node immune cell access experiments \hspace{4cm} 40
   3.2.5 Confocal imaging \hspace{4cm} 41
   3.2.6 In vitro dextran association and junction staining \hspace{4cm} 43
   3.2.7 LEC monolayer transport \hspace{4cm} 45
   3.2.8 Statistical analysis \hspace{4cm} 45
### CHAPTER 4. Lymphatic-draining nanoparticles deliver Bay K8644 payload to lymphatic vessels and enhance their pumping function

#### 4.1 Introduction

#### 4.2 Materials and Methods

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2.1 BayK-NP synthesis and characterization</td>
<td>76</td>
</tr>
<tr>
<td>4.2.2 Isolated vessel studies</td>
<td>77</td>
</tr>
<tr>
<td>4.2.3 In vivo functional analysis</td>
<td>79</td>
</tr>
<tr>
<td>4.2.4 Functional effects and side effects of BayK-NP treatment</td>
<td>80</td>
</tr>
<tr>
<td>4.2.5 Lymphatic ligation surgical model</td>
<td>82</td>
</tr>
<tr>
<td>4.2.6 Effects of BayK-NP treatment on vessel function and lymphedema development</td>
<td>83</td>
</tr>
<tr>
<td>4.2.7 Immunological effects of BayK</td>
<td>85</td>
</tr>
<tr>
<td>4.2.8 Statistical analysis</td>
<td>86</td>
</tr>
<tr>
<td>4.2.9 Animal use</td>
<td>86</td>
</tr>
</tbody>
</table>

#### 4.3 Results

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3.1 BayK-NP synthesis and characterization</td>
<td>87</td>
</tr>
<tr>
<td>4.3.2 BayK-NP effects ex vivo and in vitro</td>
<td>90</td>
</tr>
<tr>
<td>4.3.3 In vivo functional experiments</td>
<td>94</td>
</tr>
<tr>
<td>4.3.4 Nanoparticle formulation effects on BayK toxicity</td>
<td>97</td>
</tr>
<tr>
<td>4.3.5 Single vessel ligation lymphedema model characterization</td>
<td>99</td>
</tr>
<tr>
<td>4.3.6 BayK-NP for the treatment of lymphedema</td>
<td>102</td>
</tr>
</tbody>
</table>

#### 4.4 Discussion

#### 4.5 Conclusions

### CHAPTER 5. Concluding Remarks and Future Directions

#### 5.1 Conclusions

#### 5.2 Contributions to the Field

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.1 Enhanced LN drug delivery with lymph-directed NO</td>
<td>119</td>
</tr>
<tr>
<td>5.2.2 BayK-NP enable in vivo, lymphatic application of BayK</td>
<td>120</td>
</tr>
<tr>
<td>5.2.3 A novel approach to the treatment of diseases of lymphatic dysfunction</td>
<td>121</td>
</tr>
</tbody>
</table>

#### 5.3 Future Directions

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3.1 NO application to enhance delivery of therapeutics to LNs</td>
<td>122</td>
</tr>
<tr>
<td>5.3.2 Probing the mechanisms of NO’s effects on LEC barrier function</td>
<td>123</td>
</tr>
<tr>
<td>5.3.3 BayK-NP applications in basic science and disease treatment</td>
<td>124</td>
</tr>
</tbody>
</table>

### REFERENCES

127
LIST OF FIGURES

Figure 2.1 Structure and function of the lymphatic system. 13
Figure 2.2 Size-based distribution of injected species from the interstitium. 21
Figure 2.3 Size-based access of lymph-borne species to the LN. 28
Figure 3.1 Production and characterization of lymphatic-draining SNO-NP, and potential effects of delivered NO. 47
Figure 3.2 Lymphatic transport is regulated by molecule size, and a tracer panel can probe this differential transport. 50
Figure 3.3 Active transport of microparticles from injection site is unaltered by SNO-NP co-injection. 53
Figure 3.4 LN cell gating strategy. 54
Figure 3.5 LN-targeted NO delivery by SNO-NP, but not by SNAP, increases penetration of passively draining 30 nm tracer into the dLN. 56
Figure 3.6 Confocal images of LN draining forelimb tracer injections. 57
Figure 3.7 NO effects on vasculature. 58
Figure 3.8 Effect of SNO-NP treatment on 30 nm tracer positivity and ZO-1 expression in LECs in vitro. 60
Figure 3.9 SNO-NP treatment increases the association of passively drained 30 nm tracer with LN cells in vivo, but not of smaller 5 nm tracers. 62
Figure 3.10 SNO-NP treatment increases association of peptide-conjugated NP-S-S-CSIINFEKL with LN cells but does not affect antigen cross-presentation or cell activation. 65
Figure 3.11 NP-S-S-CSIINFEKL characterization. 66
Figure 4.1 Nanoparticles provide a lymphatic vessel targeting advantage compared to free drug, and allow for efficient loading and controlled release of small molecule Bay K. 89
Figure 4.2 BayK-NP loading and characterization. 90
Figure 4.3 Effect of BayK-NPs on lymph pumping ex vivo in isolated rat mesenteric lymphatic vessels (RMLVs). 92
Figure 4.4  BayK-NP do not induce T cell activation in vitro.  
Figure 4.5  BayK-NP enable BayK to acutely improve lymphatic pumping in vivo.  
Figure 4.6  In vivo lymphatic function analysis.  
Figure 4.7  BayK-NP show reduced side effects and toxicity compared to free drug.  
Figure 4.8  The single vessel ligation lymphedema model shows dysfunction in intact lymphatic vessels.  
Figure 4.9  WT lymphedema functional metrics separated by swelling severity.  
Figure 4.10  Chronic BayK-NP treatment improves early lymphedema outcomes.  
Figure 4.11  Structural tail changes measured in H&E stained sections.  
Figure 4.12  BayK-NP treatment of lymphedema in obese mice.  
Figure 4.13  Lymphedema immune analysis in obese mice.
AF-647  Alexa Fluor 647
ALT    Alanine transaminase
APC    Antigen-presenting cell
AST    Aspartate aminotransferase
AUC    Area under the curve
BayK   S-(-)-Bay K8644
BayK-NP BayK-loaded NP
cDC    Conventional dendritic cell
DC     Dendritic cell
DCM    Dichloromethane
dDC    Dermal dendritic cell
dLN    Draining lymph node
DMF    Dimethylformamide
DMSO   Dimethyl sulfoxide
ECM    Extracellular matrix
eNOS   Endothelial nitric oxide synthase
H&E    Hematoxylin and eosin
i.d.   Intradermal
i.p.   Intraperitoneal
i.v.   Intravenous
LC     Langherhan’s cell
LEC    Lymphatic endothelial cell
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMC</td>
<td>Lymphatic muscle cell</td>
</tr>
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<td>LN</td>
<td>Lymph node</td>
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<tr>
<td>LV</td>
<td>Lymphatic vessel</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NHS</td>
<td>M-hydroxysuccinimide</td>
</tr>
<tr>
<td>NIR</td>
<td>Near infrared</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<td>NO$_2$</td>
<td>Nitrite</td>
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<tr>
<td>NP</td>
<td>Nanoparticle</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
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<tr>
<td>PDS-NP</td>
<td>Pyridyl-disulfide nanoparticle</td>
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<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
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<tr>
<td>PPS</td>
<td>Poly(propylene sulfide)</td>
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<tr>
<td>PPS-NP</td>
<td>Poly(propylene sulfide) nanoparticle</td>
</tr>
<tr>
<td>RCT</td>
<td>Reverse cholesterol transport</td>
</tr>
<tr>
<td>SH-NP</td>
<td>Thiolated PPS-NP</td>
</tr>
<tr>
<td>SNAP</td>
<td>S-nitroso-N-acetylpenicillamine</td>
</tr>
<tr>
<td>SNO</td>
<td>S-nitrosothiol</td>
</tr>
<tr>
<td>SNO-NP</td>
<td>S-nitrosated nanoparticle</td>
</tr>
<tr>
<td>SSM</td>
<td>Subcapsular sinus macrophage</td>
</tr>
</tbody>
</table>

xi
SV-LEC  Murine LEC cell line
THF  Tetrahydrofuran
Treg  Regulatory T cell
TRITC  Tetramethylrhodamine isothiocyanate
ZO-1  Zonula occludens-1
SUMMARY

The lymphatic system plays a critical role in both fluid balance and the immune response, and its dysfunction is implicated in a wide variety of pathologies including cardiac disease, cancer, and lymphedema. While this makes lymphatic tissues promising therapeutic targets, lymphatic drug delivery is challenging as small molecule drugs show poor lymphatic vessel uptake by any delivery route. Once drugs have entered lymphatic vessels, access to therapeutically relevant cells within the lymph node is further restricted by cellular and physical barriers. There is a clear need for delivery methods to enhance drug access to therapeutic targets in lymphatic tissues, including both lymphatic vessels and lymph nodes. As such, the overall objective of this work was to employ engineered nanoparticle systems to enhance and control drug delivery to the lymphatics to enable the study and therapeutic modulation of 1) the lymph node and lymph node-resident cells and 2) intrinsic pumping in collecting lymphatic vessels. To this end, two nanoparticle platforms were advanced: one in which the highly reactive small molecule nitric oxide was chemically conjugated to nanoparticles and applied to modulate macromolecule penetration into lymph nodes, and one in which a calcium channel modulator was encapsulated and applied to improve lymphatic vessel pumping function for the treatment of lymphedema. This work revealed the benefit that biomaterial platforms provide to enable small molecule delivery to lymphatic tissues and the versatility of the employed nanoparticle platform, developing both a mechanism for enhancing drug access to typically restricted cells in the lymph node and a novel treatment for lymphedema and other diseases characterized by lymphatic dysfunction.
CHAPTER 1. INTRODUCTION

1.1 Motivation

The lymphatic system is a complex network of vasculature and immune organs that fulfills critical physiological functions, maintaining fluid balance, promoting lipid transport [1], and enabling the immune response. Lymphatic vessels (LVs) drain excess fluid, termed lymph, from the tissue interstitium and return it to the blood circulation at the subclavian vein, preventing deleterious accumulation of fluid. Additionally, lymph provides a critical transport pathway for a wide variety of solutes, proteins, and cells. A large portion of dietary lipid, for example, is transported via chylomicrons that are taken up into specialized intestinal lymphatic vessels called lacteals [2], making the lymphatic system vital in lipid absorption. Transport via lymphatic drainage is also important in the immune response due to the lymphatic system’s close relationship with the immune system. Lymph carries soluble proteins, antigen, and migratory immune cells from the periphery to lymph nodes (LNs), where resident immune cells can process and respond to soluble or presented antigen based on its size and spatial access within LNs.

Because of the multifaceted roles of the lymphatic system, lymphatic tissue function, or dysfunction, is involved in a wide variety of diseases. The lymphatic system’s role in lipid transport makes it of significant interest in vascular plaque deposition and atherosclerosis development [3,4], intestinal lymphatic malformations cause serious pathologies [5], and lymphatic vessel dysfunction is associated with lipedema, an adipose tissue disorder [6]. Lymphatic vessels are also the primary tissue of interest in swelling diseases like lymphatic filariasis [7] or lymphedema, in which fluid drainage via lymphatic
vessels is impaired and the intrinsic contractility and pumping capabilities of collecting lymphatic vessels are often reduced [8]. Altered lymphatic transport can additionally play subtle roles in cardiovascular disease, as lymph drainage is important in heart health and recovery after myocardial infarction [9], and in metabolic disorders like obesity [10] and diabetes [11]. The lymphatic system’s close relationship with the immune system via LNs makes lymph drainage critically important in diseases with an immunological component. In cancer, for example, LNs are important not only as a site of potential cancer metastasis, but as a site for immune cells to mobilize and respond to lymph-borne tumor antigen [12–14], and targeting resident immune cells in tumor-draining LNs with immune checkpoint blockade has been shown to promote the anti-tumor immune response [13]. Lymph drainage to LNs is also involved in initiation and exacerbation of autoimmune disease [15], and has been implicated in rejection of islet [16], corneal [17], and kidney grafts [18]. Because the lymphatic system plays important roles in so many diseases and applications, there is great interest in therapeutic regulation of lymph drainage, vessel pumping function, and LN-resident immune cells.

In spite of strong interest in therapeutic lymphatic modulation, progress is stymied by physiological barriers to lymphatic drug delivery. First, in order to enable drug efficacy, a significant concentration of drug must be achieved at the target site. In the context of the lymphatics this poses a unique challenge, as drug poorly accesses lymph from the blood circulation and many common administration routes. Instead, the most direct route into lymph is by harnessing interstitial drainage into afferent lymphatic vessels, which drains fluid and soluble factors from the interstitium after peripheral injection [19,20]. While appropriate selection of an administration route may improve lymph concentration of drug,
the ability of a molecule to drain into lymph is further restricted based on its size; small molecules, and thus many potential therapeutic candidates, are primarily cleared into blood circulation from the interstitium, while larger molecules (10-100 nm) restricted from blood access show superior drainage into lymph [19,21,22]. If an administered drug is able to reach the lymph and drain to LNs, there are further physical and cellular barriers in the LN itself that prevent the penetration of large molecules and particles into the parenchyma and the resident immune cells housed there. Large species, for example, are restricted to the LN periphery by a barrier of lymphatic endothelial cells (LECs) that line the sinus and inhibit their transport into deeper LN regions [23], and only small species can rapidly bypass this barrier by accessing the conduit system, a network of channels that bridges the LEC barrier and provides access to the LN parenchyma [24,25]. This produces a paradoxical challenge for lymphatic drug delivery, as the same molecules large enough to achieve lymph drainage are restricted from penetrating into the LN. There is thus a clear need to develop and optimize lymphatic drug delivery platforms to overcome these transport barriers and enable the application of a wider variety of drugs within the lymphatics that can target both lymphatic vessels themselves, and downstream LNs and their resident immune cells.

Engineered biomaterials represent a promising approach to enabling small molecule delivery into lymphatic tissues. Nanoparticles (NPs) are particularly useful, as they can serve as drug delivery vehicles in an ideal size range for promoting lymphatic uptake of both the NP and its payload. Although dysfunction and loss of contractility in LVs is associated with many pathologies, NP platforms to deliver therapeutics to LVs and promote their function have gone unexplored. Biomaterials for LN-targeted drug delivery
have received more attention; silica NPs [26], metallic NPs [27], and liposomes [28] have all been employed to promote lymph drainage for vaccine delivery, for example, and polymeric NPs have been delivered to tumor-draining LNs to promote anti-cancer immunity [12]. To maximize the efficacy of such approaches, however, platforms should be developed that deliberately modulate and overcome transport barriers within LNs that typically restrict the penetration of large molecules, improving drug penetration into the LN parenchyma and specifically targeting delivery to relevant immune cell subtypes. For example, a system has been developed that enables timed release of small molecule drug from its larger NP vehicle after drainage into lymph. While a drug permanently bound to its NP carrier is restricted to the LN periphery due to a barrier of sinus-lining LECs, small drug released from its NP carrier in the sinus can penetrate deeply into the LN by accessing the conduit system [29]. This highlights the promise of controlled release approaches that carefully consider LN transport barriers to improve treatment efficacy.

As such, the overall objective of this work is to develop engineered biomaterial platforms to overcome lymphatic transport barriers and improve drug delivery to therapeutically relevant lymphatic tissue targets, enabling the study and therapeutic modulation of 1) the lymph node and lymph node-resident cells and 2) intrinsic pumping function of collecting lymphatic vessels. Two model small molecule drugs are investigated; the highly reactive small molecule nitric oxide (NO) is conjugated to NP to evaluate the role of NO in lymphatic transport, with focus on its effects on drug distribution within the LN, while S-(-)-Bay K8644 (BayK), a calcium channel modulator, is delivered to its lymphatic muscle cell (LMC) target within collecting lymphatic vessels. The central hypothesis is that the transport barriers limiting lymphatic uptake and LN access of
delivered drugs can be overcome by employing polymeric nanoparticles as drug delivery vehicles, promoting the availability of drug within lymphatic tissues and, subsequently, their efficacy. This hypothesis is tested in the following specific aims.

1.2 Specific Aims

1.2.1 Specific Aim 1: Investigate effects of nitric oxide on lymph node access of biomolecules and particulates.

The working hypothesis of this aim is that nanoparticle delivery of NO promotes lymphatic accumulation of this highly reactive small molecule drug and enables its application to alter lymph node distribution and cellular uptake of co-delivered drugs and biomolecules. To explore this idea, polymeric S-nitrosated nanoparticles (SNO-NP) containing chemically conjugated NO were applied to an in vivo mouse lymphatic transport model to deliver NO to LN alongside model lymph-draining tracers and investigate spatial and cellular tracer distribution using confocal microscopy and flow cytometry. By including fluorescent tracers over a biologically relevant size range – 5, 30, and 500 nm in diameter – we were able to investigate the effects of NO application on three distinct lymphatic transport pathways after intradermal (i.d.) injection: passive transport of species with poor lymphatic uptake and high LN penetration, passive transport of species with excellent lymphatic uptake and poor LN penetration, and active transport of large species reliant on trafficking by migratory immune cells to access lymph, respectively. Lymph-directed NO treatment via SNO-NP was observed to increase the LN penetration depth of passively draining 30 nm tracer, a species which is typically restricted to the LN periphery, without a corresponding increase in its bulk LN accumulation or any impact on its transport
into LN from systemic circulation. *In vitro* modeling of tracer transport across the LN lymphatic endothelial cell (LEC) barrier showed that SNO-NP treatment increases the permeability of this cellular barrier to 30 nm tracer, revealing a potential mechanism for the enhanced LN penetration observed *in vivo*. Flow cytometric analysis of LN-resident immune cells revealed that the increased LN penetration of 30 nm tracer was also accompanied by an increase in cellular association of the tracer, particularly with B and T lymphocytes that reside in the LN parenchyma. These effects further extended to a model NP drug delivery system, which showed increased association with lymphocytes and dendritic cells (DCs) when co-delivered with SNO-NP. Overall, these results improve our understanding of the role of NO in lymph node transport and highlight the therapeutic potential of using lymph-directed NO to enhance drug access to parenchymal LN-resident cells, an outcome with significant implications for improving control of the immune response. This work is presented in Chapter 3.

1.2.2 *Specific Aim 2: Develop a nanoparticle system to encapsulate and deliver hydrophobic small molecule drug to lymphatic vessels for the treatment of diseases of lymphatic dysfunction.*

The *working hypothesis* of this aim is that small molecule drugs like calcium channel agonist Bay K8644 (BayK) have limited efficacy in LV modulation applications because of poor lymphatic uptake, and that NP delivery platforms can be employed to improve drug access to LVs, promote LV pumping function and lymph flow, and improve outcomes in diseases of lymphatic dysfunction. To investigate this hypothesis, a NP vehicle was first developed to encapsulate and provide controlled release of hydrophobic small molecule BayK. BayK was found to partition with high efficiency into the hydrophobic
core of a poly(propylene sulfide) nanoparticle (PPS-NP), forming BayK-loaded NP (BayK-NP) that released their payload over the course of several days upon dilution. The ability of this BayK-NP platform to improve lymphatic pumping function was evaluated using near-infrared (NIR) imaging technology that allowed the imaging of lymph flow through mouse tail collecting lymphatic vessels in vivo and in real time. BayK-NP injection i.d. in the tail tip was demonstrated to significantly improve LV pumping function by several metrics, including contraction amplitude and overall transport, while injection of BayK in its free formulation was ineffective. Additionally, BayK-NP simultaneously reduced systemic BayK concentration compared to the free small molecule, and reduced the frequency and severity of side effects by releasing BayK in a targeted and controlled fashion. To evaluate BayK-NP efficacy in a model of lymphatic dysfunction, a novel mouse model of lymphedema was employed in which superficial lymphatic capillaries and some deep collecting LVs are ligated, but two LVs are left intact to provide a functional route of lymph transport from the tail. These intact LVs showed impaired pumping and drainage function as lymphedema progressed, resulting in tail swelling, but treatment with BayK-NP both improved vessel function and reduced tail swelling compared to NP controls. These results highlight the importance of lymphatic drainage for small molecule efficacy within LVs, and also represent the first known demonstration that lymphedema symptoms can be alleviated by directly targeting LV pumping function. This work is presented in Chapter 4.

1.3 Significance

The work presented herein highlights the therapeutic potential and versatility of a biomaterial nanoparticle system capable of small molecule delivery via chemical
conjugation or hydrophobic encapsulation to two distinct lymphatic tissues: collecting lymphatic vessels and lymph nodes. This NP platform enables the accumulation of small molecules in lymphatic tissues after locoregional administration by routes commonly employed in the clinic, overcoming physiological barriers that would typically inhibit small molecule drainage into lymph and result in their significant systemic exposure.

In Specific Aim 1, this approach was employed to deliver highly reactive small molecule NO to LNs via SNO-NP and elucidate the effects of NO on several lymphatic transport pathways, providing insight into the potential lymphatic applications of a molecule that has previously been primarily explored in the context of the blood vasculature. This work also explored the role of NO in the modulation of drug access to LN-resident cells, providing insight into cellular targeting and a mechanism by which to regulate spatial drug distribution within the LN. Because of the physical and cellular barriers that exclude large species from the LN parenchyma where therapeutically relevant immune cells reside, many lymph-draining drug delivery platforms, like protein conjugates and NPs, are limited in their immune cell access. The ability to enhance their penetration into LNs thus has significant implications for immunotherapy, which relies on drug access to immune cells in deep LN regions for efficacy. Previous work has shown that LN penetration and immunotherapy efficacy can be improved by employing a NP platform that releases its small, mobile payload in a timed fashion after drainage into lymph [29]. The studies described herein reveal a second approach to improving drug penetration – modulating the LN itself rather than modifying the drug delivery vehicle. Importantly, the beneficial effects of NO were only observed when NO was delivered specifically into lymph via SNO-NP; a conventional small molecule NO donor was ineffective at the same
dose. This further highlights the importance of lymphatic uptake for small molecule efficacy within lymphatic tissues, and informs future vehicle design for NO application in a lymphatic context.

In Specific Aim 2, PPS-NPs were loaded with small molecule calcium channel agonist BayK, which encapsulated into the NP hydrophobic core without the need for chemical conjugation. The resulting BayK-NP platform was well-suited for lymphatic drug delivery, providing enhanced lymph drainage and controlled release of encapsulated drug without the need for environmental stimulus. When administered in the mouse tail tip, BayK-NP were found to improve the pumping function of tail collecting lymphatic vessels significantly, while administration of free small molecule BayK at the same dose was ineffective. This again highlights the critical importance of lymph drainage for small molecule efficacy in lymphatic contexts, and specifically reveals the value of intraluminal delivery of drugs that act on LVs and their surrounding lymphatic muscle cells. While some investigations have developed biomaterial tools to promote lymphatic uptake and drug delivery to LNs, this work represents the first known attempt to deliver therapeutics to LVs using this powerful approach. When applied to treat a mouse model of lymphedema, BayK-NP were found to improve LV pumping function and reduce tail swelling compared to control NP, with the most pronounced effects in animals with less severe cases of lymphedema. Current clinical approaches to lymphedema focus only on management of disease symptoms; there are no therapeutic options that target the root causes of lymphedema, and the disease has no cure. Additionally, current research into lymphedema therapeutics focus on managing the inflammatory manifestation of the disease or on promoting LV regrowth, but none have addressed the LV dysfunction associated with
disease progression. The positive results observed after BayK-NP treatment thus have great implications for the future of lymphedema treatment, and represent the first known evidence that lymphedema symptoms can be ameliorated by directly targeting LV dysfunction.
CHAPTER 2. BACKGROUND AND LITERATURE REVIEW

2.1 Introduction

The lymphatic system (Fig. 2.1) [20,30] is a network of vasculature and immune organs that plays critical roles in fluid balance, lipid transport, and in immune cell trafficking and the immune response. Its function is vital in maintaining healthy physiology, and its dysfunction is associated with a plethora of diseases. While this makes lymphatic tissues promising therapeutic targets, physiological barriers to lymphatic drug delivery necessitate careful design of drug delivery approaches and formulations that maximize drug concentration within the lymphatic system to enable modulation of tissue function and lymphatic disease treatment. This background will thus explore the critical roles of the lymphatic system in health, diseases that result from lymphatic dysfunction, and current approaches and limitations to their treatment. It will additionally discuss challenges in lymphatic drug delivery resulting from unique lymphatic physiology and explore drug delivery approaches for overcoming these barriers, with specific focus on applications in both lymphatic vessels and in lymph nodes.

2.2 Drug delivery to lymphatic vessels

2.2.1 Lymphatic structure and function

Lymph formation begins in the capillary plexus, where blunt-ended lymphatic capillaries, termed initial lymphatics, extend amongst blood capillaries. On the arterial side of the capillary, hydrostatic pressure driving fluid efflux from the blood is higher than the osmotic pressure driving fluid influx due to the presence of solutes in the blood, resulting
in the movement of fluid, along with small molecules, some proteins, and other macromolecules, into the interstitial space. While some of this fluid can be reabsorbed into the blood on the venous side of the capillary, the net result is fluid filtration into the interstitium [31], [32]. In order to maintain fluid balance, avoiding both fluid accumulation and swelling in the tissue and excessive loss of blood volume, this fluid must be removed from the interstitium and returned into circulation; the lymphatic system fills this role.

Lymphatic capillaries consist of loosely connected lymphatic endothelial cells (LECs) on a discontinuous basement membrane [33] that are connected to the surrounding extracellular matrix (ECM) by anchoring filaments [34,35] which prevent vessel collapse under pressure. These overlapping LECs produce primary valves that are highly permeable to fluid flow from the interstitium as interstitial pressure increases, and the closing of these valves prevents fluid flow back into the interstitium as lymphatic pressure rises [36,37]. These lymphatic pores are large enough to allow both fluid and a variety of molecules from the blood and interstitial space to enter the lymphatics, including small molecules, soluble antigen, and proteins like albumin. Cells, particularly migratory dendritic cells, also enter the lymphatics via these gaps between overlapping endothelial cells, following chemokine gradients and fluid flow [38]. Once in the lymphatics, this clear fluid containing cells, soluble antigen, and macromolecules is termed lymph (Figure 2.1B).

From initial lymphatics in the capillary bed, lymph is moved away from the tissue as lymphatic capillaries merge into larger pre-collecting lymphatics, and finally collecting lymphatics. These vessels are less permeable than lymph-forming initial lymphatics, and consist of a layer of LECs on the luminal side of the vessel and an outer layer of specialized lymphatic muscle cells (LMCs). Unlike the circulatory system in which fluid flow is driven
by the heart, the lymphatic system lacks a central pump to produce pressure and induce flow. Instead, lymph flow is driven by both passive forces, including the contraction of surrounding skeletal muscle [39] and artery pulsations [33,40], and active contractions of the lymphatic vessel produced by lymphatic muscle cells. Coordinated, sequential contractions drive flow in one direction, and backflow is prevented by bileaflet valves [41] that separate functional pumping units, termed lymphangions [42]. Collecting vessels eventually merge into larger lymphatic ducts that return lymph into the blood circulation.

Figure 2.1. Structure and function of the lymphatic system (modified from [20]). A) Fluid leaves the tissue interstitium and enters the initial lymphatics, flowing through larger collecting vessels en route to the lymph node. Unidirectional flow is maintained by both primary valves in the initial lymphatics and secondary valves in collecting vessels, as well as coordinated lymphangion contraction produced by lymphatic muscle cells surrounding collecting vessels. Once in the lymph node, fluid flows
through the subcapsular sinus and out through efferent lymphatics. B) Initial lymphatics are composed of overlapping lymphatic endothelial cells on a discontinuous basement membrane that produce primary valves. These valves open as interstitial pressure increases, allowing fluid and soluble factors to enter the lymphatics, while filaments that anchor LECs to the extracellular matrix prevent vessel collapse. Migratory immune cells can also traffic into lymph between LECs. C) Solutes and migratory immune cells access the lymph node via afferent lymph. In the subcapsular sinus, molecules can access conduits depending on their size. Lymph then flows through the medullary sinus and out the efferent lymphatic vessel. Antigen-presenting cells carry their antigen to B cells residing in B cell follicles and T cells in the more central T cell zone to induce immune responses. Lymphocyte trafficking to and from the lymph node can also occur from systemic circulation via HEVs, and all cell movement occurs in the context of the lymph node extracellular matrix.

2.2.2 Lymphatic dysfunction in disease

Impaired lymphatic drainage or lymphatic vessel pumping are implicated in a wide variety of diseases. For example, lymphatic dysfunction is associated with metabolic disorders, like obesity [10,43,44], diabetes [11], and lipedema [6,45]. The lymphatics additionally play a critical role in maintaining tissue health in the cardiovascular system [46,47]. For example, lymphatic vessel obstruction negatively affects cardiac function, inducing edema and causing altered electrical signaling, ventricular fibrillation, and myofibrillar degeneration [48–50]. Chronic edema can also induce fibrosis in the cardiac interstitium and cause reduced cardiac output [51], contributing to the risk of heart failure [46]. Lymphatic drainage is also critical in mediating reverse cholesterol transport (RCT) [3,52][53] in the prevention of atherosclerosis, and lymphatic dysfunction often precedes atherosclerotic plaque formation [4]. Enhancing lymphangiogenesis, the growth of new lymphatic vessels, can improve RCT and reduce cholesterol accumulation [54], while inhibiting transport via lymphatic disruption impedes clearance of cholesterol-loaded macrophages from tissue [52,54]. Functional cardiac lymphatic vessels are also critical in recovery from myocardial infarction. Enhanced lymphangiogenesis and remodeling of
cardiac lymphatic vasculature has been observed after myocardial infarction [55], and blocking the lymphangiogenic response through VEGFR3 inhibition impedes cardiac lymphatic transport, resulting in increased inflammation and edema in a post-infarct heart [56]. Enhancing lymphangiogenesis reduces inflammation and fibrosis [55], and enhancing lymph drainage in the heart after ischemia can reduce tissue damage and prevent infarction development [9]. The significant roles of the lymphatic system in cardiovascular disease progression thus suggests that improving lymph drainage is a potential approach to treating these widespread diseases.

The most common lymphatic disease, however, is lymphedema, which occurs when lymphatic drainage is insufficient to maintain physiological interstitial fluid volumes, leading to edema and structural changes in the affected tissue [57]. In primary lymphedema, lymphatic vessels do not properly develop to transport homeostatic levels of fluid, often as a result of mutations in genes critical for lymphatic development [58], such as VEGFR-3 [59] or FOXC2 [60]. Secondary lymphedema, or acquired lymphedema, is the more common manifestation of the disease, and occurs due to damage to the lymphatic vasculature such as surgical LN removal in breast cancer treatment [61] or lymphatic filariasis [62]. Vasculature disruption results in impaired lymph clearance from the drainage basin, causing fluid accumulation in the affected tissue and potentially significant swelling. Lymphedema progression is not driven by fluid accumulation alone; rather, the disease is deceptively complex in its development, and is associated with altered LEC proliferation, tissue inflammation and altered immune cell infiltration, skin thickening, and adipose tissue remodeling [63–65] in addition to swelling. Collecting lymphatic vessels in lymphedema additionally show reduced pumping function and ability to propel lymph in
both mouse models of lymphedema [66] and in human patients; lymphedematous legs show irregular contractions too weak to propel lymph [8], and lymphatic congestion lymphoscintigraphy measurements reveal lymphatic pump failure in patients with arm lymphedema after breast cancer surgery [67]. The precise driver of disease progression in this complex situation is unknown, but it is thought to be multifaceted, with each component of the pathology further worsening dysfunction. For example, damage to lymphatic vessels and fluid drainage disruption can induce DC migration to the lymphedematous tissue, where they activate, triggering a cascade of inflammatory responses that eventually result in chronic inflammation and pathological immune cell infiltration [68]. Inhibited lymph drainage and chronic inflammation result in impaired immune cell migration from the tissue [69,70]. This immune dysregulation and resulting inflammation can then also disrupt lymphatic vessel contractility, particularly through the production of iNOS by immune cells surrounding vessels [71], and inflammatory disease is often associated with lymphedema. In this way lymphedema progression is a complex feedback loop with poorly understood physiology, making disease treatment challenging.

Accordingly, there is no known cure for lymphedema. Even though the disease affects millions [72,73], symptoms are primarily managed in the clinic by compression garments to minimize swelling and manual lymph drainage, or massage, to promote lymph clearance [74]. While such interventions may provide temporary relief, they do not address the root cause of lymphedema and can only slow disease progression. More extreme and less common interventions employed in the clinic include surgical approaches like autologous LN transfer [75] to promote lymphatic vessel regrowth to bridge defected regions, or microsurgical procedures to reconnect lymphatic vessels to nonobstructed
lymphatics or local veins. These approaches face challenges such as donor site morbidity, varied patient responses, and many contraindications, and are thus underexplored and reserved for specific cases [76,77]. Surgical debulking procedures may also be employed to remove diseased tissue and reduce limb mass [78], particularly in severe cases that do not respond to compression therapy. Ongoing research in lymphedema treatment primarily focuses on lymphangiogenesis modulation or inflammation regulation. Nanofibrillar collagen scaffold BioBridge, for example, is under investigation as a tool to promote directional growth and organization of cells across a defect, promoting lymphangiogenesis and the generation of lymphatic collecting vessels [79,80], and there is evidence that targeting the inflammatory arm of lymphedema via leukotriene B4 antagonists like ketoprofin [81] and bestatin [82] can reduce edema and pathologic tissue remodeling. Such approaches are not yet part of a typical lymphedema treatment regimen.

Importantly, the interventions and investigative therapeutics described here attempt to treat lymphedema by modulating only a single facet of a complex disease, whether that is tissue remodeling (debulking), lymphatic vessel regrowth (BioBridge), or inflammation (leukotriene B4 antagonists). Although it is well established that impaired lymphatic vessel pumping function is a critical deficiency associated with lymphedema, no treatment to date has attempted to regulate the intrinsic contractility of LVs as a means to prevent lymphedema development.

2.2.3 Regulation of lymphatic vessel pumping

The intrinsic contraction of lymphatic vessels is granted by lymphatic muscle cells, which, in spite of often being referred to as lymphatic smooth muscle cells, share features
with both striated cardiac muscle and vascular smooth muscle [83]. As expected, lymphatic muscle cell contractions are electrophysiological processes regulated by ion flux; contraction strength is driven primarily by Ca2+ flux through L-type voltage-dependent calcium channels [84] and contraction frequency by T-type calcium channels [85], with additional contributions to contractility by K+ channels [86] and Cl- flux. Lymphatic vessel contraction can also be regulated by a variety of inflammatory mediators, a critical point due to the lymphatic system’s important role in the inflammatory response. Histamine, secreted by immune cells during inflammation, drives lymph formation and flow both by increasing capillary permeability and subsequent interstitial pressure [87] and through direct effects on lymphatic muscle cells [88]. Arachidonic acid metabolites are also critically involved in lymphatic pumping regulation; prostaglandins have been observed to induce lymphatic contractions, thromboxane synthesis inhibition suppresses contraction [89], and leukotriene B4 antagonism improves lymphatic function and inflammation in a mouse tail lymphedema model [82]. Perhaps the most well-studied regulator of lymphatic contractions is nitric oxide (NO), a reactive small molecule known for its inflammatory roles and its vasoactive effects in the blood vasculature that plays complex roles in lymphatic pumping. Lymphatic endothelial cells express endothelial nitric oxide synthase (eNOS), producing a basal level of nitric oxide required for normal lymphatic pumping; when eNOS is inhibited [90] or exogenous NO is added [91], normal pumping function is disrupted. NO production is thought to be tightly regulated both spatially and temporally within the lymphatics, with the pulsatile flow of lymph in vessels driving transient, cyclical production of NO, especially in the region of secondary valves, that supports coordinated contraction [92].
While there are thus many potential drugs that could be investigated for therapeutic modulation of LV pumping function, S-(-)-Bay K8644 (BayK) shows significant promise. This hydrophobic small molecule agonizes the L-type calcium channels on LMCs that are critical in regulating LV pumping function [84,93] and has been applied as a research tool to investigate LV contractility regulation ex vivo [94–96]. It has been shown that agonizing L-type calcium channels with BayK can improve functional metrics like ejection fraction [95] and contraction amplitude [93], while antagonizing with drugs like nifedipine inhibits LV function [97], highlighting the importance of this pathway for LV functional regulation. Application of BayK for therapeutic enhancement of vessel function is limited, however, by the ubiquitous expression of L-type calcium channels on non-target cells like cardiac muscle cells [98], neurons [99], and skeletal muscle [100], which causes off-target delivery of BayK to have significant side effects and toxicity [101].

2.2.4 Lymphatic vessel drug delivery challenges

Although there is great interest in delivering therapeutics into lymph and lymphatic tissues, there are physiological barriers to achieving sufficient drug concentration within lymph. Many administration routes employed in clinical or preclinical settings, including intravenous (i.v.), intraperitoneal (i.p.), or oral administration, result in high drug concentrations within the blood circulation. While this is useful for systemic drug delivery, drug access to lymph from within the blood circulation is limited [19], which may necessitate high dosing or repeated treatments to enable efficacy within lymphatic tissues. To overcome this limitation, peripheral or locoregional injection routes can instead be employed, including intradermal, subcutaneous, or intramuscular administration, to provide a lymph uptake advantage compared to systemic administration. Such
administration routes take advantage of the lymph formation process and the natural flow of fluid from the interstitium into lymph, providing enhanced access of species injected in the interstitial space to LVs and LNs draining the injection site [19,21,22]. Such approaches are commonly employed in the clinic, where peripheral injection is the preferred administration route for many vaccines [102], cancer therapeutics [103], and drugs for the management of autoimmune disease [104]. Locoregional administration not only provides improved drug access to lymphatic tissues, but also allows for selective targeting of specific LVs and LNs based on injection location. Because of the unique structure of the lymphatic system, in which vessels provide paths for unidirectional flow from a drainage basin and away from the periphery, specific vessels and the LNs to which they connect can be targeted by careful selection of an injection site “upstream.” This approach has been used to specifically drug tumor-draining LNs, or LNs whose lymph drainage basins contain a tumor and are thus bathed in tumor antigen; targeting therapeutic delivery to these LNs specifically by using locoregional administration has been shown to improve cancer treatment outcomes while enabling reduced dosing [13]. Peripheral administration thus represents a promising approach to promoting drug access to lymph, and thus to LVs and LNs through which they will drain.

After injection in the interstitial space, the biodistribution of injected molecules and particles is largely dependent on their size [19,105–107] (Fig. 2.2). Species smaller than about 100 nm in diameter are unrestricted by the interstitial extracellular matrix and can passively drain into blood or lymph. Very small molecules (<10 nm) can penetrate both blood and lymphatic endothelium, but tend to partition primarily into the blood because of its large volume and high flow rates [19,106]; this results in significant exposure to
systemic circulation, but poor lymphatic access. Larger molecules (10-100 nm) are too large to access the blood vasculature and are restricted to lymphatic uptake, significantly improving lymphatic access while minimizing loss into the blood. Very large particles (>500 nm) are matrix-restricted and cannot passively drain into lymph from the interstitium; these particles are instead taken up by migratory antigen-presenting cells (APCs) and actively transported into lymph [105]. This size-based distribution and transport has significant implications for drug delivery to lymphatic targets, as species size dictates the extent of lymphatic drainage, the resulting concentration within lymph, and subsequent efficacy at its target site.

![Figure 2.2](image)

**Figure 2.2.** Size-based distribution of injected species from the interstitium. Small species (<10 nm diameter, blue) experience significant clearance into the blood circulation and poor lymphatic uptake, while mid-size species (10-100 nm diameter, red) have improved access to lymph. Molecules larger than ~100 nm in diameter (green) cannot passively drain due to restriction by the ECM, and are instead trafficked into lymph by migratory immune cells.

Importantly, many drugs of interest for therapeutic modulation of LV function are less than 10 nm in diameter; histamine, nitric oxide, prostaglandins, thromboxane, and many ion channel modulators like BayK, nifedipine [97], and pinacidil are all small molecules, and would thus have poor lymphatic access if directly injected into the interstitial space. This is a significant limitation for LV drug delivery, as high administered
concentrations or frequent dosing may be required to achieve sufficient drug concentration within lymph and in the LV lumen.

2.2.5 Biomaterials as tools to improve LV drug delivery

Because delivery of small molecule therapeutics into lymph for therapeutic modulation of LV function is limited by small molecules’ poor uptake into lymph after injection in the interstitium, LV delivery could potentially be enhanced by using a larger drug delivery vehicle to essentially increase the size of a small payload and bias its drainage towards the lymphatics. Ideally, such a platform would be 20-100 nm in hydrodynamic diameter, the optimal size range for lymphatic uptake; contain large amounts of small molecule drug to maximize lymph concentration; provide controlled release of drug over a physiologically relevant timescale, which may vary based on the mechanism of action of the delivered drug; maintain bioactivity of the released drug, and not require chemical modification or conjugation of drug that inhibits its efficacy; and would break down into nontoxic components that are easily cleared.

Some of these requirements have been met by platforms employed to improve lymph drainage of a small payload. For example, molecular vaccines that bind to endogenous, lymph-draining albumin after injection have been shown to improve lymph drainage and LN accumulation of vaccine cargo [108]. Exogenous drug delivery vehicles have also been developed to promote lymph transport for improved delivery of vaccines and cancer immunotherapies, including silica [26], lipid-based [28], metallic [27], and polymeric NPs [12,109]. A versatile polymeric NP platform composed of a pluronic corona and poly(propylene sulfide) core (PPS-NP) has also been developed and applied for a
variety of lymph-directed drug delivery applications. At 30 nm in diameter the NP is optimally sized for lymphatic uptake, breaks down into nontoxic components, and can be employed to deliver drug conjugated to its thiolated core [110] or its functionalized corona [12,29,111]. PPS-NP have also been shown to encapsulate hydrophobic drug in their core without the need for chemical conjugation, and to release that payload slowly over the course of days upon dilution [12]. This platform may thus be a promising candidate for delivery of hydrophobic small molecules for the enhancement of LV pumping function.

Importantly, however, these platforms that harness controlled drug delivery and improved lymphatic drainage have heretofore only been employed for LN-targeting, immunomodulatory applications, not for direct therapeutic modulation of lymphatic vessel function. The few NP platforms that have been developed to promote lymphatic drainage and to intraluminally target LVs have been employed only for lymphatic imaging applications, including metallic nanoparticles conjugated to anti-podoplanin antibody for imaging of breast cancer lymphatic vessels [112] and lectins for visualization of the lymphatic vasculature [113]. While these imaging applications highlight the potential value of enhancing LV access by promoting lymph drainage, this approach has gone unexplored in a therapeutic context and has not yet been employed to enable LV-targeted drug delivery and enhance LV function using nanoparticle platforms.

2.3 Drug delivery to lymph nodes

2.3.1 Lymph node structure and function

As lymph is moved through collecting lymphatic vessels it is filtered through LNs, secondary lymphoid organs that house cells of the adaptive immune system and provide a
highly organized environment that enables antigen presentation, immune cell mingling, and the generation of immune responses (Fig. 2.1C). Lymph enters LN via afferent lymphatic vessels and flows through the LN within LEC-lined sinuses; lymph spreads first through the subcapsular sinus, passes through transverse sinuses that surround and separate lobules, and finally flows through the medullary sinuses to exit via a single efferent lymphatic vessel at the LN base [114]. Afferent lymph provides a unidirectional pathway from the periphery to draining LNs for leukocytes, as well as for lymph-borne molecules like soluble antigen which are exposed to sinus-lining macrophages as they flow through LN sinuses. While many lymph-borne species are restricted from exiting the sinus by a barrier of sinus-lining LECs, very small molecules can access transendothelial channels that bridge this barrier and provide access to the conduit system, a network of channels supported by fibroblastic reticular cells (FRCs) that allow rapid access to the deeper LN parenchyma [115]. Fluid in conduits is sampled by antigen-presenting cells that can present captured antigen directly to T cells [116], enabling rapid processing of antigen small enough to access the conduit system. Alternatively, large antigen is also carried from the periphery by antigen-presenting cells (APCs) over a longer time scale [105] and is presented to B and T lymphocytes residing in distinct locations within the LN [114]. Once activated, these cells secrete products into efferent lymph, or themselves migrate out of the LN in efferent lymph to return to the periphery, driving the immune response. LNs are also vascularized, and contain regions called high endothelial venules (HEVs) that are specialized to enable lymphocyte migration [117], allowing circulating lymphocytes to enter LNs from the blood vasculature in search of their target antigen. These constant processes of cell migration, fluid movement, and antigen transport are all supported by the
reticular network and a system of LN stromal cells that provide a scaffold for these critical LN functions to occur [114,118].

Within the LN there are distinct structural regions, and cells preferentially reside in or home to specific areas. The superficial cortex lies at the lobule apex, and includes follicles and the interfollicular cortex between. B cells home to these follicles and interact with follicular dendritic cells, proliferating and forming germinal centers within follicles upon activation. T cells home to deeper cortical regions, termed the paracortex, where they interact with a different population of antigen presenting cells [114]. Migratory dermal dendritic cells (dDCs), for example, will tend to home to more peripheral B cell follicles after activation and migration to the LN, while Langerhans cells (LCs) migrate into the LN paracortex where T cells reside [119]. The precise spatial organization of LNs and the regulated access of antigen to immune cells based on size and mode of transport provide control over the type of cells that experience antigen and the context in which they experience it, enabling cell mobilization to drive the appropriate immune response.

2.3.2 LN as therapeutic targets in disease

Because of their critical role in enabling the immune response, LNs are attractive targets for immunomodulation and the treatment of immune-related diseases. For example, LNs play a vital role in response to infectious disease. Research has accordingly shown that improving vaccine delivery to LNs can improve outcomes, enhancing the humoral response to malaria after antigen delivery [28] and to the flu after adjuvant delivery [120]. LNs have also been targeted for the induction of tolerance in the context of autoimmune disease; for example, delivery of antigen and NF-κB inhibitors to LNs and their resident
APCs has been shown to induce the generation of antigen-specific regulatory T cells and to reduce symptoms of arthritis in mice [121]. LNs also play a critical role in graft rejection, where promoting lymph drainage from the implant to draining LNs has been observed to negaively affect graft survival [122]. Inhibiting this communication with the LN has accordingly improved outcomes in islet [16] and cornea transplantation [17] and reduced immune cell infiltration in heart transplants [123].

Perhaps most notably, LNs play a critical role in the immune response to cancer. LNs that drain lymph from a drainage basin that includes a tumor, termed tumor-draining LNs (tdLNs), are both bathed in tumor antigen that drains passively via tumor-associated lymphatics and are a critical site of antigen presentation by cells carrying antigen from the tumor [14,124,125]. Targeting delivery of cancer vaccines to tdLNs has thus shown great promise [12,26], and checkpoint blockade delivery to tdLN immune cells has been shown to improve the anti-tumor immune response and animal survival even in non-metastatic disease [13]. LNs can also contain cancer themselves due to the development of lymphoma or due to cancer metastasis from a primary tumor upstream [126], making LN-targeted delivery of chemotherapeutics [127,128] for direct killing of LN-resident tumor cells an area of significant clinical interest.

2.3.3 Barriers to LN drug delivery

As previously described, drug access to lymph, and thus to LNs that receive afferent lymph, is limited by many conventional administration routes, but can be promoted by employing interstitial injection routes that optimize lymph drainage and by utilizing nanoscale drug formulations of a size that biases clearance into lymph rather than into
systemic circulation, about 10-100 nm. Once migratory cells and soluble factors have reached the LN sinus via afferent lymph, however, their ability to penetrate deeper into the LN depends significantly on their size and mode of transport. As previously described, the sinus floor is lined with lymphatic endothelial cells (LECs) that regulate the passage of lymph-borne species into the LN parenchyma (Fig. 2.3); species must either penetrate this LEC barrier to access the deeper LN regions and resident immune cells, or be rapidly transferred via the sinuses into efferent lymph and exit the LN. Large immune complexes in the sinus may be recognized by lymph-sampling subcapsular sinus macrophages (SSMs) that extend projections across the LEC barrier and directly shuttle immune complexes into B cell follicles [129], providing a limited but LEC-independent route of transport into the LN parenchyma. Large molecules that are actively transported from the periphery within migratory APCs can also be carried into the LN by these cells, which can migrate across the LEC barrier driven by chemokine gradients and home to T or B cell regions within the LN [105].

Smaller species that are passively transported within lymph from the periphery, including soluble factors, antigen, cytokines, small proteins, antibodies, and drugs and drug delivery vehicles, are restricted from LN entry primarily based on their size. Throughout the sinus floor are small transendothelial channels that bridge the LEC barrier and provide direct transport between the subcapsular sinus and the conduit system, a system of channels that provides rapid access to deeper LN regions. Access to these channels, however, is regulated by a plasmalemma vesicle-associated protein (PLVAP) diaphragm [130] that restricts entrance to the conduits to molecules less than 70 kDa [25,115]. While small molecules and proteins may thus be able to rapidly bypass the LEC monolayer, molecules
larger than 70 kDa, which includes many large proteins and drug delivery vehicles, are restricted and excluded to the subcapsular sinus (Fig. 2.3). A final route into the LN parenchyma is by direct transport across the LEC barrier via vesicular transcytosis through LECs themselves or by paracellular transport. While the occurrence of paracellular leakage of soluble species has not been well investigated in LECs in the context of the LN, paracellular transport between initial lymphatic LECs is the primary route of lymph formation, and molecule leakage between LECs of collecting LVs has been observed particularly in disease states [11]. Transcytosis, or apical uptake and basal exocytosis of soluble factors by LECs, provides another potential route beyond the sinus. The types of molecules that are subject to this pathway are poorly understood, with recent evidence suggesting that this mechanism may be responsible for the rapid access of antibodies to the LN parenchyma [118].

![Figure 2.3. Size-based access of lymph-borne species to the LN.](image)

Together, this regulation of species transport within LNs results in a clear distinction between small species, which can penetrate more deeply into the LN and access
parenchymal regions via the conduit system, and larger species, which are restricted to the LN sinus. This poses a paradoxical challenge for LN drug delivery. While drug delivery platforms in the nanoscale size range are optimal for biasing species drainage into lymph from the interstitium after peripheral injection, the size-based regulation of species transport within the LN subsequently limits their ability to penetrate the LN and access the parenchyma compared to small species. This may limit the efficacy of nanoscale vehicles that show significant association with APCs but poor access to parenchymal B and T cells [29,131]. Intelligent design of drug delivery platforms to overcome these transport barriers or to improve nanoscale species penetration into the LN thus has the potential to improve the efficacy of immunotherapies targeting lymphocytes, or of chemotherapies targeting cancers resident in the LN parenchyma.

2.3.4 Nitric oxide in the lymphatics

While NO is perhaps most well-known for its effects on LV pumping function, as previously described, it also plays many other important roles and regulates many pathways that could impact the transport of species into and within the lymphatics. Immediately upon injection in the interstitial space, NO could have a variety of local effects that impact species distribution into blood and lymph. For example, NO is well-known for its effects in the blood vasculature, where it induces vasodilation [132,133] and can modulate vascular permeability not only by classical interaction with soluble guanylyl cyclase (sGC) [134] but also by S-nitrosation of junction proteins between BECs including p120-catenin and β-catenin [135,136] that results in their downregulation and reduced association with VE-cadherin at adherens junctions. Application of exogenous NO donors SIN-1 and SNAP has also been shown to increase microvascular permeability by reducing VE-cadherin
expression in BECs [137]. If injected in the interstitial space, NO could thus affect blood capillary permeability, impacting how species partition into blood and lymph. NO application has also been shown to modulate the activation and migration of skin-resident dendritic cells [138], which has significant implications for the active transport of large species that are restricted by the interstitial ECM and rely on migratory immune cells for transport to the LN.

NO’s ability to regulate lymphatic transport extends into the LN, where its effects on blood vasculature, lymphatic vasculature, and immune cells have further potential to impact spatial and cellular access of co-administered species. For example, NO can have direct immunomodulatory effects, as stromal cells including fibroblastic reticular cell and LECs produce NO to modulate T cell proliferation [139]. Just as NO has been shown to induce vasodilation in peripheral blood vasculature, its application in the LN has also been shown to cause vasodilation and remodeling of LN vasculature [140]; such effects could impact the ability of species to access LNs from the blood circulation, providing an alternative route of entry to afferent lymph drainage. One of the most important barriers to LN parenchyma access of lymph-borne molecules is the monolayer of sinus-lining LECs, which, as previously discussed, form a continuous layer of cells that excludes molecules too large to access conduits or which are not subject to transcytosis through LECs directly. While the effect of NO on the barrier integrity of these sinus-lining LECs has not been directly investigated, studies in analogous systems reveal effects of NO application on transendothelial transport that could impact species penetration into the LN if translated to sinus-lining LECs. For example, LEC transcytosis is known to be a dynamin-dependent process [118,141]. While the direct effect of NO on LEC transcytosis has not yet been
studied, NO application has been shown to enhance dynamin-dependent endocytosis in epithelial cells [142] and blood endothelial cells [143] by S-nitrosylation of a critical cysteine residue on the dynamin protein. Outside of the LN, NO has been shown to increase the leakiness of collecting lymphatic vessels [11]. Analogous in vitro studies of collecting vessel-lining LECs show similarly enhanced permeability with NO treatment [144]; while this permeability increase was associated with reduced expression of junctional proteins, transcytotic pathways were not evaluated. Permeability of a human dermal LEC monolayer has also been observed to increase with NO treatment, and could be rescued by treatment that promoted expression of LEC junctional protein zonula occludens-1 (ZO-1) [145]. While the precise mechanism of NO’s action in these studies has not been fully elucidated, nor has the effect of NO been evaluated in LN sinus-lining LECs, this evidence in analogous LEC and BEC systems that NO can increase permeability and impact junctional protein expression in endothelial cells supports the potential for NO to modulate transcytosis and paracellular transport within the LN.

2.3.5 Biomaterials for NO delivery

While NO has a wide variety of effects within the lymphatics and is thus of great therapeutic interest in this context, there are a variety of challenges associated with lymphatic delivery of NO. First, NO is a promiscuous signaling molecule that can influence many different pathways, with beneficial or detrimental effects depending on concentration and context. In order to be therapeutically applied, NO’s delivery must be carefully spatially and temporally regulated to avoid off-target effects. NO also has a very short half-life, necessitating targeted delivery to its site of action to enable efficacy. A variety of NO donors have been employed in the clinic and in research, including NONOates [143];
nitrates[146] like sodium nitroprusside (SNP)[11,147], glycercyl trinitrate (GTN)[91], and isosorbide mononitrate; and S-nitrosothiols (SNO) like S-nitrosoglutathione (GSNO) and S-nitroso-N-acetylpentamine (SNAP). Applications of such NO donors within lymphatic tissues, however, are limited by their small size, which would result in poor lymphatic uptake upon injection in the interstitium. Nanoscale biomaterials engineered to increase the size of NO and promote its access to lymph may thus be a powerful tool to enable NO’s application within lymphatic tissues.

To overcome this limitation and enable improved lymphatic drainage and controlled delivery of NO, we have previously developed S-nitrosated nanoparticles (SNO-NP) based on a PPS-NP. These SNO-NP are 30 nm in diameter, an optimal size to promote lymphatic drainage from the interstitium, and can be loaded with high concentrations of NO in the form of S-nitrosothiols in their thiolated core under acidic conditions. This novel NO formulation has been shown to dramatically improve lymphatic uptake and subsequent LN accumulation of NO compared to small molecule NO donor SNAP[110], and is able to release its NO payload as nitrite or by transnitrosation to low molecular weight thiols[148]. This SNO-NP platform thus represents a promising tool for controlled delivery of NO to LNs in vivo, enabling the study of NO’s effects on lymphatic transport and its therapeutic application in a lymphatic context.
CHAPTER 3.  LYMPH-DIRECTED NITRIC OXIDE INCREASES IMMUNE CELL ACCESS TO LYMPH-BORNE NANOSCALE SPECIES [149]

3.1 Introduction

Lymph nodes (LNs) are complex secondary lymphoid organs consisting of high concentrations of lymphocytes supported by stromal cells, extracellular matrix (ECM), and blood and lymphatic vessels [114]. Because of their critical role in the immune response, LNs are valuable therapeutic targets for immune modulation in a variety of contexts, including infectious disease vaccination [28,120], tolerance induction [150,151], cancer immunization [12], and prevention of graft rejection [152,153]. Therapeutics delivered for these purposes are diverse, and include small molecule drugs, peptides, larger antigen, combination nanoparticle (NP) delivery vehicles, and even microparticles, covering a broad range of molecule sizes. Regardless of the disease context, in order for LN-targeted delivery to be useful, these diverse therapeutics must be able to reach the lymphatics at sufficiently high concentrations, and have access to target cells within the LN. Therapeutic delivery to LN-resident cells is complicated, however, by biological barriers that inhibit drug accumulation in LNs and subsequent access to LN-localized immune cells.

The most direct route of molecule access to LNs is via their afferent lymphatics, taking advantage of lymph drainage patterns that shuttle interstitial fluid and its soluble factors from peripheral tissue directly to LNs. This pathway is critical in the immune response, providing rapid transport of migratory immune cells, antigen, and cytokines to
waiting LN-resident lymphocytes, and can also be exploited for targeted drug delivery to LNs. In the clinic, peripheral injection into intradermal (i.d.) or subcutaneous spaces is commonly employed for a wide variety of drugs, including monoclonal antibody (mAb) adalimumab (Humira) and fusion protein etanercept (Enbrel) for the management of autoimmune diseases; common vaccines including varicella, Bacillus Calmette–Guérin, and measles, mumps, and rubella vaccines; allergen immunotherapies; and even cancer treatments, including chemotherapeutic bortezomib and mAb rituximab. Improving LN access of such peripherally administered drugs is thus an active area of research. Vaccine efficacy has been shown to be improved by antigen or adjuvant conjugation to nanoscale carriers that promote lymphatic drainage [28,108], and similarly improved outcomes are observed when NPs are used to promote co-delivery of antigen and tolerogenic agents to LNs for the treatment of autoimmune disease [121,151]. These benefits have also been observed in cancer vaccination and immunotherapy, where increased delivery of tumor antigen and adjuvants to LN improves the anti-tumor immune response [12,26]. Enhancing LN access of peripherally administered drugs thus has significant clinical implications in a variety of disease contexts.

In the context of lymphatic transport regulation, nitric oxide (NO) is of particular interest. This reactive small molecule is known for its inflammatory action and vasoactive effects within the blood vasculature, but it is also a critical regulator of lymphatic pumping, vascular permeability, and immune cell activation, all of which can regulate transport within the lymphatics (Fig. 1A). Local application of NO could impact drug partitioning into blood and lymph from the interstitial space due to NO’s enhancement of vascular permeability [134,135,154], and NO has been observed to alter the activation and migration
of skin-resident migratory dendritic cells (DCs) [138]. NO is also a critical regulator of lymphatic vessel contractility [90–92], giving it the potential to modulate transport of lymph-draining molecules and their rate of accumulation within LN. Within the LN, NO regulates many pathways that could alter intra-LN molecular transport and subsequent cellular access. For example, NO enhances the permeability of blood and lymphatic endothelium [11,134]; increases blood vascular permeability and remodels LN vasculature [140]; and critically regulates immune cell activation and proliferation [139]. Because of its many roles within the lymphatics, NO could be a promising tool with which to regulate drug access to lymphatics and lymph node-resident cells.

While NO has been applied therapeutically for the treatment of angina [155] and pulmonary hypertension [156], its short half-life and low molecular weight make targeted NO delivery to lymphatic tissues challenging. To overcome these limitations and enable concentrated NO delivery to LNs, we previously developed S-nitrosated nanoparticles (SNO-NP) and characterized their NO release and delivery both *in vitro* [148] and *in vivo* [110]. SNO-NP were shown to release NO in the form of nitrite over the course of days [110,148], and to be capable of transnitrosation of biological small molecule thiols [148]. When administered *in vivo*, SNO-NP significantly improved accumulation of NO in LN compared to a traditional small molecule NO donor [110]. Herein, we apply this LN-targeted, NO donation system to investigate the effect of NO on lymphatic transport and LN distribution of co-delivered molecules, modeled using a panel of fluorescent tracers over a biologically relevant size range. We demonstrate that while SNO-NP application did not alter active transport of large molecules from the site of injection, passive lymph drainage, or total LN accumulation of small- or medium-sized molecules, NO delivery to
LN significantly increased the penetration of typically excluded mid-size (30 nm) molecules into the LN and enhanced their subsequent association with cortical B cells and paracortical T cells. Increased access to parenchymal LN cells was observed in both model dextran molecules and an antigen-conjugated NP drug delivery system, highlighting the versatility of NO’s effects. LN-targeted NO application thus represents a promising tool for the enhancement of drug delivery vehicle access to parenchymal LN-resident cells to facilitate improved control of the immune response.

3.2 Materials and Methods

3.2.1 Nanoparticle synthesis and characterization

Thiolated NP (SH-NP) were synthesized as previously described [110]. Briefly, 500 mg of Pluronic F127 was added to 10 mL of MilliQ water under argon and dissolved by stirring at 1500 rpm. 400 µl of propylene sulfide (TCI, Tokyo, Japan) was then added under argon. After 30 min, 42 mg of thiolated initiator (synthesized as previously described [157]) that had been activated in 322 µl of sodium methoxide for 15 min was added under argon, and the mixture was stirred for 15 min. 64 µl of 1,8-diazabicyclo[5.4.0]undec-7-ene was added under argon, and the solution was capped. After 24 h, the reaction was exposed to air for 2 h to cross-link the NP poly(propylene sulfide) core, and the NP were then dialyzed in a 100,000 Da membrane (Spectrum Labs, New Brunswick, NJ) against 5 L of Milli-Q water for three d, with six water changes. NP size was measured by dynamic light scattering on a Malvern ZetaSizer instrument. In select experiments, NP were reacted overnight at room temperature with an excess of Alexa Fluor 647 C2 maleimide (Thermo Fisher, Waltham, MA) and cleaned of unreacted dye by size exclusion chromatography. SH-
NP were nitrosated immediately before use by the addition of sodium nitrite in 0.5M hydrochloric acid. Excess sodium nitrite was quenched by addition of ammonium sulfamate, and SNO-NP were cleaned using a 7kDa Zeba column (Thermo Fisher). To evaluate NO release from SNO-NP, SNO-NP in 1X PBS were incubated in a closed tube at 37°C, and SNO and NO₂ concentrations were monitored over time using the Saville and Griess assays, respectively. To characterize NP drainage to and accumulation in draining LN from a forelimb injection, Alexa Fluor 647-labeled NP were injected i.d. in the forelimbs of mice. 24 h after injection, animals were sacrificed and skin removed to expose axillary and brachial LNs. Exposed LN were imaged using fluorescence detection on an IVIS Spectrum CT instrument, and fluorescent signal was quantified using LivingImage software. LN fluorescence levels were compared to animals injected in the forelimbs with either saline or unconjugated Alexa Fluor 647-NHS ester of equivalent brightness inactivated by overnight incubation at room temperature.

NP-S-S-CSIINFEKL were synthesized from pyridyl disulfide-functionalized NP (PDS-NP). PDS-NP were synthesized as previously described [111]. First, carboxylate-Pluronic F127 was synthesized. Briefly, 23.6 g of Pluronic F127 was dissolved in 500 mL of toluene. To this solution, 2.6 mL of triethylamine and 1.4 mL of methanesulfonyl chloride were added and reacted for 18 h. The solution was filtered, concentrated under reduced pressure, and precipitated in ether. The resulting solid was dissolved in 100 mL of dimethylformamide (DMF), and 2.29 g of potassium carbonate and 2 mL of mercaptopropionic acid methyl ester was added. After 20 h, the solution was concentrated under vacuum, dissolved in 100 mL DCM, and cleaned with 8 g of activated carbon. The carbon was removed by filtration, and the remaining solution was concentrated and
The resulting Pluronic thioether propionic acid methyl ester was precipitated in ether. The resulting Pluronic thioether propionic acid methyl ester was dissolved in 200 mL of Milli-Q water, reacted overnight with 0.57 g of sodium hydroxide, dialyzed for three days against 5 L of Milli-Q water, and lyophilized to yield carboxylate-Pluronic F127. COOH-NP were synthesized as described previously using carboxylate-Pluronic F127 rather than Pluronic F127. NP were labeled by reaction in 1X PBS with Alexa Fluor 647 C₂ maleimide (Thermo Fisher) overnight, and remaining unreacted core thiols were capped by reaction with N-ethylmaleimide (NEM) until no thiol signal could be detected by the Ellman’s assay. NP were PDS functionalized by consecutive reaction with EDC, NHS, and PDS in MES buffer overnight, followed by dialysis for three days. NP-S-S-CSIINFEKL were produced by reacting CSIINFEKL peptide (New England Peptide) with PDS-NP overnight, and NP-S-S-CSIINFEKL were cleaned of unreacted peptide by size exclusion chromatography using a CL-6B resin and concentrated for use.

3.2.2 Fluorescent tracers

Red and yellow-green carboxylate-modified 0.5 µm FluoSpheres were purchased from Thermo Fisher. 10 kDa and 500 kDa amine-dextrans were labeled by reaction in PBS with Alexa Fluor 610 N-hydroxysuccinimide (NHS) ester (Thermo Fisher) or Alexa Fluor 647 NHS ester (Thermo Fisher), respectively. Alternatively, 10 kDa Alexa Fluor 647-labeled dextran (Thermo Fisher) and 500 kDa tetramethylrhodamine isothiocyanate (TRITC)-labeled dextran (Sigma-Aldrich, St. Louis, MO) were purchased. Tracers labeled with a fluorophore in-house were purified of unreacted free dye by size exclusion chromatography using a CL-6B resin and concentrated before use. All tracers were maintained under sterile conditions.
3.2.3 Biodistribution experiments

Mice were simultaneously administered both NO treatment or controls and a tracer cocktail as a single injection into the forelimb skin. Treatment groups included SNO-NP delivering 800 nmol NO, as measured by the Saville assay; dose-matched SH-NP; S-nitroso-N-acetylpenicillamine (SNAP) delivering 800 nmol of NO; or saline. The tracer cocktail included red FluoSpheres (500 nm), Alexa Fluor 647 500 kDa dextran (30 nm), and Alexa Fluor 610 10 kDa dextran (5 nm) at $1.2 \times 10^8$ spheres, 23.8 µg, and 3 µg, respectively. All components were delivered in a single 40 µl injection. At 4, 24, or 72 h after injection, animals were sacrificed and blood was harvested by cardiac puncture. Other organs, including liver, kidney, spleen, and LNs draining the forelimb injection (axillary + brachial, pooled) were harvested, weighed, and homogenized in 400 µl of 1X PBS using 1.4mm acid washed zirconium beads (OPS Diagnostics, Lebanon, NJ). The fluorescence of 100 µl of each tissue homogenate was measured in triplicate using a Synergy H4 BioTek plate reader. After compensation was applied to account for the low overlap in tracer fluorescence, the concentration of each tracer in each tissue was calculated using standard curves generated in a background of each individual tissue homogenate.

In experiments to evaluate tracer access to LNs from the circulation, fur was removed from the dorsal skin of mice and treatment (700 nmol SNO-NP, dose-matched SH-NP, 700 nmol SNAP, or saline) was injected i.d. in four locations in the dorsal skin: above the left and right shoulders and the left and right flanks. Four mice were used per treatment (in two independent experiments), and each mouse received four injections of the same treatment. Immediately, animals were given a tracer cocktail containing 238 µg Alexa Fluor 647 500 kDa dextran (30 nm), 58.6 µg Alexa Fluor 700 40 kDa dextran (10
nm), and 30 μg Alexa Fluor 610 10 kDa dextran (5 nm) by intravenous injection in the jugular vein. 4 h after tracer injection, animals were sacrificed and axillary, brachial, and inguinal LN draining the dorsal injections were individually harvested, homogenized, and their fluorescence analyzed as previously described.

3.2.4 Lymph node immune cell access experiments

In in vivo tracer access experiments, mice received 40 μl injections in both forelimbs containing both a tracer cocktail and NO treatment or controls. Treatment groups included SNO-NP delivering 800 nmol NO, as measured by the Saville assay; dose-matched SH-NP; SNAP delivering 800 nmol of NO; or saline. The tracer cocktail included yellow-green FluoSpheres (500 nm), TRITC 500 kDa dextran (30 nm), and Alexa Fluor 647 10 kDa dextran (5 nm) at 1.2 × 10^8 spheres, 23.8 μg, and 3 μg, respectively. A sample size of three mice with two forelimb injections each was used for each treatment group, and the full experiment was repeated twice. 72 h after injection, when all NO is expected to be released, animals were sacrificed and LNs draining the forelimb injection (axillary and brachial) were harvested. After 30 min incubation with 1 mg/ml collagenase D at 37°C, LN were gently separated through a 70 μm cell strainer using a 1 mL syringe plunger, and the strainer was thoroughly washed. Cells were isolated by centrifugation at 400 rcf for 5 min, and pellets were resuspended and transferred to a round bottom 96 well plate for staining. Cells were incubated with 100 μl of 2.5 μg/ml 2.4G2 (Tonbo Biosciences, San Diego, CA) for 5 min on ice, followed by incubation with Zombie Aqua Live/Dead (Biolegend, San Diego, CA) for 30 min at room temperature. Cells were then incubated with a cocktail of antibodies in FACS buffer (1% bovine serum albumin in 1X PBS) for 30 min on ice: PerCP CD45, PE/Cy7 DEC205, APC/Cy7 CD11b, BV421 CD11c, BV605
CD169, BV650 B220, BV711 CD3, and BV785 F4/80. All antibodies were purchased from Biolegend. After antibody staining, cells were fixed by incubation with 2% paraformaldehyde (PFA) for 15 min on ice, thoroughly washed, and resuspended in FACS buffer for analysis. Data was acquired using an LSRFortessa flow cytometer (BD Biosciences, Franklin Lakes, NJ), and analyzed using FlowJo software (FlowJo, Ashland, OR). Cell types were defined as follows: B cells: B220+CD3-CD11b-; T cells: CD3+B220-; conventional dendritic cells (cDCs): CD11c+B220-; plasmacytoid dendritic cells (pDCs): CD11c+B220+; subcapsular sinus macrophages (SSMs): B220-CD3-CD11b+CD11c+CD169+F4/80-; medullary sinus macrophages (MSMs): B220-CD3-CD11b+CD11c+CD169+F4/80+; medullary cord macrophages (MCMs): B220-CD3-CD11b+CD11c+CD169-F4/80+; dermal dendritic cells (dDCs): CD11c^{hi}DEC205+; and Langerhans cells (LCs): CD11c^{lo}DEC205+. Within each cell type, tracer+ populations were identified by comparison to tracer- cells from LN draining a saline injection.

For *in vivo* NP-S-S-CSIINFEKL experiments, NO treatment (800 nmol SNO-NP, SH-NP, or saline) was injected i.d. in mouse forelimbs. Alexa Fluor 647-labeled NP-S-S-CSIINFEKL were simultaneously injected alongside NO treatment in a single, 40 µl injection. Animals were sacrificed 72 h after NO treatment injection, and axillary and brachial LN were harvested and separated through a 70 µm cell strainer. Cells were stained with Zombie Aqua Live/Dead and an antibody cocktail (PerCP CD45, PE H-2K^{b} bound to SIINFEKL, APC/Cy7 CD11b, BV421 CD11c, BV605 CD169, FITC B220, BV711 CD3, and BV785 F4/80, all purchased from Biolegend) and fixed by 15 min exposure to 2% PFA as described above before analysis by flow cytometry.

3.2.5 *Confocal imaging*
To evaluate typical LN distribution of 5, 30, and 500 nm tracers, 40µl of saline containing $1.2 \times 10^8$ yellow-green FluoSpheres (500 nm), 23.8 µg TRITC 500 kDa dextran (30 nm), and 3 µg Alexa Fluor 647 10 kDa dextran (5 nm) was injected i.d. in the forelimbs of mice. 72 h after injection, animals were sacrificed, and axillary and brachial LN were individually frozen in OCT. LNs were sliced into 10 µm sections using a CryoStar NX70 Cryostat, mounted on slides, and imaged in all three tracer channels using a laser scanning confocal microscope (Zeiss 700 scanning head on an AxioObserver Z1 inverted microscope stage). Using ImageJ software (NIH), the capsule of each LN was individually traced, and the coordinates exported using a custom ImageJ macro. Images were thresholded to remove background, with thresholding values for each tracer determined using a control LN with no tracer injection. After thresholding, the coordinates of the remaining tracer+ pixels were extracted, and the minimum distance of each pixel from the LN capsule was calculated using a custom MATLAB script.

For evaluation of the effect of SNO-NP treatment on tracer LN distribution, mice were given i.d. forelimb injections containing both NO treatment (SNO-NP delivering 800 nmol NO, dose-matched SNAP, dose-matched SH-NP, or saline) and tracer cocktail (yellow-green FluoSpheres (500 nm), TRITC 500 kDa dextran (30 nm), and Alexa Fluor 647 10 kDa dextran (5 nm) at $1.2 \times 10^8$ spheres, 23.8 µg, and 3 µg, respectively). After 72 h, axillary and brachial LN draining the forelimb injection were extracted, cleaned, and imaged in all three tracer channels using a laser scanning confocal microscope (Zeiss 700 scanning head on an AxioObserver Z1 inverted microscope stage). Z-stacks were taken centered around the brightest plane in each LN. Using Zen software (Zeiss), z-stacks were converted to maximum intensity projections and images separated into individual tracer
channels. Using ImageJ software (NIH), the capsule of each LN was individually traced, and the coordinates exported using a custom ImageJ macro. Images were thresholded to remove background, with thresholding values for each tracer determined using a control LN with no tracer injection. After thresholding, the coordinates of the remaining tracer+ pixels were extracted, and the minimum distance of each pixel from the LN capsule was calculated using a custom MATLAB script.

3.2.6 In vitro dextran association and junction staining

For splenocyte association experiments, splenocytes were isolated from the spleens of C57/Bl6 mice. Spleens were gently separated through a 70 µm cell strainer using a syringe plunger to generate a single cell suspension. Red blood cells were lysed by 5 min incubation with 1 mL of ACK Lysing Buffer (Thermo Fisher), followed by dilution in 40 mL of 1X PBS and centrifugation to isolate splenocytes. Splenocytes were counted, and 1 million cells were plated in each well of a 96 well, U-bottom plate with 200ul of RPMI 1640 medium. To this cell suspension was added NO treatment (SNO-NP at 0.16 mM NO, dose-matched SH-NP, SNAP at 0.16 mM NO, or saline), followed immediately by a tracer cocktail (Alexa Fluor 647-labeled 10 kDa dextran, TRITC-labeled 500 kDa dextran, and 500 nm yellow-green spheres). After incubation for 4 h at 37°C, splenocytes were stained and fixed for flow cytometric analysis as previously described. Stains included Zombie Aqua Live/Dead, PerCP CD45, APC/Cy7 CD11b, BV421 CD11c, BV650 B220, and BV711 CD3.

For LEC association experiments, SV-LECs [158] (kindly provided by Dr. J. Brandon Dixon) were seeded at 90% confluency in collagen-coated 24-well plates and
allowed to adhere for 24 h. 400 µl of EBM containing 1 mg/ml TRITC-labeled 500 kDa dextran was added to each well alongside NO treatment (SNO-NP at 0.16mM NO, dose-matched SH-NP, SNAP at 0.16 mM NO, or saline). After 4 h of incubation at 37°C, LECs were washed with 1X PBS to remove remaining dextran, and the adherent cells were imaged using the RFP channel on an EVOS microscope. LECs that had not been incubated with dextran were imaged to obtain a background, and wells with dextran but without LECs were imaged to verify that washing successfully removed all extracellular dextran. After imaging, cells were detached from their plates by brief incubation with 0.25% trypsin and were transferred to U-bottom 96 well plates. The fluorescence of the cell suspensions in 100 µl of PBS were measured using a Synergy H4 BioTek plate reader, and cells were subsequently stained for flow cytometric analysis with Zombie Aqua Live/Dead and PerCP CD31 (Biolegend) as previously described.

To investigate LEC expression of tight junction protein zonula occludens-1 (ZO-1), SV-LECs were grown to confluency in collagen-coated 96-well plates or 8-well Lab-Tek chamber slides (Thermo Fisher) and allowed at least 48 h to form junctions. LECs were treated with SNO-NP at 0.16 mM NO, dose-matched SH-NP, or saline for 6 h. Cells were detached from 96-well plates via trypsin treatment and prepared for flow cytometric analysis. Briefly, cells were incubated with 2.4G2 for 5 min on ice, stained with Zombie Red Live/Dead (Biolegend), fixed in 4% PFA for 10 min at room temperature, permeabilized with 0.1% Triton-X for 10 min at room temperature, and stained with ZO-1 primary antibody (Thermo Fisher) or rabbit IgG isotype control and Alexa Fluor 488-conjugated anti-rabbit IgG secondary (Thermo Fisher) for 1 h each. Samples were analyzed on an LSR II flow cytometer (BD). For confocal imaging, adherent cells were fixed in 4%
PFA, permeabilized with 0.1% Triton-X, and blocked with 1% bovine serum albumin. They were incubated at 4°C overnight with ZO-1 primary antibody or isotype, then stained with secondary antibody for 1 h at room temperature. Slides were mounted in DAPI-containing Vectashield and imaged using a laser scanning confocal microscope.

3.2.7 LEC monolayer transport

5 × 10⁴ SV-LECs (P34-36) were seeded in 6.5 mm, 0.4 µm polyester transwell inserts (Corning) and allowed 48 h to settle, adhere, and form monolayers. 1 mg/ml Alexa Fluor 610-labeled 10 kDa dextran (5 nm) and 1 mg/ml TRITC-labeled 500 kDa dextran (30 nm) were added to the EBM medium in the transwell insert alongside treatment with 0.16mM SNO-NP, SH-NP, or PBS. At 0, 1, 3, 5, 8, 24, and 48 h, media from the bottom chamber of the transwell was sampled, and the presence of both 10 kDa and 500 kDa dextran was evaluated by measuring media fluorescence using a Synergy H4 BioTek plate reader. After 48 h, LECs were detached from the membranes by trypsin treatment, stained with Zombie Aqua Live/Dead, and their viability analyzed by flow cytometry.

3.2.8 Statistical analysis

All data are presented as mean ± standard error of the mean, and statistical analyses were performed in Prism 8 (GraphPad Software Inc., La Jolla, CA). Statistical significance was defined as p < 0.05 following an unpaired t-test or ordinary one-way analysis of variance with Tukey posthoc testing as appropriate. Symbols denoting p-values are as follows: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

3.2.9 Animal use
All animal procedures were performed at Georgia Institute of Technology, and were approved by the Georgia Institute of Technology Institutional Animal Care and Use Committee. All mice were female C57BL6/J, and were used at 6-10 weeks of age.

3.3 Results

3.3.1 NO delivery to draining LNs via SNO-NP

We have previously reported the generation of S-nitrosated nanoparticles (SNO-NP) capable of extended NO donation in both in vitro [148] and in vivo [110] applications. These SNO-NP are based on a polymeric nanoparticle which consists of a Pluronic corona and a hydrophobic poly(propylene sulfide) core containing large numbers of free thiols [148]. These thiolated NP (SH-NP) can be quickly loaded with NO by addition of nitrite under acidic conditions, converting thiols into S-nitrosothiols (SNO) [110,148] (Fig. 3.1A). SNO-NP provide extended NO donation, as evidenced by the gradual decrease in SNO concentration and appearance of nitrite (NO2) when SNO-NP were incubated at 37°C for several days [110,148] (Fig. 3.1B). While i.d. injection of unconjugated, small molecule (~1300 Da) Alexa Fluor 647 in the mouse forelimb did not increase the fluorescent signal in axillary and brachial LNs draining the injection above background, Alexa Fluor 647-labeled NP injection resulted in clear dye accumulation in these LN (Fig. 3.1C, D), highlighting the lymphatic uptake and LN access advantage provided by NP compared to small molecule injection [106,110]. Because of the importance of size in the advantageous lymph drainage properties of these NP, NP diameter was measured by dynamic light scattering before and after NO loading (Fig. 3.1E). Treatment with acidified nitrite did not affect the hydrodynamic radius or the polydispersity of the NP, suggesting that their critical
size and stability is retained after S-nitrosation of the core thiols. This is consistent with previous studies, in which we have observed that intradermal injection of SNO-NP results in significant accumulation of SNO in draining LN 72 h after injection [110]. Together, the lymph-draining size of these NP and the extended NO release that they provide make SNO-NP a unique tool for lymphatic-targeted NO delivery and the investigation of NO’s role in biomolecule transport.

Figure 3.1 – Production and characterization of lymphatic-draining SNO-NP, and potential effects of delivered NO. A) Schematic of NP nitrosation and potential NO effects after peripheral administration. Thiolated SH-NP can be loaded with NO by reaction with acidified nitrite, forming SNO-NP that enable NO delivery to the lymphatics. NO delivered by SNO-NP is a pleiotropic molecule that can have a variety of effects on biomolecule transport, including local effects at the site of injection, modulation of lymphatic drainage, and altered lymph node (LN) barrier penetration or immune cell activation. B) S-nitrosothiol (SNO) and nitrite (NO2) concentration in a solution of SNO-NP over time. C) IVIS imaging of AF647-labeled NP draining to
axillary and brachial LN from a forelimb injection, and D) quantification of C. E) Diameter of SNO-NP and SH-NP as measured by dynamic light scattering.

3.3.2 Fluorescent tracer characterization

To interrogate the effect of NO application on the biodistribution of co-injected molecules, we developed a panel of fluorescent tracers to probe the distinct pathways of size-based lymphatic transport (Fig. 3.2A). 5 nm tracer represents small molecules that are primarily absorbed into the bloodstream [19,106], but which have access to the deep LN parenchyma via the conduit system upon reaching the LN [24,25,115]. Mid-size molecules, represented by 30 nm tracers, show preferential lymphatic uptake, but are unable to access LN conduits, and large molecules that cannot passively drain into lymph and instead rely on active transport by migratory antigen-presenting cells (APCs) are modeled by 500 nm tracers [105,159]. These three tracer sizes were modeled by 10 kDa dextran, 500 kDa dextran, and 500 nm polystyrene spheres, respectively; tracer hydrodynamic diameters were verified by dynamic light scattering (Fig. 3.2B). After an i.d. injection, these tracers distributed in systemic tissues in accordance with their size; 5 nm tracers were primarily cleared by the kidney and 30 nm by the liver, while 500 nm spheres reliant on active cellular transport were undetected in systemic tissues even 72 h after injection (Fig. 3.2C).

Tracers also differentially accumulated in LNs draining a forelimb injection in accordance with their size, as measured by monitoring fluorescent signal in LN homogenate (Fig. 3.2D). 30 nm tracers showed highest levels of LN accumulation, with levels remaining consistent from 4-72 h. 5 and 500 nm tracers accumulated to lower extents, as is expected due to the reduced lymphatic access of 5 nm tracers and the slow, active transport of 500 nm tracers. Notably, 500 nm tracers showed minimal accumulation
at early time points and peak signal at 72 h, consistent with the time required for peripheral APCs to transport the spheres to the LN. To evaluate not only bulk accumulation of tracer in the LN, but also their spatial distribution, axillary and brachial LNs were imaged by confocal microscopy 72 h after forelimb injection of the tracer cocktail (Fig. 3.2E-G). 5 and 30 nm tracers, soluble molecules which enter the LN in afferent lymph via the sinuses, expectedly appeared in peripheral LN regions. 30 nm tracers, however, showed a stronger bias towards the LN periphery; the number of 30 nm+ pixels rapidly declined with increasing penetration depth compared to the smaller 5 nm tracer (Fig. 3.2F), resulting in a subtly reduced average penetration depth (Fig. 3.2G). 500 nm spheres appeared deep within the LN due to their transport within migratory DCs (Fig. 3.2E), and showed increased presence in these most central LN regions (Fig. 3.2F) and increased average penetration depth (Fig. 3.2G) compared to passively transported tracers. Along with distinct spatial distributions, tracers showed cellular distributions consistent with their size. Passively draining 5 and 30 nm tracers associated preferentially with CD11c+ DCs and F4/80+ macrophages, but 5 nm tracers had improved access to all cell types, including B cells in the cortex and T cells in the paracortex (Fig. 3.2H). 500 nm spheres showed significantly increased presence in CD11c+ DCs compared to other cell types (Fig. 3.2I), consistent with their reliance on active transport from the periphery. Together, the tracers employed probe distinct lymphatic transport pathways in both the interstitium and the LN, and enable studies of the effect of NO on molecule access to therapeutically relevant LN regions.
Figure 3.2 – Lymphatic transport is regulated by molecule size, and a tracer panel can probe this differential transport. A) Schematic of size-based effects on molecule drainage from the interstitium and into draining LNs (dLNs). Blue = 5 nm, red = 30 nm, and green = 500 nm molecules. B) Diameter of selected fluorescent tracers as measured by dynamic light scattering. C) Time course of tracer biodistribution in systemic organs based on their size (left), and their total accumulation in each organ (right). Tracers are cleared from systemic circulation according to their size, with 5 nm clearance in the kidney and 30 nm clearance by the liver. D) Timecourse of tracer appearance in the dLN based on their size (left) and total accumulation, measured by area under the curve (right). 30 nm tracers reach the LN more efficiently than 5 or 500 nm tracers. Passively draining tracers show significant signal in the dLN within 4 h, while 500 nm tracers show a more gradual appearance in the dLN. E) Confocal microscopy images of tracer distribution within a brachial LN 72 h after ipsilateral forelimb injection. Scale bar = 500 μm. F) Quantified distribution of tracer+ pixels in axillary and brachial LN draining forelimb injections. 30 nm tracers show a more peripheral bias than lower accumulating 5 nm tracer. Beyond 400 μm from the LN...
capsule, 500 nm signal is greater than that of passively draining tracers. G) Average tracer penetration depth. H) 5 nm tracers have improved access to LN cells compared to larger 30 nm tracers. I) Actively transported 500 nm tracers tend to appear preferentially in DCs. In G and I, broad cell types are defined as follows: B cell (B220+CD3−CD11b−), T cell (CD3+B220−), DC (CD11c+), macrophage (F4/80+).

3.3.3 SNO-NP modulation of active cell transport of large particles

NO is a potent immune modulator, and exogenous NO application has been shown to induce migration of mature DCs [138]. To investigate the effect of SNO-NP on local immune cell activation and trafficking, we administered a tracer cocktail of 5, 30, and 500 nm fluorescent tracers in the mouse forelimb alongside SNO-NP treatment or controls. At 4, 24, and 72 h post-injection, draining lymph nodes (dLNs) were dissected and either homogenized for the measurement of bulk tracer accumulation, or analyzed by flow cytometry to evaluate cellular distribution of the tracer within the LN (Fig. 3.3A). The total amount of 500 nm tracer that reached the dLN was unaltered 72 h after injection by SNO-NP treatment (Fig. 3.3B), and the total LN exposure (AUC) was similarly unaffected (Fig. 3.3C). As an additional control, another group of mice was given SNAP, a 220 Da S-nitrosothiol NO donor with a similar NO release profile to SNO-NP. Because it is a small molecule, SNAP drains inefficiently to LN from an i.d. injection and does not result in significant NO delivery to lymphatic tissues [110], and thus provides a control for NO effects at the site of injection. SNAP treatment also had no effect on 500 nm accumulation compared to the saline vehicle, indicating that strictly local NO donation also did not modulate the overall amount of tracer trafficked. To investigate the cellular distribution of 500 nm tracer within the dLN, LN cells were further analyzed by flow cytometry (Fig. 3.4). While 500 nm tracer association with CD45+ cells was clearly detectable within the LNs, neither SNO-NP nor SNAP treatment changed the frequency of these cells that were 500
nm+ compared to vehicle controls (Fig. 3.3D, E). NO treatment also did not affect the number of migratory cells, specifically dermal DCs (dDCs) and Langerhans cells (LCs), positive for 500 nm tracer within dLNs (Fig. 3.3F), suggesting that NO application did not cause increased migration of local DCs to the dLN. While 500 nm signal was observed in a wide variety of cell types, including cortical B cells, paracortex T cells, conventional DCs (cDCs), plasmacytoid DCs (pDCs), migratory dDCs and LCs, and barrier subcapsular sinus macrophages (SSMs) (Fig. 3.3G), in no cell type was the frequency of cells 500 nm+ (Fig. 3.3H) or the 500 nm mean fluorescent intensity (MFI) (Fig. 3.3I) impacted by treatment with SNO-NP or SNAP. The unchanged number, frequency, and MFI of 500 nm+ migratory cells suggest that in this context, NO injection does not impact the migration of skin-resident APCs (#, %) or their ability to take up 500 nm tracer (MFI), nor does it impact overall 500 nm access to LN-resident cell populations. Together, these data indicate that SNO-NP can be administered i.d. without significantly altering migration or behavior of skin-resident APCs, consistent with previous observations that SNO-NP injection in the skin does not cause local inflammation [110].
Figure 3.3 – Active transport of microparticles from injection site is unaltered by SNO-NP co-injection. A) Experimental schematic in tracer distribution studies. B) Percent of injection of 500 nm tracer in the dLN 72 h after injection. C) Total exposure (AUC) of dLN to 500 nm tracer over the course of 72 h. D) 500 nm tracer association with LN cells is detectable by flow cytometry. E) The percent of CD45+ cells within the dLN that are 500 nm tracer+ with SNO-NP or control treatment. F) The number of 500 nm+ dDCs and LCs found in LNs draining a tracer injection. G) Representative dot plots of 500 nm+ LN cells. H) Percent of cells 500 nm+ in dLN. I) Mean fluorescence intensity of 500 nm+ cell populations in the dLN.
Figure 3.4 - LN cell gating strategy. A) Gating for cell types. B) Representative examples of tracer+ cell gating within each cell type.
3.3.4 LN distribution of lymph draining tracers with NO treatment

We next investigated the accumulation and distribution of passively transported 5 and 30 nm tracers in LNs draining a forelimb injection. 72 h after co-injection of tracers and SNO-NP or controls, at which point complete NO release from SNO-NP is expected, confocal imaging of axillary and brachial LNs revealed a typical distribution; both 5 and 30 nm tracers appeared most concentrated in peripheral LN regions, consistent with their passive drainage via afferent lymph (Fig. 3.5A, Fig. 3.6). In saline, SNAP, and SH-NP control treatments, 5 nm tracer appears to penetrate deeper into the LN than 30 nm tracer, consistent with its smaller size. In SNO-NP-treated LNs, however, this difference in penetration depth was not visibly clear. When the distance of each tracer+ pixel from the LN capsule was quantified, we observed that while saline, SNAP, and SH-NP injections all showed a similar trend of 30 nm tracers penetrating less deeply into LNs relative to less restricted 5 nm tracers, as evidenced by the curves’ negative slopes, SNO-NP treatment prevented this decline and increased the relative LN penetration depth of 30 nm tracers (Fig. 3.5B). These effects were not observed with SNAP treatment, highlighting the importance of NO delivery to the LN. As NO is a known regulator of vascular permeability [134,135,154], we verified that NO administration did not alter tracer clearance into circulation and resulting systemic distribution of tracer (Fig. 3.7A) or the kinetics of tracer appearance in systemic organs (Fig. 3.7B). We also investigated the effect of SNO-NP application on the access of circulating tracers to LNs (Fig. 3.7C-D). LNs treated with NO showed no change in accumulation of intravenously administered tracers, suggesting that SNO-NP do not change tracer access to LNs from the LN blood vasculature. Because of NO’s known role in regulating contractility and function of lymphatic vessels, we
investigated the effect of NO treatment on passive tracer accumulation within the LN, and found that the amount of both 5 and 30 nm tracer in dLN was unchanged by treatment at any measured time point, and the total LN exposure to tracer (AUC) was similarly unaffected (Fig. 3.5C). Together, these results suggest that the increased LN penetration of 30 nm tracer was not observed due to altered tracer accumulation in the LN.

Figure 3.5 – LN-targeted NO delivery by SNO-NP, but not by SNAP, increases penetration of passively draining 30 nm tracer into the dLN. A) 5 nm (blue) and 30 nm (red) tracer distribution in the LN 72 h after co-injection with treatment. Scale bar = 500 μm. B) Quantification of tracer distribution in the LN. **** indicates
significant difference (p<0.0001) between SNO-NP linear fit and other treatments. C) Percent of injection in dLN over time for 5 nm (left) and 30 nm (center), and their AUC. D) 30 nm tracer (red) association with SV-LECs in *in vitro* culture after 4 h. Scale bar = 200 μm. E) Fluorescence intensity of SV-LECs incubated with 30 nm dextran and treatments. F) Fluorescence of 5 nm or 30 nm tracers that pass through a transwell membrane with or without an SV-LEC monolayer after 48 h. G) Fluorescence of 5 nm dextran (left) or 30 nm dextran (right) that penetrates an SV-LEC monolayer over time. Lines represent nonlinear fits. H) Viability of SV-LECs after 48 h of incubation with tracer and SNO-NP or controls.

Figure 3.6 - Confocal images of LN draining forelimb tracer injections. 5 nm tracer is shown in blue, and 30 nm tracer is shown in red. All images are maximum intensity projections at $\gamma = 0.45$. Scale bar = 500 μm.
Figure 3.7 - NO effects on vasculature. A) Systemic distribution of passively draining tracers is unaffected by NO treatment. ns indicates no significant difference between liver, kidney, or spleen AUC values. B) NO treatment does not alter the accumulation of 5 (top) or 30nm tracer (bottom) in their primary clearance organs. Presented as SNO-NP and SNAP normalized to vehicle-treated controls (SH-NP and saline, respectively). C) Injection schematic for evaluating LN accumulation of i.v. tracers. Mice were given four i.d. injections draining to either axillary/brachial or inguinal LN, followed by an i.v. tracer injection. Circles indicate i.d. injection sites, and arrows indicate dLN. Red i.d. injection site drains to red LN (axillary and brachial), while blue i.d. injection site drains to blue LN (inguinal). D) 4 h after i.v. tracer injection, no differences in LN accumulation of tracers of any size were observed with any treatment.

To further investigate the potential mechanism for the increased 30 nm penetration observed in vivo, transport of 30 nm dextran was studied in vitro using cultured SV-LECs (Fig. 3.8A, B). When SV-LECs were incubated with fluorescent 30 nm tracer, SNO-NP-treated cells appeared to have increased tracer association compared to SH-NP controls (Fig. 3.5D). This enhanced positivity was also reflected in total fluorescence measured using a plate reader, where SNO-NP increased 30 nm association with SV-LECs compared to vehicle control (Fig. 3.5E) Flow cytometric analysis of the LECs showed an increase in their tracer MFI, but no change in the percent of cells tracer+ (Fig. 3.8C), suggesting that increased fluorescence measured via a plate reader was due to an increase in the amount of
30 nm tracer each LEC contained. While SNO-NP treatment did appear to increase 30 nm tracer association with LECs (Fig. 3.5D, E), it did not alter their distribution or expression of tight junction protein zonula occludens-1 (ZO-1); the percent of LECs expressing ZO-1 (Fig. 3.8D) and their MFI (Fig. 3.8E) were unchanged by SNO-NP treatment, and confocal microscopy showed no clear alterations in junction structure (Fig. 3.8F). Tracer transport across a LEC barrier was further investigated using a monolayer of SV-LECs cultured on the apical side of a transwell membrane insert. When fluorescent 5 and 30 nm tracers were added to the apical medium, tracer signal in the basal medium 48 h later was significantly reduced by the presence of a LEC monolayer compared to a cell-free membrane (Fig. 3.5F). While 5 nm signal was reduced only 3-fold, 30 nm signal was reduced by approximately 30-fold, consistent with a higher permeability of the LEC monolayer to 5 nm tracer than to 30 nm tracer. When LEC monolayers were treated additionally with SNO-NP or SH-NP and tracer transport across the monolayer was monitored over time, there was no difference in 5 nm signal at any time point, or in the nonlinear fit of the two curves (Fig. 3.5G). In contrast, 30 nm transport across the monolayer was increased by SNO-NP treatment, resulting in a significantly different curve fit compared to SH-NP control (Fig. 3.5G). This altered transport was not due to changes in LEC viability, which was consistent across treatment groups (Fig. 3.5H).
Figure 3.8 - Effect of SNO-NP treatment on 30 nm tracer positivity and ZO-1 expression in LECs in vitro. A) Representative images of TRITC-30nm dextran association with confluent LECs after 4 h of incubation at 37°C. Background images are empty wells (no cells) incubated with tracer and washed using an identical protocol. B) Fluorescence of LEC suspension 4 h after incubation with TRITC-30nm dextran, measured using a plate reader. Fluorescence signal increases linearly as tracer concentration increases, with a significantly nonzero slope (** **). C) The number, frequency, and MFI of 30nm+ LECs after 4 h tracer incubation, measured by flow cytometry. Data are presented as normalized to vehicle control (SNO-NP/SH-
NP, SNAP/saline). D) The percent of LECs ZO-1+ and E) their ZO-1 MFI after 6 h of treatment with SNO-NP or controls. F) Representative confocal images of ZO-1 stained LECs after SNO-NP (left) or SH-NP (right) treatment. Scale bar = 25 μm.

In addition to the spatial distribution of tracer, the cellular distribution of tracer within dLN was evaluated by flow cytometry. Treatment with SNO-NP did not affect LN size or cellularity compared to SH-NP control; total CD45+ cell counts (Fig. 3.9A) and LN cellular composition (Fig. 3.9B) remained consistent with treatment. Passively draining 5 and 30 nm tracers were clearly detectable in these CD45+ populations (Fig. 5C). While 5 nm positivity was unchanged by SNO-NP treatment, the percent of CD45+ cells that were 30 nm+ significantly increased with SNO-NP treatment compared to the SH-NP vehicle control (Fig. 3.9C, D). This increased association was not observed with SNAP treatment. Much of the increased 30 nm association could be attributed to B cells and T cells; both cell types showed increased numbers of 30 nm+ cells with SNO-NP treatment compared to SH-NP control (Fig. 3.9E), and an increase in the percent of B cells that contained 30 nm tracer was also observed (Fig. 3.9F). SNO-NP did not increase 30 nm+ cell counts or frequencies in cDCs, pDCs, or SSMs, and small molecule NO donor SNAP had no effect on tracer association by either metric in any cell type (Fig. 3.9E, F). SNO-NP not only increased the number of 30 nm+ B and T cells, but also increased the MFI of those 30 nm+ cells compared to vehicle control (Fig. 3.9G). A nonsignificant increase in 30 nm+ MFI was also observed in pDCs, centrally located cells within the LN. Overall, SNO-NP induced no alterations in 30 nm association within peripherally located SSMs, but cortical B cells showed significant increases in number, percent, and MFI of 30nm+ cells, and paracortical T cells had increased number and MFI (Fig. 3.9H). This enhanced 30 nm
association was not accompanied by increased 5 nm signal; the frequency of cells positive (Fig. 3.9I) and their MFI (Fig. 3.9J) was unchanged by either SNO-NP or SNAP treatment.

Figure 3.9 - SNO-NP treatment increases the association of passively drained 30 nm tracer with LN cells in vivo, but not of smaller 5 nm tracers. A) CD45+ cell number in dLN. B) The cellular profile of CD45+ LN cells with SNO-NP treatment and control SH-NP treatment. C) Representative examples of CD45+ LN cells without 5 and 30 nm tracers, or with tracer and SNO-NP or SH-NP treatment. D) Percent of CD45+ cells in dLN positive for passively draining tracers 72 h after co-injection with treatment. E) Number and F) percent of each cell subtype positive for 30 nm tracer
after 72 h. G) MFI of 30 nm+ cells after SNO-NP treatment, normalized to SH-NP control. *s represent significant differences between pre-normalized populations. H) Effect of SNO-NP treatment on tracer association by barrier, cortex, and paracortex cell populations. I) Percent of LN cells positive for 5 nm tracer 72 h after injection. J) Mean fluorescence intensity of 5 nm+ cells 72 h after tracer and SNO-NP injection, normalized to SH-NP control. K) Percent of cells positive for 30 nm tracer after splenocytes were incubated in vitro for 4 h with tracer and SNO-NP or SH-NP control. Results are normalized to SH-NP control.

To investigate if SNO-NP treatment had direct effects on 30 nm tracer association with immune cells, murine splenocytes were incubated with 30 nm tracer and SNO-NP or SH-NP in vitro. When splenocytes were analyzed by flow cytometry after 4 h, SNO-NP did not increase 30 nm tracer positivity in B cells, T cells, cDCs, or pDCs compared to SH-NP (Fig. 3.9K), further suggesting that the increased 30 nm association observed in vivo was not due to direct effects of NO on these immune cells.

3.3.5 NO effects on NP access and antigen presentation within the LN

With evidence that SNO-NP treatment can increase access of 30 nm dextran to LN resident cells, we next sought to extend these studies to other lymph-draining molecules and evaluate SNO-NP’s effects on immune cell function in vivo. To this end, the model peptide antigen SIINFEKL was conjugated to the corona of an Alexa Fluor 647-labeled, PDS-functionalized NP via a cysteine added to the peptide’s N-terminus, yielding NP-S-S-CSIINFEKL trackable by Alexa Fluor 647 fluorescent signal (Fig. 3.10A, Fig. 3.11A). When splenocytes were incubated with increasing doses of NP-S-S-CSIINFEKL in vitro and antigen presentation was evaluated by staining with an antibody recognizing H-2Kb bound to SIINFEKL, increasing CSIINFEKL presentation was detected with increasing delivered dose as measured by cell count (Fig. 3.10B) and MFI (Fig. 3.10C), suggesting that neither delivery via a NP vehicle nor the addition of a cysteine to SIINFEKL’s N-
terminus prevent presentation of the antigen. 72 h after injection in the forelimb, NP-S-S-CSIINFEKL positivity was clearly detectable in CD45+ cells within the dLN based on Alexa Fluor 647 signal (Fig. 3.10D). When NP-S-S-CSIINFEKL were co-administered with SNO-NP or control SH-NP, SNO-NP significantly increased the fraction of both B cells and cDCs that were Alexa Fluor 647+, and nonsignificantly increased the fraction of Alexa Fluor 647+ T cells and pDCs (Fig. 3.10E). In B cells, the Alexa Fluor 647 MFI also increased (Fig. 3.10F), suggesting that each Alexa Fluor 647+ cell contained more NP. The fraction and MFI of Alexa Fluor 647+ T cells and pDCs were insignificantly increased (Fig. 3.10E-F), an observation consistent with the lowly phagocytic nature of these paracortex-resident cells. Cells were additionally stained for SIINFEKL presentation, revealing a NP+CSIINFEKL+ population in the dLN that both contained NP-S-S-CSIINFEKL and presented the delivered antigen (Fig. 3.10G). In B cells, cDCs, and pDCs that would be expected to present the antigen (Fig. 3.11C), SNO-NP treatment did not alter the frequency of cells that were NP+CSIINFEKL+ (Fig. 3.10H), nor did it alter the MFI of those CSIINFEKL-presenting cells (Fig. 3.10I). In addition to showing no increase in antigen presentation in spite of the enhanced NP association observed, neither B cells, pDCs, nor cDCs showed changes in the frequency (Fig. 3.10J) or intensity (Fig. 3.10K) of CD40 expression, expected to increase on mature APCs including B cells [160]. Together, these results suggest that the association enhancement afforded by SNO-NP is not attributed to alteration of APC activation, and is applicable not only to flexible dextrans, but also to a nanocarrier drug delivery system.
Figure 3.10 - SNO-NP treatment increases association of peptide-conjugated NP-S-S-CSIINFEKL with LN cells but does not affect antigen cross-presentation or cell activation. A) Schematic of NP-S-S-CSIINFEKL synthesis. Alexa Fluor 647-labeled, PDS-functionalized NP are incubated with thiolated CSIINFEKL peptide overnight at room temperature, resulting in the formation of NP-S-S-CSIINFEKL. B) CSIINFEKL presentation by CD45+ splenocytes increases with increasing NP-S-S-CSIINFEKL dose after 18 h of incubation in vitro, as evidenced by increasing H-2KB:SIINFEKL+ cell counts and C) increasing fluorescent intensity in H-2KB:SIINFEKL+ cells. D) Representative density plots of CD45+ LN cells with (right) and without (left) injection of 647-labeled NP-S-S-CSIINFEKL. E) Percent of cells that are positive for NP-S-S-CSIINFEKL 72 h after co-injection of NP-S-S-CSIINFEKL and treatment. F) AF647 MFI of NP+ cells. G) Representative contour plots of CSIINFEKL-presenting, NP+ CD45+ cells in LN receiving a saline (left) or NP-S-S-CSIINFEKL (right) injection. H) Percent of cells that are both NP+ and presenting CSIINFEKL, normalized to SH-NP control. I) CSIINFEKL MFI in double positive cells. J) Percent of cells expressing CD40 and K) the MFI of CD40+ cells.
**Figure 3.11** - NP-S-S-CSIINFEKL characterization. A) NP-S-S-CSIINFEKL are cleaned of excess free CSIINFEKL by SEC, and the presence of peptide in each fraction evaluated by fluorescamine signal. B) The percent of B cells, cDCs, and pDCs that are NP+ 72 h after NP-S-S-CSIINFEKL injection increases with increasing NP-S-S-CSIINFEKL dose. Administration of 5 μg of NP-S-S-CSIINFEKL results in significant NP association without saturation in any cell type of interest. C) B cells, cDCs, and pDCs all show increased NP+H-2KB:SIINFEKL+ frequencies compared to saline controls 72 h after injection.

### 3.4 Discussion

LNs are therapeutic tissue targets of great clinical significance; because of the high concentration of lymphocytes resident in these immune organs, drug delivery to lymphatic tissues represents a promising strategy for immunomodulation and management of a wide variety of applications, including infectious disease, autoimmune disease, and cancer. The lymphatic-targeting advantages provided by peripheral injection are well-established, and peripheral injection is accordingly employed in the clinic to promote lymphatic delivery and improve patient compliance. Nanoscale drug delivery vehicles are often applied to enable delivery of smaller drugs that would otherwise have poor lymphatic uptake due to their small size. While delivering drug via a nanoscale carrier improves lymphatic uptake of the drug from the interstitium, the larger size of the carrier may paradoxically reduce its ability to penetrate into parenchymal regions of the LN, limiting drug access to resident lymphocytes. Accordingly, a system that enables timed drug release from nanocarriers within LN has been shown to improve lymphocyte uptake of the delivered drug and
subsequently improve therapeutic efficacy [29]. Improving LN penetration and lymphocyte access of the nanocarrier itself could have similar advantages.

In this study, we investigated the effect of NO on lymphatic transport and LN access of peripherally administered molecules, as NO is a well-established regulator of lymphatic transport in a wide variety of contexts (Fig. 3.1A). Using SNO-NP, which are polymeric nanoparticles that enable lymphatic uptake and LN accumulation of small molecule NO [110] (Fig. 3.1C, D), and a panel of model fluorescent tracers over a biologically relevant size range (Fig. 3.2B), we quantified LN accumulation, penetration, and cellular distribution of small, nanoscale, and microscale molecules after treatment with LN-targeted NO. We observed that although SNO-NP treatment did not affect systemic drainage (Fig. 3.7A, B) or LN accumulation (Fig. 3.5C) of passively transported 30 nm dextrans, their association with cortical B cells and paracortical T cells was enhanced by SNO-NP injection (Fig. 3.9E-G). This effect was particularly stark in B cells, in which the number, frequency, and MFI of tracer+ cells were all increased. T cells showed an increase in the number of tracer+ cells as well as MFI, but the frequency of T cells positive for tracer was unchanged by treatment, potentially owing to their less phagocytic nature. While only subtle effects of SNO-NP treatment were observed in cDCs and pDCs, which also reside in the paracortex, this could potentially be attributed to the comparatively high baseline 30 nm association with these cell types (Fig. 3.9F). The enhanced tracer presence in B cells did not appear to be due to direct effects of NO on tracer association with immune cells, as association of co-administered 5 nm dextran was unaltered (Fig. 3.9I, J), and treatment with SNO-NP in vitro did not increase tracer positivity (Fig. 3.9K). Confocal microscopy revealed that with SNO-NP treatment, 30 nm dextran penetration into the LN was enhanced.
relative to mobile 5 nm dextran (Fig. 3.5A, B). *In vitro*, SNO-NP treatment also increased the permeability of a LEC monolayer to 30 nm dextran compared to treatment with control SH-NP (Fig. 3.5G), suggesting that NO delivery by SNO-NP may improve 30 nm dextran association with resident lymphocytes by increasing its ability to penetrate the LN. Though the exact mechanism of this increased permeability is unknown, *in vitro* treatment of SV-LECs with SNO-NP resulted in increased association of 30 nm tracers (Fig. 3.5E). As LECs are known to have vesicular, transcytotic transport pathways that result in shuttling of molecules like antibodies [118] and dextrans [141] across a LEC barrier, this enhanced association could potentially suggest an increase in transcytotic transport. Additionally, NO is a known regulator of blood and lymphatic vascular permeability through its effects on cell junction proteins [134,135,144,154]. While SNO-NP treatment did not appear to affect LEC expression of tight junction protein ZO-1 *in vitro* (Fig. 3.8D-F), other junction proteins can be investigated in future work to fully evaluate the role of junctional disruption in the observed permeability increase. The increased cellular association observed in 30 nm dextran appeared to extend to peptide-loaded NP-S-S-CSIINFEKL, which after i.d. injection with SNO-NP showed enhanced association with B cells and cDCs, and subtly increased association with T cells and pDCs (Fig. 3.10E, F). This enhanced association occurred without visible effects of delivered NO on cell activation, as SIINFEKL presentation and CD40 expression by LN-resident antigen-presenting cells were unaltered by SNO-NP injection (Fig. 3.10H-K).

While SNO-NP administration increased LN penetration and lymphocyte association with co-delivered 30 nm dextran, administration of SNAP, a small molecule NO donor, had no such effects; SNAP did not result in enhanced tracer positivity of LN-
resident cells (Fig. 3.9D-F), nor did it improve tracer penetration into the LN compared to a saline-treated control (Fig. 3.5B). Due to its size, SNAP is cleared rapidly from the peripheral injection site into circulation. While some low rate of drainage into lymph may be expected, akin to the drainage observed in 5 nm dextrans (Fig. 3.2D), administration of even a high concentration of SNAP does not result in detectable SNO presence in dLN [110]. SNAP is analogous to other small molecule NO formulations currently employed in the clinic and in research, including NO donors such as S-nitrosoglutathione [135], NONOates [11,143,154], glyceryl trinitrate [91,155,161], and sodium nitroprusside [11], which due to their size have poor lymphatic access and a short half-life in circulation. SNO-NP, employed here and previously described [110,148], represent a unique tool to overcome the limitations of conventional NO donors. Their efficacy compared to SNAP suggests that LN-targeted NO delivery is critical to elicit transport changes in this context, and highlights the value of the lymphatic-draining SNO-NP system.

While NO has previously been challenging to apply in the context of the lymphatics because of its small size and high reactivity, studying the role of NO in lymphatic transport is also complicated by the multifaceted effects NO can exert. It is perhaps one of the most prolific endogenous signaling molecules, and plays dose- and context-dependent roles in vascular and lymphatic permeability [11,134,135,154], vasodilation [162], coagulation [163,164], immune cell activation and proliferation [139], and lymphatic contractility [90,91], among many other pathways. While its versatility means that NO has the potential to improve molecular transport to and within the LN in a way that could benefit drug access and efficacy, it could also inhibit such transport. For example, both endogenous [90] and exogenous [91] NO has been shown to inhibit lymphatic vessel contractions in vivo and
impair normal lymph transport. When we investigated the effect of SNO-NP and SNAP injection on the accumulation of passively draining dextrans, however, we found that neither NO donor reduced the accumulation of dextran in dLN at 4, 24, or 72 h after injection compared to controls (Fig. 3.5C). This consistent tracer accumulation regardless of NO application allows the comparison of tracer spatial and cellular distribution within the LN between groups, but it should be noted that this observation does not preclude the presence of inhibited lymphatic pumping in vivo with SNO-NP administration, which we have not directly evaluated; tracer accumulation in dLN may just be sufficiently high that it is not reduced, in this context, by impaired lymph transport. Because of NO’s role as an inflammatory signaling molecule and evidence that its application can alter the viability and migration of skin-resident APCs [138,165], we additionally evaluated the effect of NO on active transport of microparticles from the forelimb to dLN within migratory dDCs and LCs. Perhaps surprisingly, NO application did not alter the number of migratory DCs that carried tracer to the LN (Fig. 3.3F), regardless of its formulation. This is consistent with previous studies that showed i.d. administration of SNO-NP did not induce local inflammation [110] and supports the application of SNO-NP for LN-targeted NO delivery without injection site morbidity, and additionally highlights the importance of context and concentration of NO delivery.

In this work, we investigated the effect of LN-targeted NO delivery on LN access and distribution of both a 30 nm dextran tracer and a 30 nm peptide-conjugated polymer NP. Although similar in hydrodynamic diameter, these molecules are structurally distinct; model dextran is a more flexible molecule with a higher aspect ratio, while NP-S-S-CSIINFEKL are particulate in nature. In spite of their differences, both dextran and
peptide-conjugated NPs showed enhanced association with B cells, as measured by both frequency and MFI of positive cells, with SNO-NP treatment. While dextran additionally showed increased MFI in paracortex T cells (Fig. 3.9G), this effect was only subtly observed in peptide-NP (Fig. 3.10C). Peptide-conjugated NP were also found in a higher frequency of cDCs with SNO-NP administration, an effect that was not observed in the flexible dextran, and both dextran and peptide-NP showed only nonsignificantly enhanced association with pDCs, as measured by MFI. These subtle differences in distribution between dextran and NP may be attributed to the variability in their size and structure, which is known to impact lymphatic transport [21], and its impact on cellular uptake, and differences in association between cell types may be a function of both their location within the LN and their inherent phagocytic properties. B cells and cDCs, for example, are more peripherally located compared to T cells and pDCs in the paracortex, which may contribute to the increased frequency of B cells and cDCs positive for dextran or peptide-NP. More subtle effects on MFI were observed in T cells and pDCs, which are both more centrally located and less inherently phagocytic than B cells and cDCs. While the dextran and polymer NP investigated are not fully representative of the diverse drug delivery vehicles employed for LN drug delivery, observing similar effects of NO on LN cellular distribution in two similarly-sized but structurally distinct molecules is a promising start. Future work could expand NO application to other vehicles employed for lymphatic drug delivery, including metallic NP [27], liposomes [28], or silica NP [26], or investigate the upper size limit of NO’s effects.
3.5 Conclusions

In summary, we have employed a SNO-NP system to deliver NO specifically to lymphatic tissues as a tool to investigate the effect of LN-targeted NO on the lymphatic transport of co-delivered molecules over a biologically relevant size range. By monitoring the LN accumulation, distribution, and cellular association of 5, 30, and 500 nm tracers, we demonstrated that when delivered to the LN in a controlled, sustained fashion, NO can improve the penetration of nanoscale molecules into the LN and subsequently increase their association with parenchymal lymphocytes 72 h after i.d. injection. Model 30 nm dextrans showed increased association with B cells and T cells with SNO-NP administration, without alteration in total tracer accumulation in the LN or significant effects on migratory immune cell trafficking from the site of injection. The effects of SNO-NP were further extended to a peptide-conjugated NP, which showed increased association particularly with B cells and cDCs in vivo. As peripheral administration is the optimal route for lymphatic targeting and also commonly used in the clinic, enhancing the access of passively transported, lymph-draining molecules, including nanoscale drug delivery vehicles, to immune cells deep within the LN parenchyma has the potential to improve drug delivery to these therapeutically important cells and improve immunomodulatory treatments.
CHAPTER 4. LYMPHATIC-DRAINING NANOPARTICLES DELIVER BAY K8644 PAYLOAD TO LYMPHATIC VESSELS AND ENHANCE THEIR PUMPING FUNCTION

4.1 Introduction

The lymphatic system is an often overlooked component of the circulatory system that is critical in maintaining fluid balance in the body. Fluid that is lost from the blood during circulation drains from the interstitium into lymphatic capillaries, and flow is driven back into circulation by a combination of passive forces, including the contraction of surrounding skeletal muscle [39] and artery pulsations [33], and active contractions of the lymphatic vessels (LVs) themselves. Because lymphatic drainage is vital in transport and fluid balance, lymphatic dysfunction is associated with many pathologies. In the cardiovascular system, for example, the lymphatic system plays an important role in maintaining tissue health [46,47]. The lymphatic system is the primary route of reverse cholesterol transport responsible for atherosclerosis amelioration [3,52], lymphatic dysfunction often precedes atherosclerotic plaque formation [4], and alleviating lymphatic dysfunction can improve outcomes after myocardial infarction [9]. Lymphatic dysfunction is also associated with diseases like obesity [10,43], diabetes [11], and lipedema [6]. The most common lymphatic disease, however, is lymphedema, a complex disease associated with both immune dysregulation and lymphatic dysfunction that occurs when lymphatic drainage is insufficient to maintain physiological interstitial fluid volumes [57]. Secondary lymphedema, the more common manifestation of the disease, occurs due to postnatal insult to lymphatic vasculature such as radiation [166], surgical lymph node (LN) removal [61] or
lymphatic filariasis [62]. This lymphatic disruption causes impaired fluid flow and clearance, resulting in fluid accumulation, tissue remodeling, altered immune cell infiltration, and inflammation [63–65]. The contractility of lymphatic vessels in patients with lymphedema is additionally impaired; lymphedematous legs show irregular contractions too weak to propel lymph [8], and lymphatic congestion lymphoscintigraphy measurements reveal lymphatic pump failure in patients with ipsilateral arm lymphedema after breast cancer surgery [67]. These dysfunctions eventually result in lymphedema symptoms including edema, tissue remodeling and fibrosis, recurring infections, and pain.

Although lymphedema affects millions, the disease currently has no cure; patients are instead treated by compression and massage that can help manage symptoms of the disease, but do not address the root inflammation or lymphatic deficiencies that promote lymphedema progression. Ongoing research suggests that leukotriene B4 antagonists like ketoprofin [81] and bestatin [82] can reduce edema and pathologic tissue remodeling, but such therapeutics are not yet part of a typical lymphedema treatment regimen. And while it is well established that lymphatic vessel pumping dysfunction is associated with lymphedema progression, amelioration of lymphatic vessel dysfunction remains an unexplored avenue for lymphedema treatment. In addition, there are no therapeutic drugs that can directly enhance lymphatic pumping in vivo, either clinically or in animal models.

Lymphatic vessel pumping function is regulated in part by L-type calcium channels on lymphatic muscle cells (LMCs) [84,93], which have been targeted with small molecule calcium channel agonists like S-(−)-Bay K8644 (BayK) ex vivo for the study of lymphatic vessel pumping mechanisms [94–96]. BayK in particular has been shown to improve functional metrics like ejection fraction [95] and contraction amplitude [93] when applied
to isolated popliteal LVs. Though BayK is a promising regulator capable of augmenting lymphatic contractility \textit{ex vivo}, there are barriers to its \textit{in vivo} application. First, small molecule drugs like BayK are challenging to deliver to lymphatic targets. Although the most direct route into LVs is via drainage from the interstitium, small molecules injected in the interstitium are primarily absorbed into the bloodstream due to its large volume and high flow rates, resulting in rapid systemic exposure and poor lymphatic access \cite{22,110}; the resulting low drug concentration within target lymphatic vessels may limit efficacy or necessitate repeated or increased dosing. Because of the ubiquitous expression of L-type calcium channels on non-target cells like cardiac muscle cells \cite{98}, neurons \cite{99}, and skeletal muscle \cite{100}, systemic drug exposure poses a risk of dangerous off-target effects \cite{101}. A major hurdle to the use of BayK, or other L-Type calcium channel agonists, to treat lymphatic impairment is therefore the lack of appropriate drug delivery vehicles to optimize lymphatic uptake and access while minimizing systemic drug exposure and resulting side effects.

To overcome these limitations and enable the application of BayK for treatment of lymphatic dysfunction \textit{in vivo}, we herein develop a nanoparticle (NP) formulation capable of encapsulating BayK and enabling its uptake into lymphatic collecting vessels after interstitial injection. The employed NP vehicle has a hydrophobic poly(propylene sulfide) (PPS) core in which large amounts of hydrophobic small molecule drug partition without the need for drug modification or conjugation \cite{12}, and has a 30 nm diameter ideal for lymphatic drainage of both the NP and its payload \cite{106,110}. BayK-loaded NP (BayK-NP) were found to release their BayK payload over the course of several days without the need for environmental stimulus, providing extended release of a small molecule drug that
would otherwise be rapidly cleared from circulation. Using near-infrared (NIR) microscopy to image lymphatic pumping in vivo and in real-time, the effect of BayK-NP on mouse tail collecting LVs was evaluated. A single BayK-NP injection was found to improve LV function for 8 h, while no LV function improvement was observed when free BayK was administered without a NP formulation, presumably due to its poor lymphatic uptake. BayK-NP simultaneously reduced systemic BayK exposure and subsequent side effects observed with free BayK treatment, providing an additional advantage to free drug administration. We also demonstrate that when applied to a mouse tail model of secondary lymphedema [66], BayK-NP improved LV pumping pressure and reduced tail swelling compared to control NP. BayK-NP thus represent a novel approach to improving LV pumping function in vivo via intralymphatic delivery of small molecule contractility enhancers and, to the best of our knowledge, the first demonstration that directly targeting LV pumping function can improve lymphedema outcomes.

4.2 Materials and Methods

4.2.1 BayK-NP synthesis and characterization

Thiolated PPS-NP (SH-NP) were synthesized as previously described [110]. Briefly, 500 mg of Pluronic F127 was dissolved in 10 mL of degassed Milli-Q water under argon, and 400 μl of propylene sulfide (TCI, Tokyo, Japan) was added. After 30 min, thiolated initiator [157] activated in sodium methoxide was added under argon and was allowed to react for 15 min under stirring. 64 μl of 1,8-diazabicyclo[5.4.0]undec-7-ene was added under argon, and the solution was capped for 24 h. The reaction was then exposed to air for 2 h, and the resulting SH-NP were dialyzed in a 100,000 Da membrane.
(Spectrum Labs, New Brunswick, NJ) against 5 L of Milli-Q water for at least three days. In experiments where fluorescent NP were required, NP were reacted overnight at room temperature with a 6 M excess of Alexa Fluor 647 C2 maleimide (Thermo Fisher, Waltham, MA) or with IRDye 680RD maleimide (Li-cor, Lincoln, NE), and unreacted dye was removed by size exclusion chromatography on a CL-6B resin.

SH-NP were loaded with (S)-(-)-BayK 8644 by mixing 1 mg/ml BayK (Cayman Chemical, Ann Arbor, MI) with 7.5-30 mg/ml SH-NP in 5% tetrahydrofuran (THF) for 5 min, and unencapsulated BayK was removed by cleaning on a 7 kDa Zeba column (Thermo Fisher). To verify successful encapsulation, uncleared BayK-NP were run on a CL-6B size exclusion column, and BayK association with NP was evaluated by measuring BayK absorbance at 415 nm and NP signal using a modified iodine assay that detects the presence of polyethylene glycol (PEG) [110] in each fraction using a Synergy H4 BioTek plate reader. To determine BayK encapsulation efficiency, BayK-NP were cleaned of unencapsulated drug as described, then lyophilized and resuspended in acetonitrile. The amount of BayK was determined by comparing the solution absorbance at 415 nm to a standard curve, and the encapsulated drug concentration was compared to the originally added dose. BayK release from NP was evaluated by dialyzing BayK-NP in a 3.5 kDa membrane and monitoring solution absorbance over time. NP size was measured before and after BayK loading by dynamic light scattering on a Malvern ZetaSizer instrument.

4.2.2 Isolated vessel studies

To evaluate the effect of BayK and BayK-NP directly on collecting lymphatic vessel pumping, mesenteric lymphatic vessels were extracted from male Sprague-Dawley
rats as described by Davis et al. [167]. A 1 cm segment of vessel was submerged in 37°C physiological salt solution (PSS, consisting of 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl$_2$, 1.17 mM MgSO$_4$, 1.2 mM NaH$_2$PO$_4$ mM, 5.0 mM dextrose, 2.0 mM sodium pyruvate, 0.02 mM EDTA, and 3.0 mM MOPS) and cannulated in an \textit{ex vivo} lymphatic perfusion system as previously described [168,169], allowing for precise maintenance of transmural pressure at 3 cmH$_2$O throughout the course of the experiment. Contractions of the vessel were imaged using a bright-field camera at 30 fps, and vessel diameter was calculated using a custom LabView program. After allowing vessel segments to equilibrate in 37°C PSS for 15 m, vessel function was verified by applying incremental pressure steps at 3, 2, 1, 0.5, 3, 5, 8, 10, and 12 cmH$_2$O for 3 min each to ensure that the vessel was not damaged during cannulation and that normal transmural pressure dependent changes in lymphatic contractility were observed, as has been reported previously [170]. Baseline, or pre-treatment, function for each vessel was obtained by imaging for 3 min before the addition of treatment. The dose-dependent effect of BayK was tested by incrementally adding 10, 30, 100, 200, 300, and 1000 nM BayK into the vessel bath for 5 m each, while continuously monitoring vessel diameter. To evaluate the effect of BayK-NP on vessel pumping, vessel segments were sequentially treated with a low dose of SH-NP for 5 min, a high dose of SH-NP for 40 min, a low dose of BayK-NP (200 nM) for 5 min, and a high dose of BayK-NP (1000 nM) for 40 min. SH-NP treatments were dose-matched to BayK-NP treatments, providing a control for the intrinsic effects of the NP vehicle. In some vessels, pressure step testing was repeated in the last 30 min of high SH-NP and high BayK-NP dose treatment to determine if treatment altered the pressure response. Any time pressure steps were applied, the vessel was allowed to equilibrate for 30 minutes for
contractile function to return to baseline. At the end of each vessel segment experiment, the PSS bath was replaced with a calcium-free solution to induce maximal relaxation of the lymphatic muscle and the vessel was imaged for an additional 5 min providing resting diameter information and allowing calculation of vessel tone. Vessel functional metrics were calculated from vessel diameter traces using a custom MATLAB script. Parameters include frequency (the average number of contractions per min), end diastolic diameter (EDD), end systolic diameter (ESD), amplitude (the average change in vessel diameter in one contraction, EDD - ESD), ejection fraction (EF, (EDD² – ESD²)/EDD²), and fractional pump flow (FPF, Freq × EF) [93].

4.2.3 In vivo functional analysis

Mice were anesthetized with inhaled isoflurane (1.8% maintenance), and injected with 10 µl of IRDye 800CW-labeled 20 kDa PEG tracer intradermally at the midline of the tail, less than 1 cm from the tail tip. Before imaging, 5 min was allowed for flow to normalize and signal to appear in lymphatic vessels along the entire length of the tail. Vessels were then imaged using NIR microscopy as previously described [66,91]. Briefly, the system consisted of an MVX-ZB10 microscope (Olympus), a Lambda LS xenon arc lamp (Shutter Instruments), a 769 nm bandpass excitation filter, an 832 nm emission bandpass filter, and an Evolve Delta 512 EM-CCD camera (Photometrics). After allowing 5 min for dye to enter lymphatic vessels, the mouse was placed on its side and left side vessels were imaged for 5 min; the mouse was then placed on its other side and right side vessels were imaged for an additional 5 min. Imaging was performed 1.6 cm from the tail base, using a 50 ms exposure time and a 10 fps frame rate. In some experiments, the pumping pressure was measured as previously described [66]. Briefly, a pressure cuff was
placed around the tail at 1.8 cm from the base of the tail. The pressure was quickly increased to 80 mmHg, held for 5 min to allow flow cessation, and then reduced to 55 mmHg. The pressure was then reduced in 2.5 mmHg increments, with each step held for 5 s, until a pressure of 0 mmHg was reached. During the pressure application, fluorescence intensity was imaged immediately proximal to the pressure cuff. Pumping pressure was defined as the pressure at which fluorescence intensity in the vessel recovered to 50% of its maximum after flow cessation, and was calculated using a custom MATLAB script.

Functional pumping metrics, including packet frequency, packet amplitude, packet integral, and packet transport, were calculated from fluorescence images as previously described [66]. Briefly, a series of fluorescent images was imported into ImageJ (NIH, Bethesda, MD) and fluorescent signal over time for a region of interest was exported. The resulting intensity trace was analyzed using a custom MATLAB script to identify peaks, each representing a packet of lymph, and calculate functional metrics. Packet frequency is calculated as the number of packets that occurred per unit time. Packet amplitude is the change in fluorescence intensity between the packet minimum and maximum. Packet integral is the fluorescence signal within each packet, or the integral of a peak between the troughs on either side. Packet transport is the time-normalized sum of all packet integrals in a video; it represents the total fluorescence signal moving through the vessel, and is thus a metric of overall lymph transport due to intrinsic contractility of the lymphatic vessel. Amplitude, packet integral, and packet transport are presented as normalized to the vessel’s baseline fluorescence to control for variations in vessel brightness between animals.

4.2.4 Functional effects and side effects of BayK-NP treatment
To evaluate the acute and longitudinal effects of BayK and BayK-NP on lymphatic pumping *in vivo*, mice were simultaneously injected with IRDye 800CW-labeled PEG tracer and a treatment. Treatment groups included: 81 μg of BayK in 50% dimethyl sulfoxide (DMSO), 50% DMSO (BayK vehicle control), BayK-NP containing 81 μg of BayK, or dose-matched SH-NP (BayK-NP vehicle control). All mice were imaged the day before treatment to establish baseline function and immediately after drug injection (0 h), and were additionally imaged either 8 h or 15 h after injection to determine if there was as a sustained effect of treatment on lymphatic function. All imaging was performed as described above, and took less than 30 min to complete at each time point. During 0 h imaging, mouse heart rate was monitored using a rodent pulse oximeter (Kent Scientific, Torrington, CT) clipped on the front left paw. After imaging was complete, mice were carefully observed and the occurrence and severity of side effects and motor impairment were recorded. Motor impairment was characterized by unsteady movement or lack of movement, and other side effects included excessive grooming, hunching, limb tonus, shaking, or nonresponsiveness.

To investigate the role of BayK circulating in the bloodstream in the observed difference in side effect severity between BayK- and BayK-NP-treated mice, BayK concentration in the blood was measured after injection of either treatment in the tail tip in isoflurane anesthetized mice. 50 μl of blood was drawn by facial lancet before injection and at 1, 5, and 60 m after injection, and was spiked with EDTA to avoid clotting. 50 μl of acetonitrile was added to precipitate blood proteins, and blood was then centrifuged at 20000 g for 20 m. 2 μl of the resulting supernatant was sampled in duplicate and absorbance
at 415 nm was measured on a plate reader using a Take3 plate. BayK concentration was calculated by comparison to a BayK standard curve.

4.2.5 Lymphatic ligation surgical model

C57/Bl6 mice were anesthetized with inhaled isoflurane (5% induction, 1.8% maintenance) and injected with 10 µl of IRDye 800CW-labeled 20 kDa PEG tracer intradermally at the midline of the tail, less than 1 cm from the tail tip. Immediately after injection, the tail was imaged 1.6 cm from the tail base, and the dominant lymphatic vessel was determined by first appearance of fluorescent signal on the left or right side of the tail [171]. The dominant side vessels were ligated by cauterization 1.6 cm from the tail base. Superficial lymphatic capillaries were also cauterized by making a superficial ligation around ~90% of the tail circumference, taking care not to disturb the nondominant side lymphatic collectors. To verify successful surgery, NIR imaging was used to demonstrate complete cessation of flow through ligated dominant vessels and to ensure that nondominant vessels remained intact. Antibacterial ointment was applied to the wound, and animals were given buprenorphine for pain management.

Lymphatic function and swelling progression in this lymphedema model were characterized in five mice over 14 d. On day 0, pre-surgery imaging was performed on all animals, followed by immediate ligation of the dominant vessel and superficial lymphatic capillaries as described above. Functional imaging was additionally performed on day 7 and d14 after surgery, and photos of the tail were taken on day 0, 7, 11, and 14. From these photos, peak tail diameter was measured in ImageJ. On day 12, 5 mice with lymphedema and 5 wild type (WT) mice were injected with 23.8 µg of Alexa Fluor 610-labeled 500 kDa
dextran (with a hydrodynamic diameter of 30 nm) and IRDye 680RD-labeled NP in a single, 15 μl tail tip injection. Fluorescent signal of both dextran and NP at the injection site was monitored using an IVIS imaging system at 0, 2, 4, 8, 24, and 48 h after injection. After d14 functional imaging, mice were sacrificed and left and right sacral LN draining the tail injection were separately harvested and homogenized in tubes pre-filled with 1.4 mm zirconium beads (OPS Diagnostics, Lebanon, NJ). Dextran and NP fluorescence in the LN homogenate was measured using a plate reader.

4.2.6  Effects of BayK-NP treatment on vessel function and lymphedema development

Lymphedema was induced and treated in WT female C57/Bl6 mice or in obese male C57/Bl6 mice fed on a high fat diet to a weight of 54 g. On day 0, mice underwent vessel ligation surgery as previously described. 3 d were allowed for wound healing and resolution of initial inflammation. Beginning on day 3, mice received daily treatment by tail tip injection of either 67.5 μg of BayK-NP (n=10 WT, n=5 obese) or dose-matched SH-NP control NP (n=10 WT, n=5 obese). Photographs of the tail were taken on day 0 and daily beginning on day 3 for swelling analysis in ImageJ. Functional imaging, including pumping pressure analysis, was performed on day 0 (pre-surgery), day 7, and day 14. On day 7, the daily treatment was given after imaging was complete to avoid measuring acute effects of the injection. After imaging was completed on day 14, animals were sacrificed, blood collected by cardiac puncture into EDTA-coated tubes, spleens harvested, and tail sections collected for histology. Spleens were weighed and photographed. Blood was centrifuged at 2100 g for 15 min, and the plasma supernatant was stored at -80C until alanine transaminase (ALT) and aspartate aminotransferase (AST) assays were performed according to manufacturer instructions (BioVision, Milpitas, CA).
For histology, 1 cm of tail distal to the ligation was fixed in 4% paraformaldehyde for 48 h and decalcified in pH 8.0 0.5 M EDTA for 2 w, with EDTA changed 2-3 times per week. Tails were then paraffin embedded and cut into 5 µm sections. For hematoxylin and eosin (H&E) staining, slides were deparaffinized and hydrated by 15 m incubation in xylene and washes in 100% alcohol, 95% alcohol, 70% alcohol, and water. Sections were stained with hematoxylin for 1 m, washed in acid alcohol, Scott’s, and 95% alcohol, and then stained in eosin for 1 m. After sequential washes in alcohol and xylene, slides were mounted in CytoSeal 60 (Thermo Fisher). For picro-sirius red, slides were deparaffinized and hydrated, and nuclei were stained for 5 m with Weigert's hematoxylin. After washing, sections were stained for 1 h in picro-sirius red solution, washed in acidified water, dehydrated, and mounted. Epidermal thickness and dermal thickness were quantified from hematoxylin and eosin (H&E) images in ImageJ; each parameter was measured 3 times at representative locations around the tail circumference and averaged per section. Average LV area was quantified by tracing a minimum of 20 representative LVs in each section and averaging. In picro-sirius red stained sections, collagen positive pixels between the dermis and muscle fascia were quantified in ImageJ. For immunohistochemistry, sections were deparaffinized and hydrated as described, incubated in citrate buffer (pH 6) for 30 m at 90°C and for 30 m at room temperature. Sections were then permeabilized by incubation with 0.5% Triton-X for 30 m at RT, blocked in 10% goat serum for 1.5 h, and incubated overnight with primary antibodies diluted in phosphate buffered saline (PBS) (1:63 podoplanin, Abcam 11936; 1:77 CD11b, Abcam 133357; or rabbit IgG isotype control, R&D Systems AB-105-C). After washing, sections were incubated for 4 h at RT with nucBlue live cell stain (Thermo Fisher) and secondary antibodies diluted 1:500 in PBS.
(podoplanin: AF488 goat anti-hamster, Invitrogen A-21110; CD11b: AF594 goat anti-rabbit, Invitrogen A-11012). Slides were then mounted in VectaShield, sealed with nail polish, and imaged on a Zeiss AxioObserver microscope. To calculate CD11b+ density, IHC images were thresholded against isotype-stained sections, and CD11b+ density was calculated in a minimum of 10 representative regions around the periphery of the tail. These densities were averaged to yield a single average density for the section, and two sections were analyzed per tail.

4.2.7 Immunological effects of BayK

To evaluate potential immunological effects of BayK administration, splenocytes were treated with BayK in vitro. A C57/Bl6 spleen was processed through a 0.7 μm cell strainer and treated with ACK lysis buffer to lyse red blood cells. Splenocytes were plated at 1 million cells/well in a 96 well plate, with 150 μl of RPMI media. Splenocytes were treated with 0.9 μg/mL BayK (2% of the in vivo dose, and a feasible resulting LN concentration), 20 ng/mL PMA and 1 μg/mL ionomycin (a positive control known to result in T cell activation), or DMSO (vehicle control). After 4 h of incubation at 37°C, cells were washed and treated with 2.4G2 Fc block for 5 m on ice. Cells were then stained with Zombie Aqua Fixable Viability Kit (Biolegend, San Diego, CA) for 30 m at room temperature, and then stained for 30 min on ice with a panel of antibodies, including PerCP CD45, BV711 CD3, PE/Cy7 CD4, FITC CD8, Alexa Fluor 700 CD25, BV421 CD69, Alexa Fluor 647 PD-1, and PE FoxP3, all from Biolegend. Cells were then fixed by 15 m incubation in 2% PFA on ice, and analyzed on a BD Fortessa flow cytometer.
In a second group of obese mice, spleens and sacral LNs draining the nondominant, intact lymphatic vessel were collected and immune profiled by flow cytometry on d14, after 11 d of daily BayK-NP or SH-NP treatment in the tail tip and footpad ipsilateral to the dominant collector ligation. Single cell suspensions were generated by processing tissues through a 0.7 μm cell strainer, and in the spleen RBCs were additionally lysed. Cells were treated with 2.4G2 Fc block for 5 m on ice and then stained with Zombie Aqua Fixable Viability Kit (Biolegend, San Diego, CA) for 30 m at room temperature. Each sample was then halved and stained for 30 m on ice with one of two antibody panels: T cell: PerCP CD45, BV711 CD3, PE/Cy7 CD4, FITC CD8, BV786 CD25, BV421 CD69, Alexa Fluor 647 PD-1, and PE FoxP3, or DC: PerCP CD45, BV421 CD206, Alexa Fluor 647 MHCII, BV786 F4/80, FITC CD11b, PE/Cy7 CD11c, and PE CD86. Cells were then fixed by 15 m incubation in 2% PFA on ice, and analyzed on a BD Fortessa flow cytometer.

4.2.8 Statistical analysis

All data are presented as mean ± standard error of the mean, and statistical analyses were performed in Prism 8 (GraphPad Software Inc., La Jolla, CA). Statistical significance was defined as p < 0.05 following an unpaired t-test, ordinary one-way analysis of variance with Tukey posthoc testing, or repeated measures analysis of variance as indicated within figure captions. Symbols denoting p-values are as follows: * = p < 0.05, ** = p < 0.01, *** = p < 0.001, and **** = p < 0.0001.

4.2.9 Animal use

All animal procedures were performed at Georgia Institute of Technology, and were approved by the Georgia Institute of Technology Institutional Animal Care and Use
Committee. All WT mice were female C57BL/6J, and were used at 10-18 weeks of age. Obese mice used were male C57BL/6J fed a high-fat diet from 6 weeks of age, and were used at 34-37 weeks of age and 46-61 g mass.

4.3 Results

4.3.1 BayK-NP synthesis and characterization

Although L-type calcium channel agonist BayK is known to improve lymphatic vessel pumping *ex vivo* through its effects on LMCs (Fig. 4.1A), its *in vivo* effect on vessel function has not been explored in part due to its small size (356 Da). While small molecule drugs like BayK are unrestricted by extracellular matrix in the interstitium and can passively drain into both blood and lymph, they tend to partition primarily into the blood because of its large volume and high flow rates [19,106], resulting in poor lymphatic access. This effect is clearly observed in the mouse tail (Fig. 4.1B); when IRdye 680 is conjugated to a 30 nm polypropylene sulfide NP (PPS-NP), it shows clear uptake into tail lymphatic vessels, co-localizing with a known lymphatic-draining IRdye 800CW-labeled 20 kDa polyethylene glycol (PEG) tracer although there is no overlap in the dye emissions (Fig. 4.2A). When free IRDye 680 is injected, however, no visibly detectable signal is observed in lymphatic vessels. This demonstrates the clear lymphatic access advantage that a NP carrier can provide, and the typically poor lymphatic uptake of a small molecule. To overcome this barrier to lymphatic delivery, BayK was encapsulated in the core of PPS-NP. These NP are composed of a hydrophilic Pluronic corona and a hydrophobic poly(propylene sulfide) core where hydrophobic small molecule drugs like BayK partition upon mixing with NP (Fig. 4.1C) [12]. To evaluate BayK encapsulation into NP, BayK
and NP elution from a size exclusion chromatography column was monitored before and after the components were mixed; BayK could be detected by its absorbance, which peaks at 415 nm (Fig. 4.1D) and can be used to generate a standard curve to determine BayK concentration (Fig. 4.2B), and NP were detected using a modified iodine assay for the detection of PEG [110]. When BayK and NP were individually run on a size exclusion column, they showed distinct elution profiles, as expected based on their size difference (Fig. 4.1E). When the two components were mixed, however, the BayK signal clearly co-eluted with the NP, suggesting successful encapsulation and the formation of BayK-loaded NP (BayK-NP) (Fig. 4.1F). Encapsulation of BayK did not affect NP size (Fig. 4.1G), which is critical for its lymphatic draining capabilities. BayK released from BayK-NP gradually in vitro over the course of days (Fig. 4.1H), with a slightly faster release at 37°C than at room temperature; 50% release occurred at ~8 h and ~15 h, respectively. Formulation within NP clearly improved BayK retention within the dialysis membrane, as free, unencapsulated BayK was completely lost with 8 h. BayK could be encapsulated with high efficiency at tested conditions, with 90% of the drug loading into the NP core (Fig. 4.1I), and encapsulation could be tailored by adjusting the ratio of BayK:NP mixed (Fig. 4.2C).
Figure 4.1. Nanoparticles provide a lymphatic vessel targeting advantage compared to free drug, and allow for efficient loading and controlled release of small molecule Bay K. A) Schematic of Bay K’s effect on lymphatic vessel pumping. B) Appearance of injected dye in tail collecting lymphatic vessels (left) after co-injection with a lymphatic-draining PEG tracer (right). Scale bar = 3 mm. C) Schematic of pluronic-stabilized poly(propylene sulfide) NP structure and loading with hydrophobic Bay K8644. D) Absorbance spectra of BayK and PPS-NP. Elution profiles of Bay K and NP vehicle from a CL6B size-exclusion column when run separately (E) or after brief mixing in 5% THF (F). NP signal in each fraction was monitored using a modified iodine assay, and Bay K presence was determined by measuring each fraction’s absorbance at 415nm. G) NP hydrodynamic diameter before and after Bay K loading, measured by DLS. H) In vitro release profile of Bay K from BayK-NP at room temperature and physiological temperature (37°C) (n=3). Loss of free BayK through
the membrane is also shown. H) Bay K encapsulation efficiency when mixed with 30 mg/ml SH-NP.

Figure 4.2. BayK-NP loading and characterization. A) Employing two NIR filters enables simultaneous imaging of IRdye 680RD (NP) and IRdye 800CW (PEG tracer), with no spillover of signal between filters. B) Bay K absorbance at 415 nm increases linearly with increasing concentration. Standard curve in ACN. C) Encapsulation efficiency of Bay K in NP over a range of Bay K:NP ratios. Using a high NP concentration allows for highly efficient encapsulation with lower drug/NP, but drug/NP can be increased at the cost of lower encapsulation efficiency.

4.3.2 BayK-NP effects ex vivo and in vitro

To directly evaluate the effect of BayK-NP on lymphatic vessels removed from the complicated in vivo context, rat mesenteric lymphatic vessels were isolated and cannulated,
their contractile response to BayK application \textit{ex vivo} was monitored, and functional metrics like contraction frequency, amplitude, and ejection fraction were calculated (Fig. 4.3A-D). BayK was found to have dose-dependent effects on isolated vessel function when added to the bath, resulting in increased amplitude (Fig. 4.3B) and vessel function, as indicated by an increased ejection fraction, at as low as 200 nM BayK (Fig. 4.3C), while contraction frequency (Fig. 4.3A) was unaffected by up to 1000 nM BayK application. When 200 nM BayK-NP were administered, there was an immediately visible response, resulting in much less frequent but stronger contractions (Fig. 4.3E), consistent with the vessel response to 200 nM free BayK (Fig. 4.3D). Treatment with control thiolated PPS-NP (SH-NP), with no BayK loaded, induced no discernable response by vessels (Fig. 4.3F); contraction frequency and amplitude remained consistent, suggesting that the NP vehicle itself does not impact vessel pumping. Overall, treatment with BayK-NP resulted in reduced contraction frequency, increased contraction amplitude, and an overall improvement in vessel pumping efficiency as measured by the ejection fraction (Fig. 4.3G). BayK similarly showed an increase in both amplitude ejection fraction, but control SH-NP treatment did not affect any function metrics. These results indicate that BayK-NP are capable of release of bioactive BayK that can improve lymphatic vessel pumping \textit{ex vivo}. 
Figure 4.3. Effect of BayK-NPs on lymph pumping ex vivo in isolated rat mesenteric lymphatic vessels (RMLVs). Isolated RMLV A) contraction frequency, B) contraction amplitude, and C) ejection fraction after exposure to BayK. * indicates statistically significant difference compared to pre-treatment 100% function by one-way RM ANOVA (n=8). D) Representative recordings of spontaneous contractions in a RMLV before and after treatment with 200 nM Bay K, E) 200 nM BayK-NP, or F) dose-matched control SH-NP. G) Change in vessel contraction frequency, amplitude, and ejection fraction after treatment with SH-NP, 200 nM BayK-NP, or 200 nM Bay K. Presented as normalized to pre-treatment baseline. * indicates significant difference between treatment value and a pre-treatment value of 100% by t-test with Holm-Sidak correction (n=7-8). H) Expression of activation markers by CD4+ T cells and I) CD8+ T cells after in vitro treatment with BayK or activation with PMA and ionomycin for 4 h. In H and I, * indicates significant difference from DMSO control by one-way ANOVA with Tukey’s multiple comparison testing (n=3).

Because BayK activates calcium channels that play an important role in immune cell function [172,173] and immune infiltration and dysregulation is critical in lymphedema development and progression [63], the effect of BayK on T cell activation in
*in vitro* was evaluated (Fig. 4.3H, I). When murine splenocytes were treated for 4 h with phorbol myristate acetate (PMA) and ionomycin, there was a clear upregulation of activation markers in both CD4+ T cells (CD69, PD-1) and CD8+ T cells (CD25, CD69, PD-1). Although BayK activates similar pathways, treatment with 0.9 μg/ml BayK for 4 h did not induce any increase in expression of these markers (Fig. 4.3H, I), and T cell viability and regulatory T cell (Treg) marker expression were similarly unaffected (Fig. 4.4). This suggests that BayK may be applied *in vivo* without overt direct immune modulatory effects on T cells.

Figure 4.4. BayK-NP do not induce T cell activation in vitro. A) T cell gating strategy. B) T-cell viability. C) Treg frequency of CD4+ cells. D) MFI of Treg markers. In all panels, * indicates significant difference from DMSO control by one-way ANOVA with Tukey’s multiple comparison testing.
4.3.3 *In vivo functional experiments*

With evidence that BayK-NP application can improve pumping function of isolated vessels, we next investigated the effect of BayK-NP injection on vessel function *in vivo*. Upon tail tip injection of IRdye 800CW-labeled 20 kDa PEG, collecting lymphatic vessels in the tail could be immediately and clearly imaged by NIR microscopy (Fig. 4.5A); simultaneous injection with NP is not expected to affect drainage of the PEG or subsequent function calculations, as the PEG does not associate with NP upon mixing (Fig. 4.6A). Vessels were imaged from the left or right side of the tail, and tracking fluorescent intensity at a region of interest revealed clear contraction peaks, consistent with the movement of lymph in “packets” (Fig. 4.5A, Fig. 4.6B). These traces were then analyzed to yield several metrics of vessel pumping function, including packet frequency, packet amplitude, packet integral, and packet transport, the amount of fluid pumped per minute and a critical metric of vessel function [66]. To investigate the effect of both free and NP BayK formulations on vessel function *in vivo*, vessels were imaged following a tail tip injection containing 80 μg of BayK in 50% dimethyl sulfoxide (DMSO), 50% DMSO, BayK-NP loaded with 80 μg of BayK, or dose-matched SH-NP with no drug loaded. While BayK administration did not affect vessel pumping by any functional metric immediately after treatment (0 h) compared to the DMSO vehicle control (Fig. 4.5B-E), BayK-NP increased packet amplitude, packet integral, and packet transport compared to its SH-NP vehicle control (Fig. 4.5C-E). An immediate decline in frequency was also observed with BayK-NP treatment (Fig. 4.5B); this is consistent with the reduction in frequency and increase in amplitude and overall fluid transport seen with BayK treatment in the literature [95] and in isolated vessel experiments (Fig. 4.3G). Following acute functional imaging, mice were
again imaged at either 8 h or 15 h after BayK administration; only one additional time point was used per animal to avoid potential effects of repeated injection on treatment clearance from the tail tip. 8 h after BayK or BayK-NP application, no effects on frequency were observed (Fig. 4.5B). BayK-NP treatment alone resulted in increased packet integral and subtly increased amplitude compared to control NP, but free drug administration had no such effects (Fig. 4.5C-D). By 15 h post-treatment, effects of a single BayK-NP injection were no longer observed (Fig. 4.5B-D). These results highlight the importance of lymphatic access to enable BayK efficacy in vivo; functional improvement was only observed when BayK was formulated into a NP, while free BayK was ineffective. These results also suggest that a single BayK-NP injection has lasting effects on vessel function that persist for at least 8 h, but which diminish within 15 h.
Figure 4.5. BayK-NP enable BayK to acutely improve lymphatic pumping in vivo. A) Schematic of analysis process. Vessels are imaged from the left or right side of the tail. ROIs are selected on visible vessels and fluorescent intensity traces are analyzed to obtain pumping metrics. Scale bar = 2 mm. B) Packet frequency, C) normalized packet amplitude, D) normalized packet integral, and E) normalized packet transport immediately after (0 h), 8 h after, and 15 h after tail tip injection of Bay K, BayK-NP, or their vehicle controls (DMSO and SH-NP, respectively); all metrics presented as separate left and right vessels normalized to respective vehicle controls. Outliers were identified by ROUT (Q=1%) and removed before analysis. Significance tested by unpaired t-tests between Bay K formulation and appropriate vehicle control.
Figure 4.6. In vivo lymphatic function analysis. A) BayK-NP do not associate with PEG-IR dye upon mixing, as determined by SEC. When run separately on a CL6B SEC column, NP and PEG-IR dye show distinct elution profiles. When the two are mixed for 30 minutes at 37°C and the mixture analyzed by SEC, no association is observed and distinct elution profiles are maintained. NP presence was monitored using Ellman’s assay for thiol detection, and PEG-IR dye signal was measured by fluorescence at the dye’s optimal excitation and emission, 770/795nm. B) Examples of intensity traces obtained from NIR imaging when peaks are normal (center) or inverse (right).

4.3.4 Nanoparticle formulation effects on BayK toxicity

Because of BayK’s small size, it is expected to rapidly access circulation upon intradermal injection. This was confirmed by measuring the concentration of BayK in blood following tail injection of BayK or BayK-NP. Free BayK was observed to access the blood within minutes, resulting in a significantly higher acute blood concentration than the same dose of BayK in a NP formulation (Fig. 4.7A). This rapid systemic access may contribute to known side effects of BayK administration, including altered heart rate [174], motor impairment, and behavioral changes [101,175]. While no clear effect of BayK administration on mouse heart rate, as measured by pulse oximeter in anesthetized mice, was observed (Fig. S5), potentially due to the overwhelming effect of anesthesia, free
BayK administration did result in more severe side effects than the same dose of BayK-NP (Fig. 4.7B,C). While BayK-NP caused minor side effects, like hunching, excessive grooming, or impaired motor coordination, mice given free BayK showed more severe side effects and significant mobility challenges (Fig. 4.7C). NP delivery thus not only enables lymphatic uptake of a typically ineffective small molecule, but also significantly reduces drug side effects by preventing rapid drug access to circulation and providing gradual release. To investigate the systemic effects of chronic BayK-NP administration, mice were treated daily with BayK-NP for 11 d. Mice were then sacrificed, and serum and spleens harvested for analysis. Even though NP-sized molecules are cleared primarily by the liver [22], no evidence of liver toxicity was observed after chronic BayK-NP treatment; alanine transaminase (ALT) and aspartate aminotransferase (AST) levels were unchanged compared to NP or untreated controls (Fig. 4.7D,E). Spleen mass was also unchanged by BayK-NP treatment (Fig. 4.7F), suggesting that chronic BayK-NP administration does not induce systemic immune side effects.
4.3.5 Single vessel ligation lymphedema model characterization

To evaluate the effect of BayK-NP in a chronic lymphatic dysfunction model, we employed a single lymphatic vessel ligation model in which the dominant collecting vessels of the tail are ligated by cauterization along with superficial lymphatic capillaries.
around the circumference of the tail, leaving only the nondominant collecting vessels intact [66] (Fig. 4.8A). This surgery resulted in immediate cessation of flow through ligated vessels, but still allowed drainage past the ligation site in intact vessels (Fig. 4.8B). Although the ligation wound healed rapidly, tail swelling distal to the ligation was consistently observed, as indicated by an increase in tail diameter immediately distal to the wound in the weeks following surgery (Fig. 4.8C-D). This swelling was accompanied by significantly impaired pumping function in the intact lymphatic collectors by every metric, including contraction frequency, amplitude, and packet transport at d7, and packet integral and packet transport at d14 after surgery (Fig. 4.8E). There was additionally a significant correlation between amplitude and tail diameter and between packet transport and diameter, and nonsignificant correlations in frequency and packet integral (Fig. 4.8F), suggesting that function by these metrics decreases with increasing tail diameter as has been shown previously [66]. Interestingly, at both 7 and 14 days post-surgery, vessel pumping pressure was lost (Fig. 4.8E) (the pumping pressure prior to surgery in these mice was 20.5 ± 8.4 mmHg). Molecule clearance from the tail was also impaired. When fluorescent dextran and NP, both 30 nm in hydrodynamic diameter, were injected in the tail tip 12 d post-surgery, both molecules showed increased retention in ligated tails compared to wild type (WT) tails (Fig. 4.8G) due to accumulation distal to the ligation as measured by IVIS (Fig. 4.8H). This reduced clearance resulted in significantly reduced accumulation of both dextran and NP in sacral LN draining ligated vessels compared to those draining intact vessels (Fig. 4.8I), even though no significant difference between dominant and nondominant-draining sacral LNs was observed in WT animals. It is important to note that during the course of experiments, the ligated lymphatic collectors
remained occluded but the intact collectors remained patent; injected NP could be clearly visualized draining past the ligation site even 14 d post-surgery (Fig. 4.8J). The single vessel ligation model is thus well-suited for investigation of BayK-NP application, as an intact vessel still remains available for drug efficacy.

Figure 4.8. The single vessel ligation lymphedema model shows dysfunction in intact lymphatic vessels. A) Schematic of surgical induction of lymphedema. The dominant tail lymphatic collectors and superficial lymphatic capillaries are cauterized, but nondominant vessels are left intact. B) This surgery results in immediate cessation of flow through the ligated dominant vessels (top), while maintaining flow through intact
nondominant vessels (bottom). Red arrow: wound location; yellow arrow: cessation of flow past ligation; green arrow: dye accumulation distal to ligation; blue arrow: dye flow past ligation. Scale bar = 2 mm. C) Representative images of tail before surgery (d0), 7, and 14 days after ligation. D) Tail diameter distal to ligation in individual mice over two weeks following vessel ligation. * indicates statistically significant difference from d0 diameter by one-way RM ANOVA (n=5). E) Vessel function metrics on d7 and d14 after ligation surgery, normalized to pre-surgery baseline function. One pumping pressure outlier was identified by ROUT (Q=1%) and removed before analysis. * indicates statistically significant difference compared to d0 100% function by one-way RM ANOVA (n=5). F) Vessel function metrics show significant (amplitude, packet transport) or weak (frequency, packet integral) correlation with tail diameter. G) Fluorescence signal in the tail after dextran or NP injection in the tail tip in WT or lymphedema mice, quantified from IVIS images. * indicates statistically significant difference between WT and lymphedema clearance by two-way RM ANOVA (n=4). H) Representative IVIS images of 30 nm dextran localization in the tail over time, showing dye accumulation immediately distal to vessel ligation in lymphedema animals. I) Dextran and NP accumulation in sacral LN draining the dominant and nondominant vessels in WT and lymphedema mice. * indicates statistically significant difference by one-way ANOVA with Tukey’s comparison (n=4). J) NIR imaging of 680-NP uptake into lymphatic vessels of ligated tail 14 days after surgery, top view. Red arrow: ligation site. Blue arrow: NP transported by intact vessels past ligation site. Green arrow: dye accumulation distal to ligation. Scale bar = 2 mm.

4.3.6 BayK-NP for the treatment of lymphedema

To evaluate the effect of BayK-NP in a chronic lymphatic dysfunction model, lymphedema was induced in 20 WT mice by ligation of dominant lymphatic collectors and superficial lymphatic capillaries, leaving only the nondominant side collectors intact. Mice were then treated daily with 68 μg of BayK-NP or control SH-NP starting d3 post-surgery through d14, with daily tail swelling measurement and lymphatic functional imaging performed on d0 (pre-surgery), d7, and d14. Frequency, amplitude, and packet transport were reduced after ligation in both treatment groups, as expected, but BayK-NP treatment did not change these metrics compared to SH-NP control at either d7 or d14 (Fig. 4.9A). Tail swelling was monitored over the course of the experiment by measuring peak tail
diameter distal to the ligation, and diameter was normalized to d3 diameter to provide a post-surgery, pre-treatment baseline and control for differing severity of the ligation. Though all mice showed expected swelling after d3, no differences in tail diameter with BayK-NP treatment appeared when all mice were considered (Fig. 4.10A). To evaluate differences in BayK-NP efficacy in mice with severe swelling and mice with mild swelling, animals were separated into low swelling (> 1.3 fold) and high swelling (< 1.3 fold) groups based on the maximum increase in tail diameter after surgery on d0. In low swelling animals, BayK-NP treatment reduced tail diameter at d7 compared to NP control (Fig. 4.10B), while treatment had no effect in high swelling animals (Fig. 4.9B). Similar trends were seen in vessel pumping pressure, which was subtly increased on d14 in animals treated with BayK-NP compared to those treated with control NP (Fig. 4.10C). These effects were more pronounced in mice with low swelling at d14 (Fig. 4.10D), but absent in mice with severe swelling (Fig. 4.9C). No additional differences emerged with treatment when frequency, amplitude, packet integral, and packet transport were similarly separated by swelling severity (Fig. 4.9D-E). These results suggest that BayK-NP treatment can improve lymphedema outcomes, reducing swelling and improving vessel pumping pressure, and that these effects are most pronounced in animals with less severe lymphedema.
Figure 4.9. WT lymphedema functional metrics separated by swelling severity. A) Vessel functional metrics frequency, amplitude, packet integral, and packet transport for all lymphedema mice with BayK-NP or control NP treatment. Statistics indicate function comparison to d0 by two-way RM ANOVA. Differences between treatment groups were nonsignificant. B) Peak tail diameter of high swelling WT mice, normalized to d3 diameter. C) Pumping pressure of high swelling WT mice. D) Vessel functional metrics for low swellers, animals with less than 1.3x change in peak tail
diameter from d0. E) Vessel functional metrics for high swellers, animals with greater than a 1.3x change in peak tail diameter from d0.

Figure 4.10. Chronic BayK-NP treatment improves early lymphedema outcomes. A) Peak tail diameter of WT mice normalized to d3 diameter, a post-surgery, pretreatment baseline. One d14 outlier was identified by ROUT (Q=1%) and removed before analysis. B) Peak tail diameter of low swelling WT mice, normalized to d3 diameter. * indicates significant difference from NP control by t-test (n=6). C) Vessel pumping pressure of intact vessel in WT lymphedema mice. * indicates significant difference by t-test. D) Pumping pressure of low swelling WT mice. E) Sirius Red staining for collagen in mouse tail sections before (top) and 14 d after (bottom) lymphedema induction. F) Quantification of collagen+ area between the muscle fascia and dermis. G) Representative images of CD11b staining in mouse tail dermis at d14 after BayK-NP treatment, SH-NP treatment, or in naïve (no surgery) animals. H) Quantification of percent CD11b+ area. In F and H, BayK-NP and NP groups were compared by unpaired t-test (n=5).

In addition to effects on limb swelling and LV pumping function, lymphedema is associated with chronic remodeling of affected tissue [65,176,177]. We thus investigated the effect of BayK-NP treatment on tissue structure in WT swollen tails on d14 after surgery. Staining sections with picro-sirius red for collagen imaging revealed increased
collagen deposition in lymphedematous tails compared to naïve animals, consistent with previous studies [178,179], and showed a reduction in total collagen+ area with BayK-NP treatment (Fig. 4.10E-F), suggesting that BayK-NP treatment can inhibit the remodeling associated with lymphedema progression. BayK-NP treatment did not, however, appear to alter skin thickness (Fig. 4.11B-C) or average LV area (Fig. 4.11D) as measured from H&E stained sections. Immune cell infiltration was additionally investigated, and no significant difference in CD11b+ density was observed between treatment groups (Fig. 4.10G-H).

Figure 4.11. Structural tail changes measured in H&E stained sections. A) Representative H&E stained sections from naïve tails (left) or d14 post-surgery (right). B) Epidermal thickness, C) dermal thickness, and D) average cross-sectional area of tail LVs in naïve, BayK-NP treated, or SH-NP treated mice. In B-D, BayK-NP and NP groups were compared by unpaired t-test.
Given that in mouse models, obesity has been shown to impair lymphatic function and exacerbate lymphedema pathology [43,66,180,181], we sought to also test BayK-NP treatment in diet-induced obese mice. Similar results were observed in obese mice treated with BayK-NP or control NP after lymphedema induction. While functional metrics were unaffected at any timepoint, with the exception of frequency at d14 (Fig. 4.12A-D), tail diameter increase after d3 was nonsignificantly reduced by BayK-NP treatment at both d7 and d14 (Fig. 4.12E). This was accompanied by a loss in detectable pumping pressure in control mice at d14 that was prevented in BayK-NP treated mice (Fig. 4.12F). Because BayK is a calcium channel modulator, it has the potential to modulate activation in immune cells expressing L-type calcium channels [172,173,182]. To evaluate whether chronic BayK-NP treatment had immunological effects in obese animals, spleens and sacral LNs draining the nondominant tail collectors were profiled after sacrifice on d14. No significant difference in activation of CD4+ or CD8+ T cells was observed with BayK-NP treatment (Fig. 4.13C), and macrophage polarization (Fig. 4.13D) and DC activation (Fig. 4.13D) were similarly unaffected in both the sacral LN and in the spleen. Overall, these results suggest that even chronic administration of BayK-NP at the employed dose does not result in significant immunological changes in the immediate draining LN or systemically, consistent with in vitro observations (Fig. 4.3H-I, Fig. 4.4).
Figure 4.12. BayK-NP treatment of lymphedema in obese mice. A) Frequency, B) amplitude, C) packet integral, and D) packet transport in obese mice before surgery (d0) and after d7 and d14. * indicates significant difference by two-way RM ANOVA. E) Peak tail diameter of obese mice normalized to d3 diameter, a post-surgery, pre-treatment baseline. Significant differences evaluated by t-test. F) Pumping pressure of intact vessel in obese mice. Significance tested by t-test.
Figure 4.13. Lymphedema immune analysis in obese mice. A) DC gating strategy. B) T cell gating strategy. C) Percent of CD4+ and CD8+ T cells expressing activation markers. D) F4/80+ macrophage occurrence and activation. E) Percent of DCs expression activation markers CD86 and MHCII. Significance tested by t-test.
4.4 Discussion

Collecting LVs are attractive therapeutic targets because of the important role of LV function in a variety of pathologies including heart disease, obesity, and lymphedema. However, no pharmacologic approaches have been developed to enhance lymphatic pumping \textit{in vivo}. This is likely because delivering agents to LMCs to improve the function of collecting LVs presents numerous delivery challenges. First, administered drugs typically have poor access to lymphatic tissues from the systemic circulation. As such, locoregional routes of administration offer advantages with respect to providing an administered therapeutic access to local collecting LVs draining the injection site. This is because after drainage into LVs, lymph-borne agents have improved access to LMCs that surround the LV wall just outside the lumen. Second, small molecule drugs, which represent a large number of potential therapeutics investigated to date that target pathways regulating LV function [20,89,96,183], show poor accumulation within lymph after locoregional administration due to their short half-life of tissue retention and rapid clearance via blood capillaries [19,106]. To overcome this limitation, a variety of controlled delivery and release technologies have been employed to improve lymphatic uptake [12,26–29]. The biomaterial PPS-NP platform employed in this work is a versatile system well-suited for the application; the NP are modular, comprised of nontoxic polymers, can encapsulate and release hydrophobic drug without the need for chemical modifications or environmental stimulus, and have been previously shown to improve the delivery of small molecules to lymphatic tissues [12,29,110,149]. Of note, however, most approaches that harness controlled delivery and release approaches to increase lymphatic uptake do so with the intent to target the LN and the immune cells resident there. The work
here described represents a logical, but novel, extension of this idea, taking advantage of enhanced lymphatic drainage to target lymphatic vessels themselves rather than downstream LN.

We observed that small molecule BayK administered i.d. was only capable of improving the pumping function of draining LVs when in a NP formulation, a finding consistent with previous LN-targeting work. This highlights the importance of lymphatic uptake for vessel-targeting drug efficacy. Of note, BayK acts on L-type calcium channels expressed on LMCs rather than acting directly on the lymphatic endothelial cells (LECs) that line the vessel lumen. BayK may reach these target cells by diffusion from the vessel lumen after intraluminal release from the NP vehicle, as lymphatic vessel walls are relatively thin, or could be released during NP degradation if NP are taken up by phagocytic LECs. Regardless of the mechanism, the observed efficacy of a drug that acts on LMCs suggests that a reasonable drug concentration is achieved at LMCs after intraluminal delivery, a promising result for delivery of other contractility-modulating drugs. The PPS-NP delivery vehicle employed in this work is a versatile platform that could be easily extended to deliver other hydrophobic drugs or modified on the NP corona to deliver hydrophilic agents, and applied for drug delivery to either LECs or LMCs as shown in this work.

In addition to improving LV pumping function in vivo, BayK-NP also reduced the severity of side effects observed after BayK administration (Fig. 4.7C). Targeted or controlled drug delivery, including using NP platforms, has long been lauded as a method to increase drug concentration at the target site, allowing for lower or less frequent dosing while reducing systemic drug concentration and resulting off-target effects. BayK-NP are
no exception, and provide these advantages. Upon injection into the interstitium, small molecules drain quickly into the blood circulation, where they typically have short half-lives and are cleared rapidly by the kidney [22]. In the case of BayK, this resulted in a rapid but brief spike in the concentration of drug in the blood (Fig. 4.7A). Because L-type calcium channels are expressed on a variety of off-target cells, this exposure resulted in significant side effects; at their most severe, animals would become nonresponsive and show involuntary muscle contractions. BayK-NP likely reduce BayK concentration in the blood in several ways. First, NP are cleared slowly from the site of injection, draining into lymph gradually. Simultaneously, BayK-NP do not release their entire BayK payload immediately upon injection; rather, the payload is released over the course of several days (Fig. 4.1H). While BayK released from BayK-NP, whether at the injection site or after lymphatic drainage, can still eventually access the blood, the reduced side effects associated with the NP formulation may be due to the measured drug release that keeps concentration from spiking. Future work can further explore these benefits of BayK-NP administration, and can investigate the lowest effective BayK-NP dose to further reduce toxicity.

Animal models of lymphedema development are currently limited. Models including rabbit ear and dog hindlimb models, in which LVs are disrupted and tissue removed to halt flow and induce pathological swelling, have been investigated, but suffer from complexity and inconsistency [184,185]. A commonly used model is the rodent tail model [82,186,187], in which all initial and collecting lymphatic vessels in the tail are severed, resulting in swelling, immune infiltration, and tissue remodeling. This double vessel ligation model has been employed in many lymphedema studies focused on
characterizing the morphological changes associated with disease progression, for which it is well-suited. The model has also been used to investigate lymphedema treatment by targeting immune regulation or lymphangiogenesis; total cessation of lymph flow out of the tail induced by severing lymphatic vessels around the full tail circumference provides an excellent environment to study the ability of lymphangiogenesis to bridge the gap and restore lymph drainage. This model has limited application in studies of collecting vessel pumping function, however, as it leaves no collectors intact for analysis. For this reason, we instead employed a novel lymphedema model in which the dominant lymphatic collectors and superficial lymphatic capillaries in the mouse tail were selectively cauterized [66], leaving nondominant lymphatic collectors intact (Fig. 4.8J) but with reduced pumping function (Fig. 4.8E). These intact collectors are the target of the BayK delivered in this study, as they are capable of taking up the injected NP and productively responding to BayK to improve pumping function and lymph flow, unlike severed vessels. In addition to being well-suited for investigations of BayK administration and LV function modification, this model is arguably more representative of the human case, in which secondary lymphedema can result from incomplete disruption of vessels from a drainage bed [188], and shows similar swelling and morphological changes compared to the double vessel ligation model [66].

While daily treatment with BayK-NP clearly affected lymphedema progression, as evidenced by reduced tail swelling at d7 in animals with less severe disease and subtly improved pumping pressure at d14, the lack of temporal alignment of these outcomes highlights the significant complexity of this disease. The fact that improved LV function at d14 was not associated with a reduction in tail swelling may suggest that by d14, other
factors affecting lymphedema progression overwhelm the benefit of improved pumping pressure. Immunological changes, for example, play an important role in lymphedema development [63,189–191]. While infiltration of CD11b+ immune cells did appear to increase in lymphedematous tails compared to naïve tails, chronic treatment with BayK-NP did not alter CD11b+ stain density in tail sections (Fig. 4.10H). This lack of difference is consistent with the observed unchanged tail diameter at day 14, and may suggest that transient swelling or vessel function improvements induced by BayK-NP are insufficient to overcome the pathological immune infiltration associated with lymphedema. Further, in double vessel ligation models, dermal LEC proliferation has been observed to occur within two weeks after ligation surgery [186]. If lymphangiogenesis begins to occur naturally during disease resolution, it may reduce the benefit of improved vessel pumping function at later time points. These complexities, paired with the fact that BayK-NP treatment only improved tail swelling in animals with less severe cases of lymphedema, may suggest a potential benefit of early, or even preventative, BayK-NP treatment, which can be explored in future studies.

Interestingly, there was also no significant change in other vessel functional metrics like frequency, amplitude, packet integral, or packet transport with BayK-NP treatment (Fig. 4.9), even though changes in swelling and collagen deposition were detected. Since swelling is associated with reduced vessel function, particularly amplitude and packet transport (Fig. 4.8F), this is perhaps surprising. In these experiments, repeated, acute improvements in vessel function after BayK-NP administration must thus be sufficient to temporarily improve lymph drainage, reducing tail swelling and improving vessel pumping pressure, although these effects together do not necessarily increase vessel function by
other metrics. This may suggest that pumping pressure changes, which were detected when other metrics were unaffected, may be a powerful metric to evaluate in future studies. Similar results were observed when BayK-NP were applied in a single vessel ligation lymphedema model in obese mice; although there was no significant improvement in contraction amplitude, packet integral, or packet transport, tail swelling was subtly reduced at day 7 and 14 and pumping pressure was subtly improved at day 14 (Fig. 4.12). Obesity is clearly associated with lymphedema onset [192], and is one of the most common lymphedema comorbidities. While the observed effects of BayK-NP treatment in obese animals are not statistically significant, these promising early data are consistent with WT results and suggest that targeting lymphatic vessel function may be a promising approach to lymphedema treatment even in the complex environment of obesity.

This work represents, to our knowledge, the first attempt to alleviate lymphedema symptoms by directly targeting LV pumping function, and reveals the great potential of such an approach. These results also highlight the complexities of lymphedema development and the many opportunities for further development of biomaterial tools to enhance LV pumping function. For example, a single injection of BayK-NP was found to improve pumping function for less than 15 h; additional benefits may be achieved by increasing the timescale of release or improving NP retention for more sustained drug delivery. The bias of BayK-NP efficacy towards animals with below average swelling severity may also suggest that early intervention may further improve efficacy. At later time points (day 14), the subtle improvement in vessel pumping pressure induced by BayK-NP treatment was insufficient to reduce tail swelling. As discussed, this may be attributed to the many facets of lymphedema development besides LV function, including collateral
vessel growth and immune infiltration and modulation. While targeting LV function shows promise for treating lymphedema symptoms, to maximize benefit future approaches could employ combination therapies to simultaneously address lymphatic function, lymphangiogenesis, and immune modulation.

### 4.5 Conclusions

In summary, we advanced a NP platform for the delivery and release of small molecule drug BayK 8644 into LVs at functional concentrations. By employing NIR microscopy to image pumping function of mouse tail collecting lymphatic vessels *in vivo* in real-time, we demonstrated that BayK-NP injection significantly improved LV pumping function by several metrics, with effects lasting ~8 h, while injection of free small molecule BayK could not elicit such effects. The NP formulation also lowered BayK concentration in the blood, and reduced the severity of side effects observed due to off-target BayK delivery compared to the small molecule formulation. When applied to treat a murine model of lymphatic dysfunction in which tail lymphedema is induced by ligation of all tail LVs but nondominant collectors, BayK-NP reduced tail swelling, inhibited collagen deposition, and subtly increased LV pumping pressure in intact LVs compared to NP control. This work highlights the importance of lymphatic drainage in the efficacy of drugs that act on LVs and develops a novel NP platform to achieve this lymphatic drainage. This also represents, to the best of our knowledge, the first evidence that directly promoting pumping function in dysfunctional LVs can improve lymphedema outcomes, highlighting a novel approach to treating this complex disease and other diseases of lymphatic dysfunction.
CHAPTER 5. CONCLUDING REMARKS AND FUTURE DIRECTIONS

5.1 Conclusions

The lymphatic system is a target of significant therapeutic interest as it plays a critical role in a wide variety of diseases. Lymphatic vessels drain fluid from the tissue interstitium, making them critical in fluid balance maintenance, and lymph nodes which house high concentrations of immune cells are involved in the immune response. Delivery of therapeutics to LVs and LNs, however, is complicated by physiological barriers that limit drug uptake into LVs from the interstitium and subsequent penetration into LNs and access to the resident immune cells there. In this work, we thus sought to develop engineered biomaterial platforms to promote small molecule drug delivery to lymphatic tissues, including both LVs and LNs, and employed these platforms to study lymphatic transport modulation and to treat currently incurable disease.

First, we applied a versatile polymeric nanoparticle, SNO-NP, to deliver highly reactive small molecule NO into lymphatic tissues. We harnessed its larger size and unique NO conjugation scheme to enhance lymphatic drainage of a payload that would otherwise be rapidly cleared into systemic circulation upon injection, and to investigate the effect of NO on a variety of size-based lymphatic transport pathways. Lymph-directed NO application was found to promote large molecule penetration into LNs, improving access of species like dextrans and NP drug delivery vehicles to LN-resident immune cells in the LN parenchyma, from which such species are typically restricted. We additionally developed a NP platform capable of encapsulating large amounts of hydrophobic small molecule drug without the need for chemical conjugation or modification of the payload, and applied it to deliver L-type calcium channel agonist BayK into lymph and promote the
pumping function of collecting lymphatic vessels. We demonstrated that only BayK in a NP formulation was capable of achieving sufficient concentrations within lymph to improve LV function; alone, the small molecule drug was cleared into the blood circulation, resulting in significant side effects and no benefit to lymphatic transport. BayK-NP were accordingly applied in a mouse model to treat the most common disease of lymphatic dysfunction, acquired lymphedema, and were found to improve vessel pumping function and reduce swelling compared to control animals. Together, this work provides a new method of modulating drug access to LN-resident immune cells, introduces a novel approach to lymphedema treatment, and highlights the significant usefulness of lymph-directed drug delivery, informing and inspiring future approaches to the treatment of lymphatic-related disease.

5.2 Contributions to the Field

The overall impact of this work was to develop and apply tools to improve small molecule drug delivery to lymphatic tissues, including both lymphatic vessels and lymph nodes. While lymphatic tissues represent promising therapeutic targets because of their significant involvement in diseases ranging from arthritis to cardiovascular disease to lymphedema, the efficacy of small molecule drugs administered to treat lymphatic disease is limited by physiological barriers that reduce drug access to and accumulation within lymphatic tissues, resulting in insufficient drug concentrations in the target tissue. In this work, we overcame these limitations by applying biomaterial tools to 1) deliver NO to LNs, modulating exclusive cellular barriers to promote large molecule penetration and access to LN-resident immune cells, and 2) enable BayK delivery to LVs to improve vessel pumping function for the treatment of lymphedema. This work is impactful in the field of drug
delivery, highlighting a novel approach to accessing LN cells and the first known efforts to treat lymphedema by targeting collecting lymphatic vessel function.

5.2.1 Enhanced LN drug delivery with lymph-directed NO

Chapter 3 of this work detailed the application of SNO-NP to deliver NO to lymphatic tissues and investigate its role in size-based lymphatic transport pathways. This work has cemented SNO-NP as a powerful platform to enable lymph-directed NO delivery through 1) its 30 nm size that promotes lymphatic drainage from the interstitium, 2) a unique core structure that enables chemical conjugation of a high concentration of an otherwise highly reactive small molecule, and 3) gradual NO donation without required environmental stimulus.

We found that application of SNO-NP in the mouse forelimb increased LN penetration depth of co-delivered species, including dextrans and polymeric NP, that are typically restricted to the periphery of the LN. Their association with therapeutically relevant immune cell populations, including lymphocytes and DCs, was subsequently enhanced. Notably, these results were only observed when NO was delivered via SNO-NP; a conventional small molecule NO donor did not affect LN penetration, highlighting the value of SNO-NP for future use in investigations of NO in the context of the lymphatics. There is significant interest in the fields of drug delivery and immunotherapy in improving drug access to LN-resident immune cells due to their great therapeutic significance in contexts like cancer and autoimmunity. Previous efforts in this space have thus focused on improving lymphatic drainage and LN accumulation or on multistage drug release platforms that allow small molecule drugs to leave their large, periphery-restricted vehicles.
and more readily penetrate into the LN [29]. The results described herein present a novel approach to improving drug access to immune cells by directly modulating LN barriers and promoting nanoscale species penetration into deep LN regions. This technique could be applied to directly improve penetration and immune cell access of larger therapeutics, like proteins and antibodies, or to improve penetration of large drug delivery vehicles like nanoparticles, antibody-drug conjugates, or other platforms to improve their efficacy without the need for sophisticated release schemes.

5.2.2 *BayK-NP enable in vivo, lymphatic application of BayK*

Chapter 4 of this work described the development and application of a NP platform for delivery of hydrophobic small molecule BayK to its LMC target to promote LV pumping function for the treatment of lymphedema. The BayK-NP platform employed is well-suited to the application, as it 1) improves lymphatic uptake of small molecule payload after i.d. injection compared to the free small molecule, 2) can encapsulate BayK in its hydrophobic core in gentle conditions with tailorable efficiency and loading capacity, and 3) releases BayK over the course of several days upon dilution, providing extended release without endocytosis, pH, or other environmental requirements.

Although small molecule BayK is known to improve LV pumping function *ex vivo*, *in vivo* it is cleared rapidly into the systemic circulation upon i.d. injection, resulting in poor lymphatic access and low drug concentrations within LVs and surrounding LMCs. This makes free BayK ineffective at improving LV pumping function *in vivo*, and has limited previous investigations into its value as a therapeutic. The BayK-NP platform was herein shown to overcome these limitations and enhance lymphatic drainage of its BayK
payload, enabling sufficient drug concentration at the target site within LVs to significantly improve their pumping function. This represents the first demonstration, to our knowledge, of vessel function enhancing drug delivery to LVs via cutaneous administration. This novel NP platform not only enables the study and application of BayK within the context of the lymphatics, but lays the groundwork for future in vivo study of additional therapeutics that could not previously be investigated, and thus represents a significant boon to the field.

In its free form, small molecule BayK induces significant side effects upon i.d. administration, including behavioral and neurological challenges, due to its rapid access to systemic circulation and off-target L-type calcium channels. In addition to improving lymph drainage and enabling LV function enhancement, the BayK-NP formulation was also shown to reduce acute BayK clearance into the blood and subsequent occurrence and severity of side effects. This enabled chronic treatment with an otherwise toxic drug, and highlights the importance of selectively increasing drug concentration within the target tissue. The PPS-NP platform may be extended to other toxic drugs, enabling their application within the lymphatics while reducing systemic exposure and toxicity.

5.2.3 A novel approach to the treatment of diseases of lymphatic dysfunction

The BayK-NP platform described in Chapter 4 was applied by daily i.d. injection in mice with acquired tail lymphedema, and this treatment was found to improve the pumping function of intact lymphatic vessels and to reduce tail swelling severity compared to control animals. Currently, lymphedema is managed in the clinic by massage and compression; no treatment exists that targets the complex root causes of lymphedema, and patients can often expect lifelong symptoms and chronic disease management. While there
is some ongoing research into potential therapeutic approaches, the current focus is on modulating the inflammatory manifestation of lymphedema [81,82] or on controlling lymphatic vessel regrowth to bridge regions of damage to the vasculature [79]. Although LV dysfunction is known to be associated with lymphedema progression, targeting LVs for lymphedema treatment is a currently unexplored avenue. The work described herein represents the first known efforts to deliver a therapeutic to directly improve LV pumping function \textit{in vivo}, and the first demonstration that such an approach can improve lymphedema outcomes. Direct targeting of LV function represents a paradigm shift in the field of lymphedema therapy, and lays the groundwork for similar investigations with other drugs or for combination with therapeutics that modulate lymphangiogenesis or immune activity to maximize treatment efficacy.

5.3 Future Directions

5.3.1 \textit{NO} application to enhance delivery of therapeutics to LNs

Future work with SNO-NP should further investigate its applications within the lymphatics, and particularly its ability to improve LN penetration of large molecules and drug delivery vehicles. In this work, we demonstrated that lymph-directed \textit{NO} application via SNO-NP improved LN penetration without observed effects on resident immune cell activation or antigen presentation. \textit{NO} could thus be investigated in the context of vaccine delivery, where SNO-NP could be applied to promote cellular access of large antigen, adjuvant, or either species delivered via NP to improve their lymph uptake; similar approaches could be taken to improve delivery of immunotherapeutic agents that require immune cell access for efficacy. In addition to the ability of SNO-NP to improve
therapeutic access to resident immune cells, the effect of improved LN penetration on drug access to and efficacy against tumor cells within the LN could also be investigated. NP-conjugated chemotherapeutic irinotecan, for example, has shown improved efficacy when its LN penetration and subsequent access to tumor cells within the LN was improved [29]. As many other chemotherapeutics are also small molecules that benefit from delivery vehicles to improve lymph drainage and LN access [12,127,128], there is great potential for co-delivery of NO to promote LN penetration of such NP and their chemotherapeutic payload not only to resident immune cells, but also to tumor cells in the LN in the case of lymphomas or metastatic disease.

5.3.2 Probing the mechanisms of NO’s effects on LEC barrier function

In this work, small molecule NO was found to promote LN penetration of species that are typically restricted to the LN periphery, like large dextrans and NP drug delivery vehicles. While preliminary in vitro testing revealed that SNO-NP application could increase the permeability of a LEC barrier to a model 30 nm dextran, the mechanism for that enhanced permeability has yet to be fully elucidated. Future work could apply both in vitro transwell studies and in vivo lymphatic transport models to investigate the effect of SNO-NP on individual LEC transport pathways, including vesicular transcytosis and paracellular transport, by evaluating fluorescent molecule transport across barriers during simultaneous treatment with transport pathway modulators like dynamin inhibitors or adrenomedullin, or by evaluating LEC junctional changes after SNO-NP treatment. These investigations would improve our understanding of LEC response to NO, a significant boon to the field as LECs have received little attention in this context compared to extensively investigated blood endothelial cells. This work would also provide insight into the
mechanisms governing LEC regulation of molecule penetration into LNs, and would have significant implications in the field of LN physiology and drug delivery.

5.3.3 BayK-NP applications in basic science and disease treatment

Enabling small molecule access to and efficacy within LVs by employing a lymph-draining NP vehicle is a novel approach to lymphatic drug delivery that has many potential applications in basic science to improve our understanding of lymphatic function. While a calcium channel agonist was investigated in this thesis work due to its significant ex vivo efficacy and hydrophobicity, there are many other drugs that have only previously been investigated for lymphatic efficacy ex vivo and could benefit from similar formulation. Pinacidil, for example, is a hydrophobic potassium channel activator that improves LV contractility ex vivo, but which is also limited in its in vivo uptake into LVs due to its small size [86], and while a thromboxane analog U-46619 has been shown to enhance LV contractile activity in isolated vessels [193], it also has the potential for off-target effects in the blood vasculature if delivered systemically. The PPS-NP platform here employed could enable lymph drainage of such drugs and, when combined with in vivo imaging technologies, allow investigation of drug effects within the complicated in vivo environment to expand our understanding of LV function. This platform could also be extended to deliver hydrophilic agents by conjugation to the NP corona, dramatically expanding the variety of drugs available for investigation.

Future work in this field could not only employ PPS-NP as a tool for basic science investigations, but also further develop the platform’s potential as a therapeutic for diseases of lymphatic dysfunction. First, BayK-NP could be further optimized to promote efficacy;
dosing, treatment schemes, and drug loading per NP could be easily tuned, and studied to identify schemes that maximize the LV response and minimize off-target side effects. This NP platform could also benefit from more sustained drug release and retention within LVs to improve tissue exposure to drug. In Chapter 4, a single injection of BayK-NP was found to improve LV function for less than 15 h, in spite of the fact that the drug was released from NP over the course of days. This may be impacted by the fact that upon drainage into lymph, NP, or other soluble factors, spend only a brief period of seconds to minutes within the LV lumen, limiting the amount of drug that can be released at the target site within lymph and resulting in significant amounts of wasted therapeutic. Preliminary results suggest that conjugation of LEC-targeting moieties to the corona of our versatile PPS-NP platform can promote their adhesion to LV-lining LECs \textit{in vitro} and \textit{in vivo}, increasing the length of time that NP are retained within LVs. Future work could expand upon this idea, identifying targeting moieties and investigating their effect on the efficacy of delivered therapeutics.

Diseases of lymphatic dysfunction are typically multifaceted and complex, with a variety of pathological manifestations and driving factors. Lymphedema, for example, is associated with a loss of LV pumping function, but also with pathological immune infiltration, tissue remodeling, and altered LEC growth and lymphangiogenesis. Future work could thus employ LV function-enhancing NP as a combination therapeutic, investigating the benefit they can provide when delivered alongside other emerging treatments like lymphangiogenesis enhancers or immunosuppressants. The versatility of the PPS-NP platform would allow its application to modify multiple pathways simultaneously, either by encapsulation of multiple drugs or by delivery of drugs with
multiple benefits. This NP platform could also be applied to simultaneously treat a combination of locations including the cutaneous injection site, where drug could promote lymphangiogenesis or regulate local immune cell activation; LVs, where pumping function could be enhanced; and even downstream LNs, where the activation of LN-resident immune cells could be modulated. Lymph-directed drug delivery shows great promise for improving lymphatic disease outcomes, and provides a plethora of opportunities for continued investigation.
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