Elucidating the Role of Lymphatics in the Pathogenesis of Chronic Inflammatory-Erosive Arthritis

By

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Submitted in Partial Fulfillment of the Requirements of the Degree Doctor of Philosophy

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2014
Biographical Sketch

Yawen Ju graduated from China Pharmaceutical University in 2006 with a B.S. in Pharmacy. In 2008, she enrolled in University of Rochester School of Medicine and Dentistry, and later joined Dr. Edward Schwarz’s lab to pursue her PhD degree in the Department of Pathology and Laboratory Medicine. Her research on rheumatoid arthritis diagnosis and therapy has generated 5 publications and 3 national conference presentations.

Articles


* Equal contribution

**Presentation**

• **American College of Rheumatology, Nov.2012**, Washington DC. Poster "Selective iNOS inhibition increases the lymphatic pulse and drainage from arthritic joints in TNF-Tg mice"

• **American College of Rheumatology, Nov.2011**, Chicago. Poster "Development of Contrast Enhanced Ultrasound Imaging and Quantification of Lymphatics in Draining Lymph Nodes of WT and TNF-Tg Mice with Inflammatory Arthritis"

• **American College of Rheumatology, Nov. 2010**, Seattle. Poster "Modeling Osteoclast Precursor Master Fusogens and Mononuclear OCP Donors with Raw Cell Line Clones with Raw Cell Line Clones"
Acknowledgements

First, I want to thank my advisor, Edward Schwarz, who has given me great support during my PhD studies. Prof. Schwarz is a fantastic adviser who not only led me in research directions, but also helped me identify significant problems for investigative research. I learned much from him including research topic selection, experimental techniques, paper writing and scientific presentation. I enjoyed working with him very much over the past 5 years. He gave me close supervision, but also gave me plenty of room to work in my own way. He is the best advisor I have ever had.

My thanks also go to committee members, Dr. Lianping Xing, Dr. Keigi Fujiwara, and Dr. Lin Gan, whose provocative questions helped me keep moving forward towards my research goals. I thank Dr. Christopher Ritchlin for his in-depth opinions on rheumatoid arthritis and clinical approaches. I also want to thank Dr. Ronald Wood, Dr. Chao Xie, Dr. Jie Li, Dr. Igor Kuzin, Echoe Bouta, Dr. Grace Chiu, and Dr. Homaira Rahimi for critical feedback and suggestions on my work. I feel fortunate that I had the opportunity to be a member of the Schwarz group.

I also want to thank my parents, Zhonghua Ju and Hui Tian, and my husband Ding Liu. They have given me tremendous support without which this journey would have been much harder.
ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease in which patients often suffer from arthritic flare. Using longitudinal contrast-enhanced (CE)-MRI to study knee arthritis in tumor necrosis factor-transgenic (TNF-Tg) mice, we observed that the popliteal lymph nodes (PLN) firstly “expand” in size and contrast enhancement, and then suddenly “collapse” during arthritic flare. Since CE-MRI is too costly for phenotyping and longitudinal analyses of PLN, our aim was to develop ultrasound (US) methods that could replace MRI. In our initial study, we demonstrated a significant correlation between PLN volumes determined by US vs. MRI. However, since PLN collapse is more closely associated with lymphatic draining function than volume, we evaluated CE-US methods to distinguish changes in lymphatic transport, which was shown as a biomarker of arthritic flare. Unfortunately, delivery of the contrast agent prior to US significantly impairs lymphatic function, making it unsuitable for phenotyping PLNs. Thus, we went on to develop power Doppler (PD) US methods to phenotype PLN with greater accuracy and cost effectiveness vs. CE-MRI.

Another important prior observation we made is that arthritic flare is associated with the loss of lymphatic pulse. From other models of inflammation, lymphatic pulse is known to be controlled by endothelial nitric oxide synthase (eNOS), and inhibited by inducible NOS (iNOS) expressed in Gr-1+ cells. To test the hypothesis that eNOS/iNOS dysregulation is responsible for the loss of lymphatic pulse during arthritic flare in TNF-Tg mice, we performed IHC and in vivo pharmacological intervention studies with selective and non-selective iNOS inhibitors. The IHC results demonstrated that large
numbers of iNOS expressing Gr-1+ cells exist in collapsed PLN. By evaluating the lymphatics with NIR-ICG imaging, we observed that the specific iNOS inhibitor L-NIL increased lymphatic pulse and afferent lymphatic drainage in TNF-Tg mice. Additionally, the micro-CT results showed that bone erosions were ameliorated in L-NIL treated TNF-Tg mice compared with placebo. Collectively, these results suggest a model that the accumulation of iNOS-expressing Gr-1+ cells accelerates the onset of flare in the setting of inflammatory arthritis via inhibition of lymphatic drainage, and identifies this pathway as a potential target for RA therapy.
Contributors and Funding Source

This work is supervised by a dissertation committee consisting of Dr. Edward Schwarz (advisor), Dr. Lianping Xing, Dr. Keigi Fujiwara, and Dr. Lin Gan. All of the experiments and analyses in this thesis were performed by the author with the following exceptions: the CE-MRI scans were performed by Pat Weber in the RCBI Core facility, and the analysis was done by author. The micro-CT scans and analyses were both performed by Michael Thullen and the author. The electron microscopy was performed by Karen Bentley. The real time intravital immunofluorescent lymphatic imaging in Figure 4.3A and Figure 4.3B were obtained by my lab mate Dr. Jie Li.

This work was supported by research grants from the National Institutes of Health PHS awards (R01s AR048697, AR053586 and AR056702; P01 AI078907; and P30 AR061307).
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<td>B-in cells</td>
<td>B cells in inflamed nodes</td>
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<td>B-mode</td>
<td>brightness-mode</td>
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<tr>
<td>CCP</td>
<td>cyclic citrullinated peptide</td>
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<td>CD11b</td>
<td>cluster of differentiation molecule 11b</td>
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<td>CE-MRI</td>
<td>contrast enhanced-magnetic resonance imaging</td>
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<tr>
<td>CIA</td>
<td>collagen-induced arthritis</td>
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<tr>
<td>DMARD</td>
<td>disease-modifying antirheumatic drugs</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>FPsi</td>
<td>mean signal intensity of the fat pad</td>
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<td>GC</td>
<td>guanylate cyclase</td>
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<tr>
<td>Gd-DTPA</td>
<td>gadolinium-diethylenetriaminepentaacetic acid</td>
</tr>
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<td>GPI</td>
<td>glucose-6-phosphate isomerase</td>
</tr>
<tr>
<td>ICG</td>
<td>indocyanine green</td>
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<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
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<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
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<td>LEC</td>
<td>lymphatic endothelial cells</td>
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<td>LN</td>
<td>lymph node</td>
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<tr>
<td>L-NAME</td>
<td>L-NG-nitroarginine methyl ester</td>
</tr>
<tr>
<td>LNcap</td>
<td>lymph node capacity</td>
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<td>LNCE</td>
<td>lymph node contrast enhancement</td>
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<td>L-NIL</td>
<td>N6-(1-iminoethyl)-L-lysine, dihydrochloride</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>LNvol</td>
<td>lymph node volume</td>
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<tr>
<td>LS</td>
<td>lymphatic sinuses</td>
</tr>
<tr>
<td>LSsi</td>
<td>mean signal intensity of the lymph node sinus</td>
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<tr>
<td>LYVE-1</td>
<td>lymphatic vessel endothelial receptor 1</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<td>NIR</td>
<td>near infrared</td>
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<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
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<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
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<tr>
<td>OA</td>
<td>osteoarthritis</td>
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<td>Pax7</td>
<td>paired box protein 7</td>
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<td>PD</td>
<td>power Doppler</td>
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<td>PLN</td>
<td>popliteal lymph node</td>
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<td>RA</td>
<td>rheumatoid arthritis</td>
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<td>RF</td>
<td>rheumatoid factor</td>
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<td>ROIs</td>
<td>region of interests</td>
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<td>RT</td>
<td>room temperature</td>
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<td>SMA</td>
<td>smooth muscle actin</td>
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<td>standardized sinus volume</td>
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<tr>
<td>SV</td>
<td>sinus volume</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>TNF-Tg</td>
<td>tumor necrosis factor-transgenic</td>
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<td>US</td>
<td>ultrasound</td>
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<td>WT</td>
<td>wild type</td>
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Chapter I

Introduction
1.1 Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a chronic, systemic inflammatory disorder affecting many tissues and organs but it primarily attacks synovial joints. Inflammation can be found in the joint lining synovium, which is known as synovitis. The inflamed synovium, called pannus, invades and destroys bone and cartilage resulting in irreversible joint damage (2). RA has a worldwide prevalence of 1 to 2%, mostly women, and approximately 1.5 million Americans have RA (3). Although RA-like disease can occur in children, referred to a juvenile RA, it usually affects people between 25 and 50 years of age. The significant mortality/morbidity caused by RA includes a shorten life span by 3 to 15 years, serious disability, pain, fatigue, weight loss, fever and other complications. The direct annual health care costs of RA disease in patients has been estimated to be $8.4 billion per year, with an additional $10.9 billion per year in associated costs (4).

Although the etiology of RA remains unknown, several risk factors have gained broad acceptance. The first evidence that suggested genetic disorder in RA was presented by Stastny et al (5), who revealed that the over-expression of human leukocyte antigen-DR gene of the major histocompatibility complex (MHC) was highly associated with the disease. The over-expression of specific RA-associated genes is thought to lead to an autoimmune response by presentation of low-affinity autopeptides that are not efficient in generating a T cell response under normal conditions (6). Another risk factor is rheumatoid factor (RF), which is an autoantibody against the Fc portion of IgG. RF is present in 75% of RA patients. This leads to autoimmunity and results in the formation of very large immune complexes (7). In addition, the autoantigen, glucose-6-phosphate isomerase (GPI) and cyclic citrullinated peptide (CCP) and their antibodies, anti-GPI and
anti-CCP, are in high concentrations in the synovial fluid of RA patients (8-9). Passive
transfer of anti-GPI induces arthritis in healthy animals (8), leading to a link between this
autoantibody and the pathology of RA. To inhibit bone erosion, relieve pain and improve
the life quality of RA patients, treatments include non-biologic and/or biologic disease-
modifying anti-rheumatic drugs (DMARD), most of which are immunosuppressive (10-
11). Poor prognosis factors, which include positive serum RF and anti-CCP
autoantibodies, family history of RA, and poor functional status are important indicators
to treat RA patients. Monotherapy or combination of non-biologic DMARDs, such as
methotrexate, leflunomide, sulfasalazine, hydroxychloroquine and minocycline are
commonly used in patients of the poor prognosis with moderate disease activity, or those
with high disease activity but without features of poor prognosis (11-12). In patients with
high disease activity and poor prognostic factors, the biologic DMARDs, such as anti-
tumor necrosis factor (TNF) therapy, are started (11-12). When TNF-α antagonists fail or
complications occur, a different biologic DMARDs may be used, including treatments
that inhibit B-cells, the IL-6 receptor, and T-cell costimulation (11).

Despite major advances in RA therapies, they have flaws that cannot be ignored.
For example, many patients are non-responsive or have inadequate responses: in anti-
TNF therapy, 42% and 33% of patients can achieve a good response and a moderate
response, respectively, while 25% of patients have no response to anti-TNF therapy (13).
In B-cell depletion therapy, only 12% of patients responded, but 51% did not present
complete amelioration (14). There are also significant concerns about immunosuppression caused by these drugs and the contraindication in patients with
known infections (15-16).
Thus, it is urgent to elucidate the mechanism of arthritic flare and identify novel drug targets for therapy. With an increased understanding of the etiology of RA, including the role of the lymphatic system, we can open doors to non-immunosuppressive therapy for this terrible disease.

1.2 Lymphatics in RA

Lymphocytes and Cytokines

Inflammatory-erosive arthritis, such as RA, is triggered by the activation of the immune response. B cells are considered as an important player in the pathogenesis of RA, because B cells in RA are thought to secrete RF and anti-CCP autoantibodies, both of which are considered as serological markers for the diagnosis of RA (7, 17-20). However, there is evidence showing that B cells contribute to pathogenesis of RA in mechanisms other than antibody production. First, B cells have been shown to regulate the structure of lymphoid tissues and lymphangiogenesis (21). Secondly, the translocation of B cells from follicles to sinuses in popliteal lymph nodes (PLNs) blocks lymph transportation, leaving the ankle and knee swollen in TNF-Tg mice (22). This finding was caused by a subpopulation of B cells in the PLNs, B cells in inflamed nodes (B-in) cells (CD23+/CD21hi), that migrated from B follicles to sinuses as arthritic flare occurred. Lymphatic drainage was decreased with the translocation of B-in cells, onset of synovitis, and bone erosion. Thirdly, B-cell depletion therapy via the anti-CD20 antibody, Rituximab, is used in RA patients who do not respond to the conventional therapies (14, 23-25). Interestingly, even though the anti-CD20 antibody shows significant clinical
benefits, the patients do not always have reduction in serum autoantibody level including IgM, IgG, IgA and RF. Therefore, it is thought that the efficacy of B cell depletion therapy is dependent on removing the B-in cells that clog lymph node (LN) sinuses. However, resting B cells, as antigen-presenting cells, induce inflammation by presenting arthritis-associated antigens to T cells (26), and secrete various cytokines, including IL-10, TGFβ-1, IL-2, IL-4, TNFα, IFNγ, or IL-12 (27). So there may be multiple mechanisms by which B cell depletion therapy is beneficial.

High concentrations of cytokines and chemokines in the lymph of RA patients were found by cannulating lymphatics. RA patients showed several folds elevation of TNF, IL-1, IL-6, IL-15, GM-CSF, IL-8, and MIP-1 in lymph compared to lymph fluid from healthy controls (28). Among these cytokines, TNF and IL-16 are generally believed to be the master cytokines in the pathogenesis of chronic inflammatory arthritis. TNF plays an important role as it is the inducer of other pro-inflammatory cytokines, such as IL-1, IL-6, IL-8 and GM-CSF (2). The role of TNF on inducing RA was demonstrated by a RA animal model, the TNF-Tg mouse, which was created by over-expression human TNF-α (29). Moreover, anti-TNF monoclonal antibody therapy is widely used on RA patients who are refractory to other DMARDs (11-12). TNF dependent or independent induction of IL-1 has found in many chronic inflammatory models (30). Arthritis can be induced within 24 hours by intra-articular administration of recombinant IL-1 at doses as low as 1ng (31). The anti-IL-1 or IL-1 receptor antagonist has shown the potent inhibition of cartilage and bone damage in RA patients (32-33).

Lymph Node
The presence of altered LN size and function in RA has been documented in clinical studies (34-36) via various imaging modalities (37-39) and the analysis of afferent lymphatic flow (28). Overall frequency of enlarged LNs in patients with active RA has been shown to be 82%, while no enlargement of LNs were seen in osteoarthritic control patients. Furthermore, RA patient’s LN size decreased following anti-inflammatory therapy (40). Since the regional LNs are the primary targets for preventing the spread of local inflammation, the changes in the size, number and fatty composition of the LNs correlate with the severity of synovitis in the joints (41). In another study, the investigators found enlarged PLNs by magnetic resonance imaging (MRI) in 100% (21 of 21) of RA cases versus 44% (32 of 73) in osteoarthritis (OA) cases, and the LNs were larger and displayed less fatty changes than the PLNs in the OA knees (42). Another obvious change in the PLNs in TNF-Tg mice is lymphangogenesis, leading to the expanded sinuses (49). Similar expansion of lymphatic vessels in the PLNs of RA patients was observed and the area of lymphatic vessel endothelial receptor 1 (LYVE-1)+ signal was much larger than OA patients (unpublished data). These observations indicate that PLNs play a pivotal role in the pathogenesis of chronic inflammatory arthritis.

**Lymphatic vessels and lymph drainage**

A common symptom of RA of the ankle is a swollen joint filled with synovial fluid that contains excess pre-inflammatory cytokines (28) that should be removed via lymphatic vessels. The lymphatic circulation begins with blind ended (closed at one end) highly permeable superficial lymphatic capillaries formed by endothelial cells with button-like junctions (43). Then the lymph, the fluid within the lymphatic system, is
drained to larger contractile lymphatic vessels called collecting lymphatics, which have endothelial cells, intraluminal valves, and smooth muscle cells. The collecting lymphatics accumulate lymph fluid and become larger as they enter LNs. Collecting lymphatics leading into the LNs are considered afferent lymphatics while vessels leaving the LNs are referred to as efferent lymphatics. The smooth muscle layer of collecting lymphatics may be occasionally interrupted where unidirectional valves divide the vessel into multiple little chambers. The smooth muscle exhibits spontaneous and phasic contractions that enable each chamber to act as a “primitive heart” to pump lymph (44-45). Due to the contraction of smooth muscle, the afferent lymphatic vessels will transfer lymph in a pulsatile manner from the ankle to the draining LNs. Besides lymph fluid, dendritic cells, macrophages, and lymphocytes are also transported by lymphatic vessels from inflamed sites to LNs (46). The pulse of lymphatic vessel in chronic arthritis mouse model (1 pulse/min) is dramatically smaller than the vascular pulse from the heart (550-600 pulses/min), and is similar to the lymphatic pulse of wild type (WT) mice but lower than the lymphatic pulse of acute arthritis mouse model (5 pulses/min) (47). Even if the draining function of afferent lymphatic vessels seems to not change in chronic arthritis compared to WT mice, increased lymph is still detected in the draining LNs of distal joints of TNF-Tg mice (37, 48-49). This parallels clinical data as accelerated lymphatic flow was also found in 20 RA patients by collecting lymph from the cannulated lymphatics. RA patients showed almost double lymphatic flow rates compared to healthy controls (28). These consistent findings raise our interests to study the role of LNs and lymphatics in RA pathogenesis.
1.3 The TNF Transgenic Mouse (TNF-Tg) Model of Chronic Inflammatory-Erosive Arthritis

TNF-α is a member of TNF ligand superfamily, which raised attention in the last three decades because it is a pre-inflammatory cytokine that is associated in numerous inflammatory diseases, including RA, psoriasis, psoriatic arthritis, ankylosing spondylitis and inflammatory bowel disease (50-51). During inflammatory reactions, TNF is primarily produced by myeloid lineage cells, such as macrophages and monocytes, but it is also widely made by other cells, such as B cells, T cells, NK cells, Kupffer cells, glial cells and adipocytes (52).

Various animal models of arthritis have been developed, including collagen-induced arthritis (CIA) (53), adjuvant arthritis (54), streptococcal cell wall arthritis (55) and serum-induced arthritis (56). However, each of these models has a pathobiology of arthritis that differs from the synovitis and joint destruction observed in RA patients, which limits their applications. Moreover, they represent acute synovitis, which does not facilitate the evaluation of long-term anti-inflammatory therapies. Twenty years ago, George Kollias' laboratory generated the TNF-Tg mouse model of arthritis with a human TNF transgene in which the AU rich 3' UT region, which shortened mRNA half-life, was replaced by the stable β-globin 3' UT, resulting in over-expression of TNF (29). TNF-Tg mice develop erosive polyarthritis with swelling of the ankle joints occurring at 6 to 8 weeks of age and impairing movement. Beginning at 6 weeks of age and continuing throughout their life (<1year), hyperplasia of the synovial membrane as well as cellular inflammatory infiltrates of the synovial space can be seen in nearly all joints examined. Pannus formation, articular cartilage destruction and massive destruction of bone and
fibrous tissue are observed in the advanced stages of disease (29). Anti-TNF therapy efficiently prevents development of this disease in these animals (29). For our purposes, the TNF-Tg mouse is an ideal model because the etiological mechanism is evident and the chronic course of the synovitis and joint damage mirror RA closely.

We have recently demonstrated that PLNs are the most sensitive biomarker for knee joint arthritic flare in the TNF-Tg mouse model of RA (49). Longitudinal contrast enhanced-magnetic resonance imaging (CE-MRI) of PLNs and knees demonstrated that prior to knee flare PLNs display an “expanding” phenotype as evidenced by strikingly large CE and paracortical sinuses that are mostly void of cells, which appear to protect the adjacent knee from arthritis via efficient lymphatic drainage. Suddenly, arthritic flare, featured by bone erosion and joint swelling, appears to follow PLNs “collapse,” which is characterized by decreased volume and CE. The non-CE regions correspond to “clogged” sinuses that are completely filled with B-in cells (49), and closely resemble LNs shutdown during an immune response (49).

1.4 Medical Imaging and RA

MRI and RA

MRI is a medical imaging technique broadly used in both research and clinic settings to image the detail internal structure of body. MRI generates images via aligning the hydrogen nuclei (protons) of tissues with the strong magnetic field. Radio frequency magnetic fields are applied to alter the alignment of this magnetization and cause a rotating magnetic field, which causes the protons to induce an alternating
electrical current detectable by the receiver wires in an imaging coil placed near the patient or animal (57). Contrast agent can be injected intravenously before the scan to visualize the blood vessels. The commonly used agents for contrast enhancement are gadolinium-based compounds, which show brighter areas of contrast enhancement in image (58). MRI has been now used commonly in RA studies and offers excellent images of many tissues, such as the synovium, bone, and LN (Figure 1.1A) (59). Because of the high resolution of the MRI images, bone erosion in arthritis has been evaluated via MRI, which shows a greater sensitivity than radiography (60). By quantifying the amount of synovial fluid in the joint, CE-MRI can be used to identify patients with early RA (61). Due to the comprehensive evaluation of MRI on the changes of RA patients, it provides a reliable and efficient tool to diagnosis and study RA.

However, considering its high cost and limited access, MRI is not very popular in the study of the RA animal models. Before our group developed the CE-MRI to evaluate the PLNs and synovitis in TNF-Tg mice, the only study of MRI on an RA murine model was from the Hirsch group (62). Their study showed a high resolution MRI, including knees, synovium and LNs. They tried to diminish the signal in region of interests (ROIs) by using the contrast ultrasmall superparamagnetic iron oxide contrast agent rather than gadolinium, which made it difficult to tell the ROIs from the background tissue. Therefore, quantifying ROIs on the 3D stacks of MRI images had its challenges and limitations.

**Ultrasound**

Compared to other musculoskeletal imaging modalities (i.e. MRI, x-ray),
ultrasound (US) has several remarkable advantages, including real-time imaging, easy accessibility, cost-efficiency, and the lack of radiation (63). The use of US in RA patients provides high-resolution images of joints and surrounding tissues, and can be performed and interpreted by a rheumatologist in real-time, thus allowing it to become an increasingly common method for imaging in most rheumatology departments (Figure 1.1B) (64-65). To assess synovitis and degree of joint damage in RA, both gray scale US and power Doppler (PD) US have proven to be useful (66-71). The scoring system for gray scale US, which ranks the synovitis as normal, minor, moderate and severe synovitis, is generally accepted for semi-quantifiable purposes (68). Similarly, gray scale or PD-US can also be used to evaluate tenosynovitis (72) and bone erosion (69, 73). Two sets of semi-quantifiable scoring systems have been established for PD-US, which visualizes blood flow, using either the area of the PD signal (69) or the maximal degree of PD activity (67). CE-US has also been employed in RA diagnosis, which has greater sensitivity versus PD-US at detecting vascularity, synovitis and tenosynovitis (74-75).

Consistent with our MRI findings, PLN changes in patients and mouse models of RA have been observed via US. It was previously found that there was a significant relationship between the LN volume (LNvol) between MRI and US, showing agreement between the two modalities (76). Furthermore, the PD signal in the draining LN in RA patients relates to disease activity, and US is able to detect differences in cortical hypertrophy in these draining LNs (77).

With the clinical success of US in RA patients, US has been used in some RA animal models (78-79). A scoring system based on gray scale and PD-US on knees and ankles in the CIA mouse model has been established with a strong correlation between
histology and US score (78). Another group used US to evaluate the synovitis in the antigen-induced arthritis rabbit model to measure the capsule thickness and employed CE-US to measure the synovium thickness, which was found to significantly and positively correlate with the histology synovitis score (80). Both studies demonstrate the feasibility of US as an outcome measure of arthritis in small animals. Thus, in order to expand the utility of US to assess inflammatory arthritis in mice, we performed studies to establish an US measurement that can faithfully phenotype expanding versus collapsed PLNs in TNF-Tg mice as a biomarker of arthritic flare.

1.5 Nitric Oxide Synthases in Inflammatory Conditions

Nitric Oxide and Nitric Oxide Synthase

In biological systems, nitric oxide (NO) is an important intracellular messenger, which modulates smooth muscle tone (81-83), insulin secretion (84), angiogenesis (85-86) and neural activity (87-88). The production of NO is catalyzed by nitric oxide synthase (NOS) from L-arginine (89). Currently, three types of NOS have been reported to catalyze this reaction: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) (90). The mechanism of how the NOS is activated is not fully understood. However, several studies suggest that calmodulin, a small calcium binding protein, is required to activate the enzymes (91-92). eNOS and nNOS are classified as constitutive NOS. Their ability to produce NO is highly controlled by intracellular Ca2+ concentration, because CaM binds to eNOS and nNOS only when the concentration of Ca2+ is elevated (93-94). Since the binding between iNOS and CaM is so tight, iNOS
could not be efficiently activated in the absence of Ca2+ (94-95). Instead, the expression of iNOS is induced by the inflammatory cytokines and microbial products, such as TNF, INFα, INFβ, INFγ and lipopolysaccharide, which would produce a significantly large amount of NO (94-101).

**eNOS and lymphatic vessel contraction**

As the major subtype of NOS in the endothelium cells, eNOS plays a vital role in the regulation of the contraction in blood and lymphatic vessels (102-104). In a few seconds after shear stress increasement, eNOS protein activity increased due to the changes of cytosolic calcium and eNOS protein phosphorylation(105). Later on, mRNA stabability and protein expression of eNOS are evoked by fluid shear stress (105-107). Shear stress to endothelial cells is increased when the flow in vessel is high, but decreases when the vessel diameter increased (108). Meanwhile, pressure and cyclic stretch from the fluid flow would also increase the activity of eNOS and the eNOS mRNA level (109-110). Stress-stimulated endothelial cells relax the smooth muscle cells by turning on eNOS expression to produce the relaxation factor NO (111-112). When lymphatics were treated with the non-specific NOS inhibitor L-NG-nitroarginine methyl ester (L-NAME) in vivo, the lymphatic pulse increased, while the end diastolic diameter and the end systolic diameter were decreased (104). Similarly, the lymphatic pulse was also accelerated in eNOS/-/- mice compared to WT mice, while no difference on lymphatic pulse was observed in the iNOS/-/- mice (102). Therefore, it is likely that the rhythmic lymphatic pulse is controlled by eNOS rather than iNOS. eNOS expression was dynamically manipulated by the changes of shear stress, which led to a cyclic contraction.
in the lymphatic vessels (113).

**iNOS and lymphatic vessel**

iNOS is an important source for NO, especially in the inflammatory conditions. iNOS expression has been shown to be induced by pro-inflammation cytokines, such as TNF-α, IF-γ and IL-β (114-115). Studies demonstrated that iNOS induced under inflammation impair lymphatic pumping in multiple acute inflammation animal models (102, 116-117). Liao et al developed an intravital image method to observe the lymphatic contraction in mice (102). She observed that, under acute inflammation, the contraction frequency of iNOS-/- mice decreased to 30 pulses/min compared to 40 pulses/min in WT and eNOS-/- mice, and the iNOS was expressed by CD11b (cluster of differentiation molecule 11b)+/Gr-1+ cells. Also, an iNOS inhibitor was shown to recover the lymphatic pumping in the inflammation animal model stimulated by IL-β1 (116). Similarly, an iNOS inhibitor and its downstream guanylate cyclase (GC) inhibitor dramatically restored the lymphatic contractile in 2,4,6-trinitrobenzenesulfonic acid-treated animals (117). Therefore, several groups have reported the iNOS inhibition effect on lymphatic contraction in acute inflammation models. However, a chronic inflammatory model, such as TNF-Tg mice, has never been studied. Previously, our group found that lymphatic pulse significantly decreased in TNF-Tg mice with collapsed PLNs compared to expanding or WT PLNs. CD11b+ Gr-1+ cells were also found to accumulate in the afferent lymphatics to collapsed PLNs (118). These results led me to investigate the iNOS dysregulation of lymphatic contractile in TNF-Tg mice.
iNOS and inflammatory arthritis

Several studies have demonstrated that the induction of iNOS pathway contributes to the pathogenesis of inflammatory arthritis. For instance, increased circulating NO levels were found in the serum, urine, and synovium fluid of RA patients (119-120). Also, much higher expression of iNOS was found in the synovial tissues of RA patients, compared to less expression in the OA joints and the absence in normal joints (121-124). As a critical regulator of inflammation, NO plays an important role in the chronic inflammatory arthritis in several aspects (125): 1) significantly elevated NO derived from iNOS pathway mediates the apoptosis in the RA joints (126); 2) over-expression of NO accelerates the angiogenesis and the formation of pannus in RA joints (127-128); 3) a major complaint of RA patients is edema of the joint, which was partly due to NO-induced microvascular permeability (127). However, no one has studied the pathogenesis of inflammatory arthritis regarding the regulation of iNOS on lymphatic drainage function, which we have demonstrated to be an important factor for the pathogenesis of the disease. Thus, we aimed to evaluate the possible ameliorating affects of iNOS inhibition on lymphatic drainage in TNF-Tg mice.

1.6 Experimental Goals

The primary goal of this research was to clarify the role of lymphatics during the pathogenesis of inflammatory-erosive arthritis. Although RA is a chronic systemic disease, patients can have acute episodes of inflammation in selective joints, with increased joint pain and fatigue, known as arthritic flares. However, the mechanism responsible for arthritic flare is unknown. Since the PLN is the most sensitive biomarker
for knee joint arthritic flare in TNF-Tg mice (129), CE-MRI is used frequently to evaluate PLNs in our experiments. Due to its very high costs and the limited access to MRI machines, CE-MRI has not gained broad acceptance. However, US is used broadly as a more practical and cost-effective method to study LNs in animal models and RA patients. Thus, we aimed to validate in vivo US to phenotype expanding PLNs vs. collapsed PLNs in TNF-Tg mice. The results indicate that CE-US is reliable to evaluate the onset of inflammatory-erosive arthritis by measuring the relative PLN volume, but is not practical to phenotype expanding PLNs vs. collapsed PLNs in TNF-Tg mice.

It is already known that the PLN collapse is due to the translocation of B-in cells from B cell follicles to the sinuses in PLNs, so the pathway of lymph fluid is blocked, leading to the poor drainage of synovial fluid from inflamed joints. The cytokines in synovial fluid will then stimulate the formation and activity of osteoclasts, which causes severe bone erosion. Sufficient lymphatic drainage, which is partially controlled by an intrinsic lymphatic pulse, is an important mechanism to limit joint inflammation and destruction of arthritis. It is already known that arthritic flare in TNF-Tg mice coincides with the loss of the lymphatic pulse afferent to draining LNs, causing their collapse. It has been demonstrated in other models that the lymphatic pulse is driven by eNOS, but inhibited by iNOS expressing in Gr-1+ myeloid cells (102). Based on these studies, I hypothesized that the loss of lymphatic pulse, which leads to arthritic flare via decreased lymphatic drainage, is due to the elevated iNOS from accumulated Gr-1+ cells. The results in this dissertation shows that large numbers of iNOS expressing Gr-1+ cells exist in collapsed PLNs of TNF-Tg mice with flaring inflammatory-erosive arthritis, and that the specific iNOS inhibitor L-NIL increases the lymphatic pulse and afferent lymphatic
drainage. Our hypothesis is further confirmed by the long-term treatment of L-NIL, in which the flaring TNF-Tg mice showed decreased bone erosion with L-NIL compared to placebo group.

Taken together, this thesis suggests a model in which the accumulation of iNOS-expressing Gr-1+ cells accelerates arthritic flare in the setting of inflammatory arthritis by inhibition of lymphatic drainage. Furthermore, selective iNOS inhibition is effective in ameliorating arthritic flare by improving lymphatic drainage.
Figure 1.1
Figure 1.1. Clinic applications of MRI and US on RA patients. (A) MRI of a representative knee from a RA patient shows expanding PLN (arrows) adjacent to highly vascular synovitis (arrow head) (59). (B) A representative dorsal long-axis view of first metatarsophalangeal joint from a RA patient presents hypoechoic synovial tissue and anechoic synovial fluid are shown (arrows) (65).
Chapter II

Materials and Methods
Animals:

The 3647 line of human TNF global transgenic mice were generated by Dr. George Kollias (Institute of Immunology, Alexander Fleming Biomedical Sciences Research Center, Vari, Greece) (29). In this mice strain, 3’-region of human TNF gene were replaced by the 3’-region of human β-globin gene. The line 3647 of TNF-Tg mice contains one copy of the transgene, which differs from the most commonly used TNF-Tg mouse line (line 197) which contains 5 copies of the TNF transgene. The TNF-Tg mice were bred as heterozygous at C57BL/6 background. Their WT littermates were used as healthy controls in the experiments. The TNF-Tg mice used in this study were more than 3.5 month old. Typically, the PLN starts to expanding at 3 month old, and falls into collapsed from 5 month old or older. iNOS-/- mice on C57BL/6 background, which are also called B6.129p2-Nos2<sup>tm1Ian/J</sup>, were purchased from Jax lab (stock # 002609). I used both female and male mice in the experiment. Mice were housed in a barrier facility at University of Rochester Medical Center. The mice were kept in the cages with 24 hours access to food and water, and 12 hours light-on/day. All the animal experiments were approved by University of Rochester Committee for Animal Resources.

CE-MRI and MRI data analysis:

All MRI scans were performed in a 3T Siemens Trio (Siemens Medical Solutions, Erlangen, Germany) as described previously (37, 48). Briefly, TNF-Tg mice were anesthetized with intraperitoneal ketamine (60 mg/kg) and xylazine (4mg/kg), with their knee and ankle inserted into customized knee and ankle coil. After a pre-contrast MRI
scan, gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA) contrast agent (Omniscan, Amersham Health, Norway) was injected via orbital venous plexus at 0.500 mL/kg diluted in sterile saline. After 5 minutes to allow for Gd-DTPA to circulate, a post-contrast scan was performed to image the CE of the knees and PLNs. The other leg can be scanned as soon as 24 hours later after the Gd-DTPA was metabolized.

There were several parameters that were used in analysis of CE-MRI scans, including ankle synovial volume, knee synovial volume, LNvol, lymph node contrast enhancement (LNCE) and lymph node capacity (LNcap). To quantify these parameters, Amira (Mercury Computer Systems, USA), a software for image analysis, was used. The 3D stacks of pre-contrast scans were aligned with post-contrast via automatic registration. A 3D stack of images was generated from subtracting pre-contrast scan from the post-contrast scan using the arithmetic module. PLNs and Synovial volumes were segmented by manually drawing ROIs on the 3D stack of post-contrast scan. The edge of PLN and surrounding fat pad tissue were determined based on signal intensity >1500 arbitrary units. The knee or ankle synovial volumes were quantified via the Tissue Statistics module based on the volumes of contrast enhancement in ROIs, which are the voxels above the threshold of 3.5 times of muscle signal intensity. LNCE is defined as the LN signal intensity divided by muscle signal intensity, and LNcap= LNCE *LNvol.

Ultrasound of PLN

PLNs were imaged with a high-resolution small-animal ultrasound system (VisualSonics 770 with 704 scanhead). Each mouse was anesthetized with ~2%
isoflurane in oxygen. Hair was removed from ankles to hips using a depilatory cream. The mouse was placed in the supine position on the 40°C heated imaging platform with paws taped to surface electrodes for heart rate monitoring and respiratory rate synchronization.

To segment and quantify PLN volume via 3D ultrasound, the ROIs of PLN and the surrounding fat pad were selected manually in Amira. The mean signal intensity of the fat pad (FPsi) was computed using the Tissue Statistics module. To eliminate the fat pad from the PLN, selected areas in which the signal intensity was over FPsi were subtracted and any resultant empty inclusions (“holes”) within the PLN were filled. The SurfaceGen module was used to arrange the labeled pixels as a bounded surface for subsequent 3D visualization and volumetric quantification with the Surface View and Tissue Statistics modules.

**US contrast studies**

When phenotyping collapsed vs. expanding PLNs using contrast enhanced-ultrasound, three-dimensional images of the PLNs and surrounding triangular fat pads were acquired before and 7, 30 and 60 minutes after saline or US contrast agent DEFINITY® (Lantheus Medical Imaging, USA) injection into the ipsilateral footpad. The FPsi was computed as described before. Lymph node sinuses (LS) in the PLNs were segmented to derive the sinus volume (SV) at all time points; and the sinuses were defined as regions with greater signal intensity than FPsi. The mean signal intensity of the LS (LSsi) was also calculated using Amira. Based on the background signal FPsi, the
SV was normalized to FPsi to derive the Standard SV (SSV), which is equal to SV * LSsi/FPsi.

**Electron microscopy:**

The muscle with the afferent lymphatic vessel to the PLN was identified by injecting Evan's blue in the footpad and was then excised and fixed overnight at 4°C using a combination fixative of 2.5% glutaraldehyde and 4.0% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The specimens were rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) and post-fixed with buffered 1.0% osmium tetroxide. The tissue was dehydrated gradually in 70%, 95% and 100% ethanol, transitioned into propylene oxide, infiltrated with EPON/Araldite epoxy resin, followed by embedment in fresh resin and polymerization for 2 days at 70°C. To identify the lymphatic vessel in the specimen, the epoxy embedded block was cut serially into one micron slices and stained with toluidine blue. Then, the specimen was trimmed of excess surrounding tissue and thin sectioned at 70 nm with a diamond knife using an ultramicrotome. These thin sections were placed onto mesh carbon coated nickel grids, and stained with 5% uranyl acetate in distilled water and Reynold’s Lead Citrate Solution. A Hitachi 7650 Transmission Electron Microscope with a Gatan 11 megapixel Erlangshen digital camera was used to image the grids.

**In vivo immunofluorescence:**
To capture the in vivo images the cells in lymphatic vessels of TNF-Tg mice, intravital immunofluorescence microscopy was developed by our lab. After injecting anti-CD11b FITC (eBioscience, USA) and anti-Gr-1 FITC (eBioscience, USA) antibody into the footpad of TNF-Tg or WT mice 2 hours prior to imaging, the mice were anesthetized with isoflurane and the hair on the lower limb was removed with a depilatory cream. Immediately before imaging, tetramethylrhodamine–conjugated dextran beads (Lifetechnologies, USA) were subcutaneously injected into the footpad to visualize the afferent lymphatic vessel to PLN. The skin was opened above the blood vessels from footpad to PLN, thus the afferent lymphatic vessels parallel to the blood vessel would be exposed as well. The lymphatic vessel was first located by the 5x objective lens of a fluorescence microscope (Zeiss Axio Imager M1m), and then the images of cells in lymphatic vessels were captured under the 10x objective lens. Time-lapse photographs were obtained at 3 frames per minute. The moving cells were highlighted red by using the “image calculator” function of ImageJ, which defines the cells in each frame.

**Immunohistochemistry**

For fluorescent IHC analysis, PLNs were dissected and freshly frozen in Tissue-Tek® O.C.T compound (Sakura Fineteck, USA). For lymphatic vessel sections, 0.5% Evan’s blue was injected into footpad to indicate the lymphatic vessels towards PLN before the dissection. The sections of PLNs or lymphatic vessels were cut at 6-μm–thick using Cryojane tape transfer system (Leica, Germany). Before staining, the slides were
recovered to room temperature (RT) for at least 30 minutes. The sections were fixed in 4% PFA for 10 minutes at RT or acetone/ethanol for 5 minutes in -20°C, followed by washing with PBS 3 times at 5 minutes each. The blocking buffer (3% BSA in PBS) was applied for 30 minutes at RT. The fluorescence-conjugated antibodies, including anti-mouse LYVE-1 Alexa Flour 488 (eBioscience, USA), mouse anti-iNOS/NOS type II FITC (BD Biosciences, USA), anti-mouse Gr-1 PE (eBioscience, USA), and monoclonal anti-α-smooth muscle actin (SMA) FITC (Sigma, USA), were diluted in blocking buffer and incubated with sections overnight at 4°C. For unconjugated primary antibodies, such as anti-GC α1, α2 or β1 (Abcam, USA), Myf5 (Santa Cruz, USA) and Pax7 (paired box protein 7) (Developmental Studies Hybridoma Bank, USA), the secondary antibody was applied for 1 hour at RT. After washing with 0.1% PBST for 3 times, the sections were mounted by Prolong Gold anti-fade agent with DAPI (Lifetechnologies, USA). The images were taken under Zeiss Axio Imager M1m (Zeiss, Germany) and analyzed by AxioVisionRel (Zeiss, Germany).

NIR-ICG

We have developed a near infrared (NIR) imaging system tailored specifically for characterization of murine lymphatic function. The target area was excited with NIR illumination provided by a tungsten halogen non-dichroic MR16 light bulb in a conventional voltage regulated microscope light source. The NIR blocking filter, placed between the bulb and end proximal end of a fiber optic ring illuminator, was replaced with an excitation filter suitable for indocyanine green (ICG) fluorescence excitation (FF01-769/41-25, Semrock Inc, USA). The ring was placed immediately below the lens
(Zoom 7000 macro, Navitar Inc., USA). A fluorescence emission filter without a mounting ring (FF01-832/37-25, Semrock, USA) was placed behind the lens and in front of the sensor of a high sensitivity 1.4 megapixel CCD camera (Prosilica GC1380, Allied Vision Technologies, USA). Control of the camera, as well as the NIR excitation and background illumination, was done using LabVIEW (National Instruments, USA). The camera and ring illumination system were placed at a fixed distance from the mouse. The mouse was placed in the supine position on the 40°C heated temperature-regulated surface (THM 1000, Indus Instruments, USA). The mouse was anesthetized with continuous delivery of ~2% v/v isoflurane in oxygen. Hair was removed from ankles to hips using a depilatory cream. ICG (Akorn Inc., USA) was dissolved in ddH2O at 0.15 μg/μl. After stretching the leg straightly and taping the footpad, 10 μl of the ICG solution was injected subcutaneously into the mouse footpad using a 10 μl syringe (Hamilton, Switzerland) with a 30-gauge needle. ICG images from footpad to knee joint were recorded for 1 hour after injection and again at 24 hours after ICG injection. A fixed square ROI was placed on the lymphatic vessel, and the signal was recorded. The lymphatic pulse was also calculated by the software. After the images of time lapse were exported as PNG, the fluorescence intensity of ICG in footpad could be quantified via ImageJ software (NIH, USA). 24 hr clearance of ICG is quantified as the percent difference in ICG signal intensity at the footpad 1 hour after administration and 24 hours later. The movies were generated from the images of the time lapse by QuickTime (Apple, CA).

**Micro-CT**
The knee joints were scanned by micro-CT by using a Scanco Medical VivaCT 40 cone-beam CT powered by 55-kVp and 142 micro-amps source (Scanco USA, Inc., USA). The machine was calibrated weekly with a density phantom and monthly alignment test. The mice were anesthetized with ~2% v/v isoflurane in oxygen and placed into a holder inside of the micro-CT. The knee joints were scanned at an isotropic resolution of 17.5 μm. It took 13 to 26 minutes to scan one animal, depending on limb alignment. 3D images were reconstructed using Scanco 3D software. The patella was hand-contoured by the operator and analyzed for bone volume with a Scanco threshold of 220.

**L-NIL treatment**

TNF-Tg mice with collapsed PLN were selected by CE-MRI. To evaluate the immediate effects of the iNOS inhibitor L-NIL (Cayman, USA, CAS 159190-45-1), mice were anesthetized with ~2% v/v isoflurane in oxygen, and injected with ICG in the footpad. After a half hour, the afferent lymphatic vessel to PLN would be filled with ICG, and the basal lymphatic pulse was recorded. Then, L-NIL was given at 4mg/kg for each leg subcutaneously in the footpad. As a control, TNF-Tg mice were given general NOS inhibitor L-NAME (CAS 51298-62-5, Cayman, USA) at 4mg/kg or saline of an equivalent volume. The lymphatic pulse and clearance was monitored by NIR-ICG after treatment. To verify the therapeutic potential of L-NIL in long-term treatment, plain water, 100ng/ml of L-NIL or 100ng/ml L-NAME, which were changed everyday, were given to TNF-Tg mice with collapsed PLNs in drinking water for 6 weeks. NIR-ICG was
performed to calculate the lymphatic clearance before and after treatment. To compare bone erosion, the patella volumes in knee joints were measured by micro-CT before and after treatment.

**Statistical analyses**

PLN volume, SSV, iNOS+/Gr-1+ cells, lymphatic pulse and clearance between groups were tested by two-sided t-test with a significance level of p<0.05. Linear regression was performed to compare the PLN volume measured by US and MRI. All statistic analysis above was performed by Microsoft Excel software (Microsoft, USA) or the GraphPad PRISM software package (GraphPad Software, La Jolla, USA).
Chapter III

3D and Contrast-Enhanced Ultrasound versus MRI
Quantification of Popliteal Lymph Node Volume and Drainage as Biomarkers of Inflammatory-Erosive Arthritis in Mice
3.1 Abstract

RA patients can have acute episodes of pain and inflammation in selective joints, with increased joint pain and fatigue, known as arthritic flares. The Popliteal Lymph Node (PLN) was demonstrated as the sensitive biomarkers for arthritic flare in TNF-Tg mice. To identify expanding vs. collapsed PLNs in TNF-Tg mice, Contrast Enhanced (CE)-MRI was developed as a reliable approach for the phenotyping. However, it is not broadly accepted by researchers due to the high cost. Thus, it is necessary to develop a method to replace CE-MRI for phenotyping PLNs. First, we demonstrated the feasibility of ultrasound (US) to visualize the PLNs in mice. The high quality image of US showed a significant correlation with the PLN volumes measured by CE-MRI. However, since there was no significant difference in lymph node volume (LNvol) between expanding and collapsed PLNs due to high variation, CE-US was a potential method to distinguish expanding vs. collapsed PLNs via evaluating the changes of lymphatic drainage in PLN. As expected, a significant increase in Standard Sinus Volume was shown following the US contrast agent DEFINITY® injection afferent to WT and expanding PLNs, but not collapsed PLNs. The LNvol after the DEFINITY® injection were dramatically increased in WT, but not in expanding or collapsed PLNs. Unfortunately, we found that delivery of DEFINITY® prior to US significantly impairs lymphatic function by damaging the lymphatic vessel and causing lymphatic leakage. Thus, other US approaches, such as Power Doppler, warrant future investigation for this purpose.
3.2 Introduction

Arthritic flare, a common feature of RA, is an episode of worsening inflammation during the chronic pathogenesis. However, flare is such a complex symptom that there are no standard criteria or markers to define flare for health care professionals (130-131). Thus, it is urgent to find an early marker for flare. Recently, draining LN enlargement has been recognized as a hallmark of joint inflammation in RA, and can be quantified with MRI (42). We have recently demonstrated that PLNs are the most sensitive biomarker for knee joint arthritic flare in the TNF-Tg mouse model of RA (49). To investigate these findings, we performed a prospective study in which TNF-Tg mice with bilateral ankle arthritis were followed with CE-MRI every 2-weeks until they presented with knee synovitis, which revealed two distinct phases of disease progression. The first, characterized as the PLN “expansion” phase, is associated with increased, but relatively stable synovial volumes without bone erosions, and large LNcap values, which indicate an expanded, fluid-filled node. Subsequently, a yet to be identified event triggers the PLN “collapse” phase, in which LNcap values decrease rapidly due to parallel reductions in both PLN volume and CE, while synovitis worsens, as highlighted by higher synovial volume values. Consistent with synovitis presentation, knees that drain to an expanding PLN have no evidence of focal erosions, whereas knees adjacent to collapsed PLNs display extensive bone loss. The non-CE regions correspond to “clogged” sinuses that are completely filled with B-cells (49), B-in cells (IgM+, CD220+, CD23+, and CD21+), and closely resembles LN shutdown during an immune response (49). To summarize, expanding PLNs appear to protect the adjacent knee from arthritis, while collapsed PLNs
cause erosion due to the insufficient clearance of pathologic inflammatory cells and cytokines from the joint space.

The importance of CE-MRI to a biomarker of inflammatory-erosive arthritis initiation, progression, and response to therapy has recently been demonstrated in murine models (22, 37, 49, 132). Despite its potential value, this approach has not gained broad acceptance due to its very high costs (money, time and labor), and limited access to MRI machines.

Gray scale and PD US have been used to evaluate the synovitis in RA patients (66-67, 69-71, 133). In addition, blood vessels in synovitis or tenosynovitis were also quantified by CE-US, which showed higher sensitivity than the PD US (74-75, 77). The application of US on murine models was demonstrated by the successful images of gray scale and PD US on knees or ankles of CIA mice model (78). Similar results were observed by another group who evaluated the synovitis in antigen-induced arthritis (AIA) rabbit model by gray scale US and CE-US (80).

Further, investigators have been evaluating US as a more practical and cost-effective method to study LNs in animal models (78, 80), and RA patients (74-75, 77). Based on these promising results, we aimed on phenotyping the collapsed and expanding PLN in TNF-Tg mice with varying degrees of arthritis by comparing the results obtained using both US and MRI modalities.

To determine whether US imaging of PLN would be a substitute for MRI as an early marker of arthritic flare, I tested that (1) US is capable to measure the volume of PLN in TNF-Tg mice; (2) contrast-enhanced US could phenotype expanding and
collapsed PLN as accurate as contrast-enhanced MRI. At last, I discussed the possibility of lymphatic damage due to US contrast agent DEFINITY®.
3.3 Results

3.3.1 Strong correlation between PLN volumes determined by MRI vs. US

The mouse was placed in the supine position on the 40°C heated imaging platform with paws taped to surface electrodes for heart rate monitoring and respiratory rate synchronization (Figure 3.1A). The PLN was identified in brightness-mode (B-mode) by adjusting the scanhead up or down to position the PLN at the plane of focus (red arrow in Figure 3.1B), and then scanned in 3D-mode with a step size of 0.032 mm. The 3D US image data were used to quantify PLN volume by manual segmentation of the LNs and surrounding fat pads quantification with the Surface View (Figure 3.1C) and Tissue Statistics modules. A linear regression analysis was performed on the volumes generated from both imaging modalities (Figure 3.2A). We also determined the intra and inter-observer reliability of our US PLN volume measurement, which showed insignificant variability ($p = 0.8399$ and $0.8096$ respectively) (Figure 3.2A).

To give a broader illustration of the correlation between MRI and US measurements, 3 PLNs were chosen to represent the smaller, middle and larger PLNs, and their 3D rendered images generated by MRI and US are presented (Figure 3.2 B-G).

3.3.2 Development of contrast enhanced ultrasound imaging and phenotype the collapse vs. expanding PLNs

After the footpad injection, fluorescence and Evan’s blue dye was shown to travel from footpad to PLN through the lymphatic vessel, and then arrive in the PLN sinuses (47, 134-135). Thus, we hypothesize that US contrast agent DEFINITY® would enter
PLN sinus after footpad injection, showing contrast enhancement under a B-mode US scan.

In TNF-Tg mice, there was a <10% change in FPsi within animals before and after contrast agent injection (Table 3.1), demonstrating the remarkably consistent echogenic signal of this tissue, and its appropriateness for normalization in longitudinal studies. This FPsi proved useful as a threshold value to quantify SV (Figure 3.3A-B). The CE in PLN was determined as the voxels that were brighter than FPsi. The SV in PLN was calculated based on the volume of CE before and after US contrast agent DEFINITY® injection. The time course study shows that SV 7 minutes after injection and reached the maximal value at 30 minutes post-injection (Table 3.1). Moreover, DEFINITY® CE demonstrated the functional differences between expanding and collapsed PLNs. Afferent footpad injection resulted in a significant increase in SSV (5.58-fold) and 85% increase in PLN volume in WT mice; and a 2.9-fold increase in SSV and 16% increase in PLN volume in expanding TNF-Tg PLNs (Figure 3.4). In contrast, collapsed PLNs in the older TNF-Tg mice did not display an increase in SSV and PLN volume following afferent DEFINITY® injection. These results are consistent with the established phenotypes of these different LN, as PLN expansion is known to occur primarily through to accumulation of afferent lymph, while collapsed PLNs have very limited draining function. Our saline injections proved that the CE is not due to the vehicle, and that injection of an echogenic media is required for this US imaging approach, as SV could not be calculated without CE (Figure 3.3C-D).
3.3.3 CE-US induces lymphatic vessel damage.

Unfortunately, the severe deformation was found in the endothelial cells of the afferent lymphatic vessel after DFINITY injection. The lymphatic vessel leakage was observed on the second day after contrast enhanced US (Figure 3.5B), while, as an untreated control, the other legs from the same mice had intact lymphatic vessel highlighted by NIR-ICG imaging (Figure 3.5A). Four weeks after the treatment, the lymphatic vessel pulse (Figure 3.5C) and lymphatic clearance (Figure 3.5D) decreased in the leg with DFINITY® injection compared to the control leg (n=3, *p<0.05). We also observed PLN collapse shortly after CE-US evaluation of expanding PLN, which we have not previously observed in young (5-months-old) TNF-Tg mice. The MRI image of the leg with DFINITY® (Figure 3.6B) and control leg (Figure 3.6A) are shown. LNCE was significantly decreased in PLN with DFINITY® injection compared to control (n=3, p<0.05) (Figure 3.6C). Previously, we found that PLNs with LNCE<4.5 a.u. have larger synovial volume while the PLNs with a LNCE>4.5 a.u. have moderate synovial volume (129, 136). Thus, we used 4.5 a.u. as the threshold to phenotype expanding vs. collapsed PLN. In our CE-US study, PLNs with DFINITY® were all presented as collapsed (LNCE<4.5 a.u.). In contrast, all PLNs from the control group were expanding (LNCE>4.5 a.u.). To better understand this phenomenon, we performed transmission electron microscopy on lymphatic vessels afferent to the PLNs that received DFINITY® and control (Figure 3.7). The results showed endothelial cell detachment, degenerative changes to smooth muscle cells, large vacuoles and obvious intraluminal protrusions in the DFINITY® injected tissue (Figure 3.7A–B). In the control group, the endothelial cells displayed a normal ultrastructural morphology with minor vacuoles, and
no abnormalities in the smooth muscle or connective tissue (Figure 3.7C) were observed compared to WT controls (Figure 3.7D). Thus, while CE-US may be useful for PLN phenotyping, the above findings raise concerns about potential side-effects and warrant further investigation.
3.4 Discussion

US proved to be a very facile approach to assess murine PLNs (Figure 3.1B), since it is readily identified in B-mode after locating the triangular fat pad. A strong relationship between PLN volumes measured by MRI and US was found using a linear regression ($R^2 = 0.844$, $P<0.0001$) (Figure 3.2A). However, vertical placement of PLN in US image is a potential source of variability, perhaps as much as 10 percent, and may result from mechanical compression with the scanhead. To reduce this variation, a lower frequency scanhead could be used resulting in a greater focal depth and distance from the head. In addition, the scanhead position should be adjusted so that the PLN is centered consistently in the plane of focus (Figure 3.1B). Of note is that smaller PLNs are less susceptible to scanhead compression error, as suggested by the stronger linear relationship with the volume obtained from MRI.

Unexpectedly, the slope was not 1.0 between PLN volume measured via US vs. CE-MRI, despite the strong correlation between measurements on the same LN across animals on the two instruments (Figure 3.2A). To test the hypothesis that we might be compressing the LNs during US imaging, we varied the vertical position of the scanhead in an attempt to compress the LN. In a representative test, the LN showed a volume of 6.86 mm$^3$ at a depth of 7 mm, and a volume of 6.39 mm$^3$ at a depth of 5 mm, resulting in a ~10% difference in LNvol. This is not large enough to account for the differences we observed (slope = 1.45). Thus, other factors must also contribute to the differences that we observed and accuracy of the absolute volume measurements attainable with these non-invasive approaches remains a limitation. Another error with the US measurement is the roughened surface (Figure 3.2E, F, G), which occurs due to our inability to fill
surface holes. This should be addressable in the future with the evolution of superior surface rendering software applications. However, since the primary outcome measure of this biomarker is to predict RA progression by PLN volume, we conclude that larger PLN imaged in MRI will also be larger during US imaging. The three largest and the three smallest LNs (Figure 3.2A) clearly differ from one another, and the rank order of size derived from each modality is the same for these six observations. The LNs in the middle of the range are closely bunched and may not differ significantly from one another in each group. There is also an issue with reconstituted images. Even if, according to the volume calculation by the image software Amira, Figure 3.2C, F are present smaller PLN comparing to Figure 3.2D, G, they look similar in reconstituted images. It is possible that Figure 3.2C, F show a flat-shaped PLN, while Figure 3.2D, G present a round-shaped PLN. However, the reconstituted images only present one side of PLN.

Although palpable draining LN have long been recognized as a symptom of RA, their value as a quantitative biomarker of disease initiation, arthritic flare and response to therapy has only recently been appreciated (77). However, if this biomarker is to be broadly utilized, it needs to be assessed by practical means such as US, which can be readily performed during the office visit. For this reason, US imaging has recently been evaluated as an alternative to MRI to assess various musculoskeletal conditions. In some cases, such as detecting psoriatic arthritis of fingers and toes in patients with psoriasis (137) and detection of bone erosions in gouty arthritis (138), US has been shown to be just as effective as MRI. However, in other cases such as predicting the development of
RA from undifferentiated peripheral inflammatory arthritis, MRI assessment of bone edema, synovitis and erosion pattern proved to be more useful (139).

We also demonstrated the possibility of phenotyping the lymphatic drainage function in murine PLNs efferent to normal and arthritic joints via in vivo 3D ultrasound imaging. The use of echogenic contrast agent vs. saline was found to be critical for segmenting the lymphatic vessels within the PLN, but similar changes in expanding LNvol are detectible following injection of either agent. Since no increase in SV and LNvol occurs in collapsed PLN following afferent injection with DEFINITY®, we find that dynamic US imaging is a feasible approach to phenotype draining LN function.

However, the local injection of DEFINITY® caused damage to the endothelial cells in the lymphatic vessel (Figure 3.7A-B). The gas imbedded in DEFINITY® lipid bubbles contained C₃F₈. This gas was widely reported to decrease corneal endothelial cells in number and increase permeability (140-142). Combining with the high frequency US scan, C₃F₈ containing US contrast agent caused endothelial surface area damage in blood vessels, including swelling of cells, widening of the space between cells, and necrosis (143-145). Similarly, our study also showed US contrast agent caused the irreversible damage of lymphatic vessel endothelial cells, leading to impaired lymphatic drainage in TNF-Tg mice at relatively younger age (less than 5 month old). As a result, CE-US is not a practical method to phenotype expanding and collapsed PLNs due to its permanent impairment in lymphatic vessel. It is possible that the purpose of phenotyping PLNs could be approached by other US method, such as PD-US. Thus, in my following studies, I continued to use the CE-MRI to phenotype PLNs in TNF-Tg mice.

Recently, Echoe Bouta in our lab developed a US method to phenotype collapsed
vs. expanding PLNs in TNF-Tg mice (146). She demonstrated that PD-US can efficiently quantify the blood flow in PLNs of TNF-Tg mice. Moreover, she presented that PD-US was able to phenotype PLNs, because expanding PLNs have a significantly higher normalized PD volume comparing to collapsed PLNs. Thus, PD-US is a safe and cost-effective substitute to CE-MRI to phenotype murine PLNs as a biomarker of arthritic flare.
3.5 Conclusions

In this study we have shown a strong correlation between US and MRI in measuring relative PLN volume. We also show that CE-US is capable to phenotype collapsed PLNs vs. expanding PLNs as well as MRI. However, the US contrast agent is toxic to lymphatic vessels. Thus, CE-US is not suitable for the longitudinal studies, such as the PLN comparison of pre- and post- treatment. Taken together, US studies demonstrated that it is an efficient way to detect the onset of inflammatory-erosive arthritis including the enlargement of PLNs. However, CE-MRI is a more practical way to evaluate arthritis flare since MRI contrast agent does not destroy lymphatic vessels.
Figure 3.1
Figure 3.1. High resolution ultrasound imaging with 3D reconstruction and volumetric analysis of murine PLN. (A) A photograph of an anaesthetized mouse positioned on the heating pad with an ECG monitor and a Scanhead 704 placed above the knee, is presented to illustrate how to image the PLN with the US machine. (B) A representative 2D US image of the PLN obtained under B-mode scan is shown to illustrate the dark PLN (red arrow) surrounded by the bright-echogenic white triangular fat pad (green arrow). (C) A reconstructed 3D image of the PLN (green) with surrounding soft tissue generated via US 3D-mode scan and Amira analysis software is presented to illustrate the volumetric analysis.
Figure 3.2

A

\[ y = 1.450x - 0.530 \]
\[ R^2 = 0.844; P<0.0001 \]

US (PLN volume mm\(^3\))

B

2.66 mm\(^3\)

C

7.63 mm\(^3\)

D

9.58 mm\(^3\)

E

2.07 mm\(^3\)

F

5.50 mm\(^3\)

G

7.64 mm\(^3\)
Figure 3.2. Strong correlation between PLN volumes determined by MRI vs. US. (A) A linear regression analysis was performed by plotting the PLN volume (mm$^3$) measured by MRI (Y-axis) versus US (X-axis). The slope and highly significant $R^2$ value are also presented. The 3D images of representative small, medium and large PLN generated independently by MRI (B, C, D) and US (E, F, G) are presented to illustrate their similarities. The colors for each PLN correspond to the colored dots in D.
Figure 3.3

A  Pre-contrast

B  After contrast
Figure 3.3. Footpad injection of DEFINITY increases lymphatic sinus volume in draining PLN. 2D US images of an draining PLN in a 4 month old TNF-Tg obtained before and 30 min after a contrast agent (A,B) or saline (C,D) injection in the afferent footpad are shown. The lymphatic sinus volume (SV) was quantified by identifying the voxels within the PLN whose signal was greater than the mean fat pad signal intensity (FPsi). Note that these PLN regions pre-contrast (A) are expanded post-contrast (B), while the decreased signal throughout the LN following saline (vehicle control) injection demonstrates the specificity of DEFINITY contrast enhancement.
Table 3.1

<table>
<thead>
<tr>
<th></th>
<th>FPsi</th>
<th>SV (mm$^3$)</th>
<th>LSsi</th>
<th>SSV (mm$^3$)</th>
<th>Fold change of SSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-contrast</td>
<td>147</td>
<td>0.09</td>
<td>157.88</td>
<td>0.10</td>
<td>1</td>
</tr>
<tr>
<td>7min</td>
<td>147</td>
<td>0.19</td>
<td>158.92</td>
<td>0.21</td>
<td>2.13</td>
</tr>
<tr>
<td>30min</td>
<td>154</td>
<td>0.21</td>
<td>166.82</td>
<td>0.23</td>
<td>2.32</td>
</tr>
<tr>
<td>60min</td>
<td>154</td>
<td>0.19</td>
<td>165.48</td>
<td>0.20</td>
<td>2.09</td>
</tr>
</tbody>
</table>
Table 3.1. The calculation of CE-US parameters at 7, 30, and 60 min after contrast agent injection. FPsi and SV were determined as described Figure 3.3 (A, B) in 3D US, and the mean signal intensity of the LS (LSsi) was calculated in Amira as the mean voxel signal intensity of the SV. To normalize the SV to changes in FPsi following injection, the Standard SV (SSV) was determined as (SV*LSsi)/FPsi. Note the 2-fold increase in SSV at all time points following contrast agent injection.
Figure 3.4

A

Fold change of SSV vs. pre-contrast

B

Change in PLN volume following DEFINITY
Figure 3.4. Collapsed PLN shows a significantly decreased normalized SSV compared to WT and Expanding PLN. Collapsed PLN were phenotyped by CE-MRI before CE-US. (A) A significant increase in SSV was shown following DEFINITY injection afferent to WT and expanding PLN, but not collapsed PLN (*p<0.05 vs. uninjected). (B) The volumes after the DEFINITY injection were dramatically increased in WT, but not in expanding or collapsed PLN (*p<0.05 vs. uninjected).
Figure 3.5

A B

C D

DEFINITY control

DEFINITY control
Figure 3.5. Contrast enhanced US induces lymphatic vessel damage. 4-month old TNF-Tg mice with expanding PLN were injected with 50 μl of US contrast agent DÉFINITY® in right footpads twice a week, followed by US B-mode scan for 20 minutes over the PLN, while the left legs were untreated as controls. NIR-ICG imaging was performed after US to quantify lymphatic drainage and the lymphatic pulse. Representative NIR-ICG images show that (A) the control leg had an intact lymphatic highlighted by ICG dye, (B) while the lymphatic vessel leakage (red arrow) was observed on the second day after contrast enhanced US in the contrast agent injected leg from the same mouse. The lymphatic pulse (C) and lymphatic clearance (D) decreased in the legs injected with DÉFINITY® compared to the control legs (n=3, *P<0.05).
Figure 3.6

A

B

C

\[ p < 0.05 \]
Figure 3.6. Contrast enhanced US causes PLN collapse. (A, B) Representative CE-MRI images show the difference in LNCE between control PLN (arrow in A) and PLN with DEFINITY-induced lymphatic damage (arrow in B). (C) 4 weeks after the initial treatment the PLN efferent to the DEFINITY-induced lymphatic damage collapsed, and had a significantly lower LNCE to control (n=3, p<0.05), which remained expanding (LNCE>4.5).
Figure 3.7
Figure 3.7. Lymphatic endothelial cell damage following afferent DEFINITY injection. Lymphatic damage caused by DEFINITY® was further confirmed by electron microscopy. 4 weeks later, the injected footpad received an injection of Evan’s blue dye to identify the lymphatic vessels afferent to the PLN, which were harvested and processed for transmission electron microscopy. Representative images are shown to illustrated the endothelial cells (*); smooth muscle cells (#); and lumen (&). Note the damaged lymphatic vessel in the DEFINITY® injected mice (A, B), as evidenced by the cell detachment (arrows in A), and large vacuoles and intraluminal protrusions (arrow head in B), as well as the degenerated appearance smooth muscle cell (# in B). In contrast, the lymphatic vessels exposed to saline showed attached endothelial cells and minor vacuoles (arrow head in C), but also have obvious intraluminal protrusions (C). WT lymphatic vessel displayed and intact endothelial cell layer without vacuoles and no intraluminal protrusion (D).
Chapter IV

Selective iNOS inhibition increases the lymphatic drainage from arthritic joints and ameliorates the bone erosion in TNF-Tg mice.
4.1 Abstract

Arthritic flare is an episode of pain and inflammation in selective joints during the chronic inflammatory pathogenesis of RA. Arthritic flare was found to be associated with the loss of lymphatic pulse in TNF-Tg mice, a RA model. From other models of inflammation, the lymphatic pulse is known to be controlled by eNOS and is inhibited by iNOS expressed in Gr-1+ cells. The existence of Gr-1+ cells in lymphatic vessels was demonstrated in TNF-Tg mice by in vivo fluorescence imaging. To test the hypothesis that this eNOS/iNOS dysregulation is responsible for the loss of the lymphatic pulse during arthritic flare in TNF-Tg mice, we performed IHC and in vivo pharmacological interventions studies with selective and non-selective iNOS inhibitors. The IHC results demonstrated large numbers of iNOS expressing Gr-1+ cells exist in collapsed PLNs of TNF-Tg mice. By evaluating the lymphatics by NIR-ICG, the specific iNOS inhibitor L-NIL increased the lymphatic pulse (from 0.42 ± 0.84 to 2.19 ± 0.77 pulses/min; p<0.05) and afferent lymphatic clearance (73.48 ± 8.9% with L-NIL vs. 53.25 ± 12.72% with saline; p<0.05). Additionally, the micro-CT results showed that bone erosion is ameliorated in L-NIL treated TNT-Tg (12.34±17.84% change of the patella volume) compared with placebo group (-2.19±14.50%). Collectively, these results suggest a model in which the accumulation of iNOS-expressing Gr-1+ cells accelerates the onset flare in the setting of inflammatory arthritis by inhibition of lymphatic drainage. This provides a potential target for treatment of inflammatory-erosive arthritis.
4.2 Introduction

While much is now known about immune-mediated inflammatory disorders such as RA, there is no explanation for the severity of the disease and its sudden onset commonly referred to as a “flare”. In our quest to elucidate the cellular and molecular mechanisms responsible for arthritic flare, we developed 3D CE-MRI for the mouse, and used this approach to evaluate the natural history of inflammatory-erosive arthritis in various murine models (37, 132, 147-149). The results of these studies produced several remarkable findings, including that the changes in the volume and CE of draining LNs precede arthritic flare in adjacent joints. Another discovery was the observation that knee arthritis in TNF-Tg mouse model of RA is asymmetric in which the unaffected knee drains to an expanded-contrast enhancing PLN while the contralateral knee with severe inflammatory-erosive arthritis is adjacent to a much smaller PLN that fails to take up Gd-DTPA (150). Longitudinal CE-MRI of PLNs and knees demonstrate that early on in the disease, PLNs present as an “expanding” phenotype with an intense CE and empty sinuses that are void of cells. These characters of expanding PLNs suggest an efficient lymphatic drainage which would protect the adjacent knee from arthritis. Later on, arthritic flare appears following PLN “collapse”, which is characterized by decreased volume and CE (129).

The role of lymphangiogenesis and lymphatic flow has been investigated during arthritis initiation and progression in murine models of RA (37, 147, 149-152). This research produced several remarkable findings, most notably that the rate and direction of pulsation of lymphatic vessels changes dramatically during the acute and chronic phases of inflammatory-erosive arthritis. Sufficient lymphatic drainage, which is partially
controlled by an intrinsic lymphatic pulse, is an important mechanism to limit joint inflammation and destruction from arthritis. It is hypothesized that alterations in lymphatic clearance precedes flare and that using agents that promote lymphatic transport will be beneficial in the treatment of RA patients. We recently reported that arthritic flare in TNF-Tg mice coincided with the decrease of the lymphatic pulse afferent to draining LNs (135). From other models of inflammation, iNOS has been shown to decrease lymphatic pulsing and/or flow, while eNOS has been shown to maintain the regular pulsing and/or flow of lymphatics (102). iNOS inhibitor also restored the lymphatic pulse in IL-1b and 2,4,6-trinitrobenzenesulfonic acid induced acute inflammation models (116-117). Moreover, some studies showed that the iNOS inhibitor L-NIL ameliorated bone erosion in acute inflammation arthritis models (123, 153). However, the effect of iNOS inhibitors on lymphatic contractile function is still unknown in chronic inflammatory conditions. Thus, I aimed to study whether L-NIL would inhibit the bone erosion by restoring the lymphatic pulse in chronic inflammatory-erosive arthritis model.

To determine if this mechanism controls the lymphatic pulse and lymphatic drainage in chronic inflammatory-erosive arthritis, I tested the hypotheses that: 1) Gr-1+/iNOS+ cells are present in the draining LNs of flaring TNF-Tg mice; 2) selective iNOS inhibition increases the lymphatic pulse and drainage from joints to draining LNs; and 3) selective iNOS inhibition protects the bone from erosion in the TNF-Tg mice.
4.3 Results

4.3.1 Gr-1+ cells express iNOS in lymphatic vessels of TNF-Tg mice, and the number of Gr-1+/iNOS+ cells is significantly higher in collapsed vs. in expanding or WT PLNs.

Intravital fluorescent microscopy was developed in our lab to observe the cell types and cell density in the lymphatic vessels afferent to PLNs (118). Fluorescence-conjugated antibodies, such as the CD11b and Gr-1 antibody, were injected into the footpads to stain the cells in the lymphatic vessels, which were draining the footpad. A dextran conjugated dye was used to indicate the afferent lymphatic vessels to PLNs, also by injection into the footpad.

The CD11b+ cells were immobile in lymphatic vessels afferent to a collapse PLN (Figure 4.1A-B). In contrast, in the lymphatic vessel afferent to an expanding PLN, CD11b+ cells moved very fast along the vessel and had a velocity of 186 ± 37 micrometers/second (n=9) (Figure 4.2 A-C). No CD11b+ cells were found in lymphatic vessels of WT mice (data not shown). The presence of Gr-1+ cells was also demonstrated in the lymphatic vessels of TNF-Tg mice (Figure 4.3B). The existence of Gr-1+ cells in lymphatic vessels was further confirmed by IHC and co-staining of lymphatic endothelial cell marker LYVE-1 in the PLN. A limited amount of Gr-1+ cells were co-localized with LYVE-1+ at the edge of WT PLN (Figure 4.3C), while extensive Gr-1+ cells were shown in the middle of PLN within the LYVE-1 sinuses, aligning with previous evidence of lymphangiogenesis in TNF-Tg PLN (Figure 4.3D). Thus, Gr-1+ cells were co-localized with LYVE-1+ lymphatic vessels in both collective lymphatic vessels afferent to PLNs and PLN sinuses.
Recently, Liao et al. reported that iNOS expressing Gr-1+ cells were increased around the lymphatic vessels afferent to the PLN in an acute inflammatory mouse model. They further demonstrated that iNOS+/Gr-1+ cells would cause the dilation of lymphatic vessels in vivo (102). Similarly, the Gr-1+ cells in lymphatic vessels also express iNOS in the chronic inflammatory arthritic model TNF-Tg mice (Figure 4.3E). Thus, it is possible that the loss of lymphatic pulse in the afferent lymphatic vessel to collapsed PLN is associated with the increased amount of Gr-1+/iNOS+ in the lymphatic vessel.

To further confirm this hypothesis, the amount of Gr-1+/iNOS+ was evaluated by IHC on the sections of WT, expanding or collapsed PLNs (n=4). Only a small amount of Gr-1+ cells were observed in WT PLNs, but none of them expressed iNOS (Figure 4.4). In contrast, more Gr-1+ cells were in expanding and collapsed PLNs (Figure 4.4). Moreover, the majority of those Gr-1+ cells were also iNOS+ (Figure 4.3E). Further, Gr-1+/iNOS+ cells were significantly increased in collapsed PLNs (15.7 ±1.2 cells/mm²) vs. expanding PLNs (10.8 ± 1.7 cells/mm², p<0.05), and in undetectable numbers in WT PLNs (Figure 4.4). Therefore, Gr-1+ cells expressed iNOS in TNF-Tg PLNs and the number of Gr-1+/iNOS+ cells was significantly higher in collapsed vs. expanding and WT PLNs.

4.3.2 Guanulatecyclase (GC), the primary receptors for NO produced by iNOS, are co-localized with smooth muscle cells in PLNs.

GC is a heterodimeric protein which has two subunit α and β. The GCα1β1 and GCα2β1 heterodimers are the two isoforms of NO-responding GC (154-155). To investigate the GC isoforms in the PLN, IHC for GC subunits and smooth muscle cell
marker (α-smooth muscle cell actin) was performed on frozen sections of WT PLNs, which showed that all NO-responding GC subunits, α1 (Figure 4.5A), α2 (Figure 4.5B), and β1, (Figure 4.5C) were existing in smooth muscle cells in PLNs. To identify the differential expression of GC genes in TNF-Tg mice, quantitative real-time RT-PCR revealed that mRNA level of GCα1 and GCβ1 were significantly decreased in collapsed comparing to WT PLNs (n=3, P<0.05). In expanding PLNs, there was a reduction of GCβ1 and GCα1 mRNA level vs. WT (n=3, P<0.05), but the mRNA level of GCα2 did not changes in WT, expanding and collapsed PLNs (Figure 4.5D).

It was recently demonstrated the NO-responding GC subunit was GCα1 in lymphatic endothelial cells (LEC) (156). Kajiya et al. presented that the GC function was inhibited by the knockdown of GCα1 in LEC, but not by the reduction of GCα2. They further confirmed it by the inhibition of lymphatic vessel dilation using GCα1β1 specific inhibitor on the UVB-induced skin inflammation mice. This result indicated the major isoform of GC in NO-mediated lymphatic vessel dilation was GCα1β1, other than GCα2β1. Based on this theory, our finding of the decrease of GCα1 and GCβ1 mRNA expression in collapsed PLNs may reflect a negative feedback to the increased effect of NO on smooth muscle cells. To protect the smooth muscle from continuously intensive relaxation by elevated NO, the smooth muscle cells could block the NO pathway by decreasing the expression of NO receptor GCα1β1. Since GCα2 subunit did not involve in the lymphatic vessel dilation, its mRNA level stayed unchanged as in WT, expanding or collapsed PLNs.

4.3.3 Selective iNOS inhibitor L-NIL restores lymphatic drainage afferent to
collapsed PLN by increasing the lymphatic pulse in TNF-Tg mice with arthritic flare.

L-NIL has been broadly used in vitro and in vivo to selectively eliminate the NO production from iNOS (153, 157-160). Based on the reported specificity and potency of L-NIL, we tested its immediate effects on lymphatic pulse and drainage in TNF-Tg mice. ICG imaging was performed on TNF-Tg mice with collapsed PLNs, which were phenotyped as expanding or collapsed by CE-MRI. The representative ICG images were shown as the footpads subcutaneously injected with L-NIL (Figure 4.6A-D) or saline (Figure 4.6E-G). Before the L-NIL injection, there was no lymphatic pulse in the afferent lymphatic vessels to collapsed PLNs. After L-NIL injection, the lymphatic pulse was restored and presented as the rhythmic changes of ICG signal intensity (Figure 4.6A-D). In contrast, saline injection did not cause any changes in the signal intensity of the afferent lymphatic vessels to collapsed PLNs (Figure 4.6E-G). In Figure 4.6H, the signal intensity in ROI also demonstrated the recovery of lymphatic pulse after L-NIL. There was no significant change of signal intensity in ROI after saline treatment (Figure 4.6 H).

By quantifying the lymphatic pulse described in Figure 4.6, saline did not change the lymphatic pulse in either WT or TNF-Tg mice. However, L-NIL significantly increased the lymphatic pulse from 0.42±0.84 to 2.19±0.77 pulses/minute (n=4, p<0.05) in TNF-Tg mice with collapsed PLNs, but no significant changes were observed in WT littermates with L-NIL (0.96 ± 1.42 to 3.13 ± 2.36 pulse/min; p=0.08) (Figure 4.7A). Moreover, by analyzing the ICG clearance in footpad after 24hrs, L-NIL significantly increased lymphatic clearance in TNF-Tg mice vs. saline (73.48 ± 8.9% vs, 53.25 ± 12.72%; p<0.05). In contrast, the non-specific NOS inhibitor L-NAME decreased the clearance rate in both WT (27.77 ± 13.78%) and TNF-Tg mice (9.07 ± 33.65%) (Figure
4.7B), but did not significantly change the pulse rate (Figure 4.7A). Thus, blocking iNOS significantly increased the lymphatic pulse in the afferent lymphatic vessels to collapsed PLNs, leading to increased lymphatic drainage.

4.3.4 The 6-week treatment of iNOS inhibitor ameliorates the bone erosion by increasing the lymphatic drainage in TNF-Tg mice with collapsed PLNs

L-NIL has been published to ameliorate adjuvant-induced arthritis (123). Therefore, to investigate whether L-NIL would protect bone from erosion by improving the lymphatic drainage on chronic inflammatory arthritis, L-NIL (100µg/ml), L-NAME (100µg/ml) or placebo was given via drinking water every day to TNF-Tg mice. The lymphatic clearance was compared before and after treatment by NIR-ICG imaging. The lymphatic clearance was decreased (-4.70±4.36%) in the placebo group, which was due to the normal pathogenesis of the disease in TNF-Tg mice (Figure 4.8A). Similar to the immediate effect in Figure 4.6B, the long-term treatment of L-NAME dramatically decreased the lymphatic clearance (-24.15±23.71%) (Figure 4.8A). In contrast, the L-NIL treatment significantly increased the lymphatic clearance (+5.53±2.12%, p<0.05) compared to the placebo and L-NAME group (Figure 4.8A). Therefore, the 6-week treatment of L-NIL showed a significant increase of lymphatic clearance compared to the placebo group, while the general NOS inhibitor L-NAME decreased the lymphatic drainage.

To evaluate the bone erosion after treatment, in vivo microCT was performed on TNF-Tg mice with collapsed PLNs pre- and post-treatment, followed by analysis of the patella volumes. The patella volume increased by +17.50±17.85% in the L-NIL group,
while patella volume decreased -9.06±5.68% and -5.87±4.43% in placebo and L-NAME, respectively (Figure 4.8B). The increase of patella volume caused by L-NIL was significant compared to both placebo and L-NAME groups (p<0.05), indicating L-NIL prevented bone erosion (Figure 4.8B).
4.4 Discussion

Several groups have been discovered that iNOS specific inhibitor inhibited inflammatory arthritis in RA model or OA model (123, 153, 161). All presented results on the beneficial effects of iNOS inhibitor, but did not provide any evidence to clearly explain why the iNOS inhibitor would reduce the arthritis. We suggest here one of the possible explanations for this phenomenon by exploring the changes of lymphatic drainage, which was demonstrated to significantly inhibit bone erosion, after treating TNF-Tg mice with the iNOS inhibitor.

We demonstrated that large numbers of iNOS expressing Gr-1+ cells exist in collapsed PLNs of TNF-Tg mice (Figure 4.1-4.4), and that the specific iNOS inhibitor L-NIL increased the lymphatic pulse and afferent lymphatic clearance (Figure 4.6-4.7). The long term treatment of L-NIL (6 weeks) in TNF-Tg mice with collapsed PLNs also showed that L-NIL increased the lymphatic clearance and ameliorated the bone erosion in knee joint (Figure 4.8). Additionally, we found that the receptors GC subunits for NO were widely expressed in lymphatic smooth muscle cells, and that mRNA level of GCα1 and GCβ2, were down-regulated in the sinuses of collapsed PLNs (Figure 4.5), suggesting a possibility of a protective response to avoid excessive effect of NO from iNOS.

These results indicated that selective iNOS inhibition may be used for the amelioration or reversal of the disease by improving lymphatic drainage. This new use is thought to offer minimal risk of side-effects. Current treatments for RA focus on the inflammatory process; this approach focuses on reversing impaired lymphatic function or enhancing function early in the disease process. Because decreased lymphatic transport
(lymphatic dysfunction) may be involved in the pathophysiology of other disease conditions, applications of the invention are not limited to inflammatory arthritis. Lymphedema can occur in any part of the body where lymphatic function is disturbed. Lymphedema can result from LN dissection or other cancer treatment regimens. Parasitic infestations such as filariasis, or bacterial infections such as cellulitis may result in lymphedema. Thus, the employment of iNOS inhibitor might benefit other diseases that related to the dysfunction of lymphatics.

The iNOS inhibitor L-NIL has been tested in human and animals (123, 153, 160-161). In the healthy volunteers and mild asthmatics, L-NIL was well tolerated, showing no apparent affect on heart rate, blood pressure, hematology and blood biochemistry (160). Unfortunately, the death rate was as high as 50% in the 6-week study of L-NIL on TNF-Tg mice with collapsed PLNs. TNF-Tg mice got arthritis as early as 3 months old and presented with collapsed PLNs at 7-8 months old. It vividly mimicks the chronic pathogenesis of RA much better than the other murine models. However, the TNF-Tg mice with collapsed PLN are extremely weak and their death rate is already significantly higher than age-matched littermates. In previous iNOS inhibitor study, researchers have used an acute inflammatory arthritis model CIA, which were younger than 4 months old during the studies and the mice only experienced inflammation for 3 weeks (161). Survival might be improved by setting up multi-dosage L-NIL treatment groups, thus a less aggressive but effective dosage might be found to study TNF-Tg mice with collapsed PLNs. Another option is to study the prevention or delay of arthritic flare starting on young TNF-Tg mice. For this study, L-NIL would be used on TNF-Tg mice with expanding PLNs at a younger age, which may lead to a much lower death rate.
4.5 Conclusions

The results in this chapter present an explanation for the decreased lymphatic pulse in TNF-Tg mice with collapsed PLNs; it may be that iNOS produced by Gr-1+ cells induces the relaxation of lymphatic vessels. It also suggests the possibility of targeting the inhibition of iNOS as a therapy for the treatment of erosive inflammatory arthritis, which was tested in RA animal model TNF-Tg mice in a long-term treatment.
Figure 4.1. CD11b+ Myeloid cell are stationary in afferent lymphatic vessels to collapsed PLN. Intravital microscopy was performed to quantify the velocity of CD11b+ myeloid cells in lymphatic vessels afferent to collapsed PLN by injecting FITC-conjugated anti-CD11b-antibody into footpad of anesthetized mice. After 2 hours, tetramethylrhodamine-dextran was injected into the footpad, and skin incision was made to expose the lymphatic vessel afferent to PLN. A movie was made via time lapse intravital immunofluorescent microscopy, which was performed under the 10x objective lens at 3 frames/min. To eliminate the movement due to breathing, the “StackReg” plug-in of ImageJ was used to align the images. Note that the screenshots of the movie indicate that the resident CD11b+ myeloid cells (highlighted in red) have no velocity.
Figure 4.2
**Figure 4.2.** CD11b+ myeloid cells move at a high velocity in lymphatic vessels afferent to expanding PLN. Myeloid cells were labeled by anti-CD11b-FITC and imaged via intravital microscopy as described in Figure 4.1. The myeloid cells (red arrow) travel from the footpad towards the expanding PLN in the afferent lymphatic vessels. Frame rate and distance measurements in ImageJ were used to calculate the velocity of the CD11b+ myeloid cells as 186±37 micrometers/sec (n=9).
Figure 4.3. Gr-1+ myeloid cells expressing iNOS exist in afferent lymphatic vessel and PLN of TNF-Tg mice. Intravital immunofluorescent microscopy was performed on afferent lymphatic vessels as described in Figure 4.1, using labeled antibodies against CD11b+ (A, n=5) and Gr-1 (B, n=5) (These images were obtained by Jie Li, PhD.). (C-E) Immunofluorescent microscopy was performed on fresh frozen histology sections of the PLN from WT and the expanding PLN from TNF-Tg mice, using PE-conjugated Gr-1, FITC-conjugated LYVE-1, or FITC-conjugated iNOS antibodies. DAPI was used to counterstain the nuclei (blue) in the co-localization panels. (C) Gr-1 positive cells were co-localized with the lymphatic vessel (LYVE+, indicated by arrows) at the edge of the WT PLN (n=3). Note that the lymphatic vessels in TNF-Tg PLNs are expanded in the middle of PLN due to lymphangiogenesis. (D) Gr-1+ cells are also present in the middle of the PLN, within the lymphatic vessel (indicated by arrows) (n=3). (E) Additionally, Gr-1+ cells also co-localized with iNOS-expressing cells (n>3).
Figure 4.4

[Bar chart showing iNOS+/Gr-1+ cells in PLN (cells/mm²) for WT, Exp, and Col groups. The chart indicates a significant difference between the experimental and control groups, with an asterisk (*) indicating *P<0.05 for the comparison.]

N=4, *P<0.05
Figure 4.4. iNOS expressing Gr-1+ cells are significantly increased in collapsed PLN. Histomorphometry of the immunohistochemistry that was described in Figure 4.3 was performed to quantify the number of Gr-1+, iNOS+ cells in WT, expanding and collapsed PLN, and the data are presented as the mean ± S.D. (n=4; *p<0.05). Note the significant increase in Gr+/iNOS+ cells in collapsed PLNs compared to expanding PLNs.
Figure 4.5

Relative mRNA level normalized to WT PLN

A. GC α1
B. GC α2
C. GC β1

D. Relative mRNA level normalized to WT PLN

WT PLN
Expanding PLN
Collapsed PLN

GC alpha 1  GC alpha 2  GC beta 1

* * *
**Figure 4.5. Expression of guanulate cyclase (GC) in smooth muscle cells in PLN.**

Immunohistochemistry on fresh frozen PLN histology sections was performed with labeled antibodies against GCα1, GCα2, GCβ1 and α-smooth muscle actin (SMA) as described in Figure 4.4. (A-C) Co-localization of GCs and $\square_{sm}$ in PLN. (D) The expressions of GCα1, GCα2, GCβ1 mRNA was confirmed by real time RT-PCR of total RNA isolated from PLN, and the data are presented as the mean ± S.D. Note that mRNA levels of GCα1 and GCβ1 are dramatically decreased in collapsed PLN compared to WT PLN (*p<0.05, n=3).
Figure 4.6

A. Pre-L-NIL

B. Footpad LV ROI 4min

B’. 4min

C. Footpad LV ROI 4min 10sec

C’. 4min 10sec

D. Footpad LV ROI 4min 20sec

D’. 4min 20sec

E. Pre-saline

F. 9min

G. 10min
The lymph pulse measurement after L-NIL or Saline

Signal Intensity (Arbitrary unit)

L-NIL or Saline

TNF-Tg with L-NIL

TNF-Tg with Saline

200 millisecond
**Figure 4.6. Administration of the iNOS inhibitor L-NIL increases the lymphatic pulse in TNF-Tg mice with collapsed PLN.** (A-D) TNF-Tg mice with collapsed PLN underwent NIR-ICG imaging to quantify the afferent lymphatic pulse prior to, during, and following a direct injection of L-NIL in the footpad. (A) NIR-ICG images illustrate the lack of ICG lymphatic drainage from the injection site in the footpad to the collapsed PLN at 1hr post injection. (B-D, B’-D’) NIR-ICG images of the afferent lymphatic vessel and PLN are presented to illustrate the recovery of lymphatic flow from the footpad to the PLN 4 minutes after the injection of L-NIL. To monitor the lymphatic pulse, a region of interest (ROI, indicated by red circle) was chosen on the lymphatic vessel, and the signal intensity in ROI was recorded real time. The ROI from B-D were enlarged in B’-D’. These images illustrate a cycle of a lymphatic pulse in which the changes of signal intensity in ROI are: dim (B’), brighter (C’), and then dim again (D’), indicating a contraction has occurred. (E-G) In contrast, saline injection did not increase the signal intensity in lymphatic vessel or PLN 10 minutes after injection. (H) Real time ICG signal intensity in the ROI vs. time is presented to illustrate the recovery of the lymphatic pulse (indicated by red arrows) after the iNOS inhibitor L-NIL (blue line) injection, while the saline did not affect the lymphatic pulse (red line).
Figure 4.7

A

![Bar chart showing changes in pulse rate before and after pre- and post-experimental conditions.](image)

N=4, *P<0.05

B

![Bar chart showing changes in ICG clearance.](image)

N=4, *P<0.05
Figure 4.7. The iNOS inhibitor L-NIL restores lymphatic drainage afferent to collapsed PLN by increasing the lymphatic pulse. L-NIL (4mg/kg), L-NAME (4mg/kg) and volume-matched of saline were injected subcutaneously into footpad of TNF-Tg and WT mice. NIR-ICG imaging was performed as described in Figure 4.6 to quantify the lymphatic pulse (A) and % ICG clearance (B). Note that (A) L-NIL significantly increased the lymphatic pulse in TNF-Tg mice with collapsed PLN, but had no significant effect in age-matched WT littermates. In contrast, saline and the non-specific NOS inhibitor L-NAME did not change the lymphatic pulse in either TNF-Tg or WT mice. (B) Similarly, the specific iNOS inhibitor significantly increased the lymphatic clearance compared to saline controls in TNF-Tg mice, while L-NAME decreased lymphatic drainage in both WT and TNF-Tg mice compared to saline controls.
Figure 4.8

A

Change in % ICG Clearance

L-NIL  H2O  L-NAME

N=3, *P<0.05

B

Change in Patella Bone Volume

L-NIL  H2O  L-NAME

N=3, *P<0.05
Figure 4.8. Effects of selective iNOS inhibition vs. non-specific NOS inhibition on focal erosions and lymphatic drainage in TNF-Tg mice with collapsed PLNs. TNF-Tg mice with established arthritis were given drinking water with L-NIL, L-NAME or saline. (A) NIR-ICG imaging was performed at 0 and 6 weeks, and the change in the % ICG clearance following the treatment is presented as the mean ± S.D. for the group (n=3). Note that in the H2O group, the lymphatic clearance was moderately decreased, which was due to the pathogenesis of the disease. Meanwhile, the lymphatic clearance dramatically smaller in the group treated with L-NAME compared to L-NIL and H2O. In contrast, the L-NIL treatment significantly increased the lymphatic drainage compared to both H2O and L-NAME group. (B) MicroCT was performed on the knees of the mice at 0 and 6-weeks post treatment to quantify the patella volume, and the data are presented as the change in patella volume. Note the patella volume decreased in both the H2O and L-NAME groups (Figure 4.7B, n>3), while 6-weeks of L-NIL treatment increased the patella volume significantly compared to both H2O and L-NAME groups ((Figure 4.7B,*P<0.05, n>3), demonstrating the protective effect of L-NIL on bone in the chronic inflammatory arthritis.
Chapter V

General Discussion
5.1 General conclusion and discussion

Based on previous studies in our lab, CE-MRI is a reliable method to phenotype expanding vs. collapsed PLNs, which are sensitive biomarkers for arthritic flare (49, 118, 129). However, it has not been broadly accepted by other research groups due to its extremely high cost. As a result, a cost-efficient substitute was required. In this thesis, US was shown to be a very practical approach when measuring the volume of PLNs, a conclusion made because the strong linear correlation was found between PLN volumes found by MRI vs. US. The US contrast agent DEFINITY® made it possible to phenotype PLNs via contrast-enhanced US. By analyzing the 3D image of PLNs after DEFINITY® injection to footpad, a significant increase in SSV was shown in WT vs. expanding PLNs, but not in collapsed PLNs. However, because the lymphatic sinuses are clogged in collapsed PLNs, the PLN volumes after the DEFINITY® injection did not change in collapsed PLNs, but dramatically increased in WT. However, the damage of LEC was found after DEFINITY® injection, causing lymphatic leakage and dysfunction. Thus, our group focused on using other US approaches, such as PD, to reach this aim. It confirmed later in our group that PD could be used to phenotype PLNs since collapsed PLNs showed a significantly lower normalized PD volume compared to expanding PLNs (146). As a result, PD-US is a cost, time and labor efficient alternative for CE-MRI when phenotyping PLNs.

We demonstrated that Gr-1+ cells were immobile and accumulated in lymphatic vessels with collapsed PLNs, while they were absent in WT lymphatic vessels or moving very fast in lymphatic with expanding PLNs (118). Further studies presented that some of these Gr-1+ cells were also iNOS+ cells, and that the number of double positive cells was
significantly higher in collapsed PLNs compared to those in expanding or WT PLNs. From the studies in other models of inflammation, the intrinsic lymphatic pulse is known to be controlled by eNOS, and is inhibited by iNOS expressed in Gr-1+ cells under the inflammatory conditions (102). Thus, I hypothesized that the loss of lymphatic pulse, which leads to arthritic flare via decreased lymphatic drainage, is due to the elevated iNOS from accumulated Gr-1+ cells. The specific iNOS inhibitor L-NIL immediately increased the lymphatic pulse after footpad injection. Moreover, after a 6-week treatment of L-NIL, not only the lymphatic clearance was improved, but also the micro-CT results showed that bone erosion was ameliorated in L-NIL treated TNF-Tg compared to placebo group.

The collective results from this thesis suggested a model for lymphatic contraction under normal and inflammatory conditions (Figure 5.1). Under normal conditions, eNOS in endothelial cells lying adjacent to the smooth muscle cells is induced by increased shear stress and stretch generated by lymphatic fluid. NO, produced by the eNOS, dilates the smooth muscle of lymphatic vessel. Dilation of lymphatic vessel decreases shear stress and stretch, thereby turning off eNOS and causing lymphatic vessel contraction. Thus, the rhythmic lymphatic pulse is maintained by a temporal and spatial NO gradient from shear stress/stretch-responsive eNOS (Figure 5.1A). During chronic inflammatory-erosive arthritis, iNOS expressing Gr-1+ cells enter the lymphatic vessel and secrete NO, which disturbs the NO gradient created by eNOS. Meanwhile, the sealing of cell–cell contacts becomes disconnected in the endothelium after the chronic exposure to inflammatory factors. At this time, NO from iNOS passes the barrier of endothelium and meets with smooth muscle cells, leading to the persistent dilation of smooth muscle in
lymphatic vessel. The continuous dilation of smooth muscle causes impaired lymphatic drainage via the loss of lymphatic pulse. Thus, PLNs are collapsed in chronic inflammatory-erosive arthritis animal model, followed by bone erosion due to the dysfunction of lymphatics to remove synovitis fluid (Figure 5.1B). When iNOS is blocked by the specific inhibitor L-NIL, the lymphatic pulse is controlled by shear stress/stretch-responsive eNOS again, as in the normal condition. As a result, the lymphatic drainage is significantly improved and bone erosion is ameliorated (Figure 5.1C).

The mean shear stress in lymphatic vessels in WT and TNF-Tg would be estimated according to Hagen-Poiseuille equation: \( \tau = 4\eta Q/\pi R^3 \), where \( \eta \) is the viscosity of lymph (Poise), \( Q \) is the volumetric velocity of lymph flow (milliliters per second), and \( R \) is the lymphatic vessel radius (centimeters) (162). The volumetric velocity \( Q = \pi R^3 V \), where \( V \) is the lymph flow velocity (centimetres per second) (163). Thus, Hagen-Poiseuille equation could be simplified as \( \tau = 4\eta V/R \). Bouta et al has measured the viscosities in WT, expanding and collapsed lymphatics, which were 18.1 poise, 18.4 poise and 16.1 poise respectively, while the lymph flow velocity were 0.0109 cm/s for WT, 0.0104 cm/s for expanding and 0.0073 cm/s for collapsed. The radius for lymphatics to WT PLN was about 0.0018 cm (102). The radius of lymphatic vessels to expanding and collapsed PLN was dilated to about 0.0036 cm (Figure 4.1 and Figure 4.2). Based on all the information above, the mean shear stress in afferent lymphatic vessels are 438 dyne/cm² in WT mice, 212 dyne/cm² in expanding and 130 dyne/cm² in collapsed TNF-Tg. However, these are estimated results. To make accurate values, the parameters need to be carefully measured, such as the radius of lymphatic vessels, the different velocity at different
This novel model provides us with an explanation for lymphatic dysfunction during arthritic flare. However, there are still limitations for this model. First, it is possible that the iNOS inhibitor L-NIL systemically rather than specifically ameliorated bone erosion in TNF-Tg mice. The iNOS inhibitor could contribute to other compartments of the disease when decreasing the bone erosion after 6-weeks of L-NIL treatment. For example, L-NIL would inhibit inflammation by decreasing the pro-inflammatory factors IL-1β and PGE2 (153). Further, L-NIL could inhibit angiogenesis (164), leading to reduced bone erosion by limiting pannus formation and invasion. Thus, my model provided one of the possible mechanisms for reduced bone erosion after long-term L-NIL treatment. We are the first group that discovered the possibility of iNOS+ cells travelling inside lymphatic in RA animal model. However, the source for this cell population is still unknown. From acute inflammation model, the major source for iNOS+ cells that affected lymphatic pumping was derived from bone marrow cells (102). Thus, it is possible that iNOS+/Gr-1+ cells in our model are from the same linage, but this still needs to be verified in the future.
5.2 Suggestions for future direction

In this thesis, I propose a mechanism for the loss of lymphatic pulse, which could be one of triggers for arthritic flare. Elevated numbers of iNOS+ cells were found in the lymphatics that lost their rhythmic pulse in TNF-Tg mice. The lymphatic pulse in TNF-Tg mice with collapsed PLNs was restored by treating with an iNOS inhibitor. So far, our study was focused on blocking the genesis of NO by the inhibition of iNOS. We could further confirm our theory by increasing NO from iNOS and demonstrating the arthritic flare was accelerated in TNF-Tg mice with expanding PLNs. Moreover, it is important to test the role of NO in loss of lymphatic pulse via inhibition of the NO receptor GC. We have showed the existence of NO receptors in smooth muscle cells of lymphatics. The question of which GC isoform(s) is the major player in lymphatic smooth muscle cells should be answered. Blocking GC could be performed by either using GC specific inhibitor or constitute a GC deficient animal model. Additionally, the changes of GC downstream, such as the level of cyclic guanosine monophosphate and calcium concentration, need to be measured and compared among lymphatics from WT, expanding and collapsed PLNs.

Since the major source for iNOS outside of lymphatics was demonstrated to be CD11b+/Gr-1+ derived from bone marrow cells in an acute inflammation model (102), it would elucidate our theory more clearly if we demonstrated that Gr-1+/iNOS+ cells inside of lymphatics were from bone marrow derived cells and that the depletion of iNOS+ in bone marrow cells would ameliorate arthritic flare in TNF-Tg mice with collapsed PLNs. Thus, the source of Gr-1+/iNOS+ could be determined by the synthetic transplantation of bone marrow from TNF-Tg mice with CD45.1 background to lethally
irradiated TNF-Tg mice with CD45.2 background. If Gr-1+/iNOS+ cells inside of lymphatics are CD45.1, they are derived from bone marrow stem cells. Otherwise, they are from the lineage of hematopoietic stem cells. To confirm that genetic loss of iNOS could be of benefit for arthritic flare, another experiment would be designed to transplant the bone marrow from iNOS-/- mice to lethally irradiated TNF-Tg mice with collapsed PLNs. We anticipate that TNF-Tg mice with iNOS-/- bone marrow would have better lymphatic clearance and less bone erosion in knee joints compared to the control group (TNF-Tg mice with TNF-Tg bone marrow).

Electron microscopy images have showed significant damage to lymphatic smooth muscle cells in aged TNF-Tg mice (unpublished data in Dr. Lianping Xing’s lab), which may lead to as low pulse rate. To investigate the lymphatic system under inflammatory conditions better, it is important to determine the reason for the degeneration of smooth muscle cells. We think one of the reasons is that the pool of stem cells for smooth muscle cells is depleted due to inflammation and aging, thus it is not enough to reconstitute the damaged smooth muscle cells at the later stage of chronic inflammation disease. To test this hypothesis, a biomarker for adult smooth muscle stem cells should be determined. It was published that skeletal muscle satellite cells Pax7+/MyoD+ cells could spontaneously enter an alternative mesenchymal pathway, including the smooth muscle pathway (165). I have finished a study which showed the existence of a large number of Pax7+/MyoD+ cells near lymphatic vessels afferent to PLNs (Figure 5.2B-C). It was demonstrated that there is a significant decrease in the number of double positive cells with age and with the progression of inflammatory arthritis (Figure 5.2E). The origin of lymphatic smooth muscle cells should be tested by
using Pax7-CreER crossing with a floxed reporter mouse. If the expression of reporter gene was found after tomaxifen treatment, the lymphatic smooth muscle cells are derived from Pax7+ satellite cells. Then, the lymphatic drainage could be tested in a Pax7 deficient animal model. Moreover, a method to increase Pax7 stem cells around lymphatics would help to ameliorate the arthritic flare.

In this thesis, some findings have the potential to serve as clinical therapy. A translational study to investigate the relationship between PLN and arthritic flare in RA patients is needed. This study requires the adaption of PLN phenotyping method, such as CE-MRI or PD-US, to RA patients. Also, we need to evaluate the relationship between the lymphatic drainage and arthritic flare in RA patients, which NIR-ICG could be used for. The loss of lymphatic pulse was shown to be induced by Gr-1+/iNOS+ cells, resulting in swelling and bone erosion. This result suggested a new direction to ameliorate the arthritic flare in RA patients.
Figure 5.1

A. Normal

Shear Stress increase

Shear Stress decrease

Contraction

Dilation
B. Chronic Inflammatory-Erosive Arthritis
C. Chronic Inflammatory-Erosive Arthritis with iNOS Inhibitor

Shear Stress increase → Shear Stress decrease

Dilation

Contraction
Figure 5.1. A schematic model of lymphatic pulsing under normal and inflammation conditions. (A) Under normal conditions, eNOS is induced by shear stress and stretch generated by lymphatic fluid, and turned off due to decreased shear stress and stretch at the maximum stretching point, in endothelial cells to maintain a regular lymphatic pulse via NO- regulation of lymphatic smooth muscle cell contractions. (B) During chronic inflammatory conditions, high levels of NO- produced by CD11b+/Gr-1+/iNOS+ cells that have attached to the lymphatic vessel overwhelm the eNOS NO gradient such that lymphatic smooth muscle cells are in a constant state of relaxation, therefore causing the loss of lymphatic pulse and reduction of lymphatic clearance. (C) This inhibition of the lymphatic pulse can be reverse by selective iNOS inhibition using the specific inhibitor L-NIL, which restores NO- regulated contractions by lymphatic smooth muscle cells.
Figure 5.2
Figure 5.2. Age related depletion of Pax7+/MyoD+ cells adjacent to lymphatic vessels. (A) Evan’s blue dye was injected into the footpad of 1, 4 and 8-month old WT and TNF-Tg mice (n=4) prior to euthanasia to identify the lymphatic vessel (arrow) afferent to the PLN in frozen tissue sections. (B) Multicolor fluorescent IHC for Pax7, MyoD and DAPI nuclear staining was performed on the frozen sections and a representative image of the tissue from a 1 month old WT mouse containing the lymphatic vessel (LV), the adjacent blood vessel (BV) and the region of interest (ROI; yellow box) are shown at 10X. (C-D) A 40X image of the ROI illustrates the Pax7+/MyoD+ cells (arrows in C), which were quantified to demonstrate the significant decrease in the number of double positive cells with age in both WT and TNF-Tg mice (D, n=3, *p<0.05).
References


46. von der Weid PY, Muthuchamy M. Regulatory mechanisms in lymphatic vessel contraction under normal and inflammatory conditions. Pathophysiology. 2010;17(4):263-76.


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