VITAMIN D₃ IS AN EFFECTIVE COUNTERMEASURE AGAINST NITROGEN MUSTARD EXPOSURE

by

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Dedication

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List of Abbreviations

1,25(OH)$_2$D$_3$ – calcitriol – 1,25-dihydroxycholecalciferol

1400W – Hydrochloride – iNOS inhibitor

25(OH)D – 25-hydroxyvitamin D$_3$

Dil – dialkylcarbocyanine dye

H&E – hematoxylin and eosin

IκBα – nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor

iNOS – inducible nitric oxide synthase

i.p. – intraperitoneal injection

i.v. – intravenous injection

NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells

NM – nitrogen mustard

SM – sulfur mustard

TNF-α – tumor necrosis factor-α
Vitamin D₃ is an Effective Countermeasure against Nitrogen Mustard Exposure

Abstract
by
LIEMIN AU

Exposure to mustard gas and mustard gas-related compounds has detrimental local and systemic effects¹,². In recent history, these compounds have not only been used as chemical warfare agents³ but also medicinally as chemotherapeutic agents against blood cancers such as leukemia and lymphoma⁴. Exposure to these powerful alkylating agents suppresses the bone marrow from producing circulating cells vital for survival⁵. The cellular mechanisms responsible for nitrogen mustard (NM)-induced toxicity remain unknown; moreover, there are no countermeasures to mitigate the detrimental effects of NM exposure. Here we show a skin and bone marrow connection mediated by inflammatory macrophages that is critical for NM-induced marrow toxicity. Using a murine model of topical exposure to NM, we identify inducible nitric oxide synthase (iNOS)-expressing macrophages as critical mediators responsible for exacerbating local tissue damage. These cells of the innate immune system accumulate at the site of NM exposure then exit the injured skin and traffic to the bone marrow where they contribute to bone marrow suppression, peripheral blood pancytopenia, and mortality. Direct inhibition of iNOS with a specific pharmacological inhibitor or with 25-hydroxyvitamin D₃ (25(OH)D), known to suppress macrophage inflammation⁶-⁹, protects mice from
local and systemic effects of NM. Additionally, local depletion of skin recruited macrophages not only accelerates healing of wounds inflicted by NM but also rescues mice from bone marrow suppression and blood cytopenia. In this report, we present findings which suggest that 25(OH)D is an effective and practical countermeasure to prevent detrimental effects of NM exposure. Furthermore, once assumed to be solely mediated by the direct alkylating effects of NM, our observations establish a novel and critical role of the innate immune system in linking local tissue inflammation with systemic pathologies.
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1. Background
2. Vitamin D₃ as an effective countermeasure to topical nitrogen mustard exposure
3. Preventing adverse systemic effects and observed mortality post-NM exposure with vitamin D₃
4. Rescuing mice from NM-induced toxicity in BALB/c mice with vitamin D₃
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Chapter 1

Background
1.1 Mustards

Mustard gas was first developed by Cesar-Mansuete Despretz in 1822\(^3\). Thereafter, this compound has been synthesized and characterized by countless scientists. In 1913, chemists Hans Thacker Clarke and Viktor Meyer developed a mustard formulation that had toxic properties, such as, causing extensive blistering of the skin and mucous membranes upon contact\(^{10-14}\). The Germans saw the potential destructiveness of Clarke-Meyer's compound, now known as sulfur mustard, and exploited it during World War I (WWI) against the British and Canadian soldiers\(^3\). Since WWI, the utilization of mustard gas has been continued in several wars, which are listed in Table 1.1. Consequently, soldiers and civilians exposed to mustard gas suffer from morbidity and mortality.

Table 1.1: History of mustard gas usage in wars\(^3\).

<table>
<thead>
<tr>
<th>Period</th>
<th>User</th>
<th>Weapons used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1915</td>
<td>Germans in Ypres, Belgium</td>
<td>Mustard gas</td>
</tr>
<tr>
<td>1919</td>
<td>British forces intervening in the Russian Civil War</td>
<td>Mustard gas; artillery</td>
</tr>
<tr>
<td>1923-1926</td>
<td>Spanish forces in Morocco</td>
<td>Mustard gas; a/c bombs</td>
</tr>
<tr>
<td>1930</td>
<td>Italian forces in Libya</td>
<td>Mustard gas; a/c bombs</td>
</tr>
<tr>
<td>1934</td>
<td>Soviet forces in Sinkiang</td>
<td>Mustard gas; a/c bombs</td>
</tr>
<tr>
<td>1935-1940</td>
<td>Italian forces in Ethiopia</td>
<td>Mustard gas; a/c bombs; spray tanks</td>
</tr>
<tr>
<td>1936-1937</td>
<td>Soviet forces in Sinkiang</td>
<td>Mustard gas</td>
</tr>
<tr>
<td>1937-1945</td>
<td>Japanese forces in China</td>
<td>Mustard gas &amp; lewisite; a/c bombs</td>
</tr>
<tr>
<td>1963-1967</td>
<td>Egyptian forces in Yemen</td>
<td>Phosgene &amp; mustard gas; a/c bombs</td>
</tr>
<tr>
<td>1983-1988</td>
<td>Iraqi forces in Iraq/Iran war</td>
<td>Mustard gas; a/c bombs</td>
</tr>
<tr>
<td>1987-1988</td>
<td>Iraqi forces in Iraqi Kurdistan</td>
<td>Mustard gas &amp; nerve gas; a/c bombs</td>
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</tbody>
</table>
1.1.1 Mechanism of action

Mustards are also commonly referred to as vesicants due to their ability to cause chemical burns or mucosal irritation. Different classes of vesicants include distilled mustard, mustard gas, lewisite, nitrogen mustard (NM), phosgene oxime, sesqui mustard, and sulfur mustard (SM). Although the damaging effects of these vesicants are similar, onset of symptoms may vary from an immediate effect to more delayed effect following exposure.

Common routes of exposure to mustard gas and mustard-related compounds are through inhalation, dermal contact, and ocular contact. Even though these vesicants are exposed to localized areas, absorption through the skin allows entry into the circulation. Given their lipophilic nature, mustards circulate in the blood stream and rapidly distribute into fatty areas in the body (i.e. liver, kidney, and bone marrow) resulting in organ toxicity and eventual malfunction and mortality.

Mustards act as non-specific DNA alkylating agents with the N7-site of guanine being the preferred site of initial attack. Consequently, electrophilic attacks on two guanine can afford bi-functional DNA adduct with both strands of DNA. These cross-links and distortions of the double helix of DNA are a cause of cytotoxicity. This results in blocking or interfering with the passage of polymerases, which leads to DNA polymerase inhibition and ultimately cell apoptosis. Due to these activities, mustard-mediated cytotoxicity found its use in cancer chemotherapy by targeting rapidly proliferating cancer cells. NM, a specific class of antineoplastic agent, is less toxic compared to sulfur mustard.
and therefore more feasible as an experimental and medical vesicant. During the 1940s, hospitals used NM to treat patients with blood cancers, such as lymphoma and leukemia; however, they are also limited by dose-dependent toxicity\textsuperscript{4}.

1.1.2 **Severity of mustard exposure**

Toxicity of mustards varies depending on the way these vesicants are introduced to the body. Topical exposure to mustards is more detrimental compared to oral or intravenous (IV) administration of mustards\textsuperscript{18}. Studies have reported that IV injections of mustards have lower mortality rate compared to topical exposure to mustards at the same dose\textsuperscript{15, 18}. The mechanisms of toxicity between these routes of exposure have not been well established, however, several factors can contribute to the detrimental effects of NM-induced toxicity including; temperature, humidity, skin hydration and body site.

The locations most sensitive to mustard exposure are warm, moist areas on thin skin\textsuperscript{19-23}. Upon mustard exposure to the skin, dermal cells undergo apoptosis causing hyperactivation of the host’s own immune system resulting in a cascade of events that is more potent in exacerbating tissue damage. Thus, mustards are the most toxic when administered through the skin\textsuperscript{15, 18}.

1.1.3 **Chemotherapy use of nitrogen mustard**

Mustards, such as NM or carmustine, are commonly administered topically or intravenously when used as chemotherapeutic agents. Different
routes of administration are determined by the type of cancer that is being treated. Intravenous administration of mustards has been used for patients receiving treatment for leukemia as well as Hodgkin’s and non-Hodgkin’s lymphomas. Furthermore, NM has also been used to treat breast and lung cancer patients. Additionally, patients with mycosis fungoides (also known as cutaneous T-cell lymphoma – CTCL) skin cancer, have 76-80% response and survival when treated with a topical delivery of diluted mustard solution directly onto their skin lesions.

1.2 Wound Healing

Wound healing is a complex process that can be divided into four overlapping stages: hemostasis, inflammation, proliferation, and remodeling (Figure 1). This process is well-orchestrated by cell-cell interaction via cytokines, chemokines, and growth factors released at the site of injury. Defects in any one of these stages may lead to chronic inflammation and persistence of wounds. For example, chronic wounds may remain in the inflammatory stage, which will cause the loss of balance between production and degradation of molecules like collagen.

1.2.1 Stages of wound healing

Initial response to a ruptured blood vessel is bleeding. Then, a fibrin clot forms at the site of injury mediated by platelet aggregation to prevent excessive bleeding. Next, platelets release growth factors that recruit innate immune cells,
initiating the second phase of wound healing: inflammation (Figure 1.1). Neutrophils, the first cellular responders, migrate into the site of injury to engulf and digest bacteria, dirt, and any potential debris in the wound bed. Subsequently, apoptosis of neutrophils leads to the release of chemotactic factors (i.e. interleukin (IL) -6, IL-8, monocyte chemoattractant protein (MCP-1), and tumor necrosis factor (TNF)-α) that recruit macrophages to the wound bed. Macrophages prevent infections by phagocytizing incoming microbes at the site of injury to allow for the next phases of wound healing, proliferation and remodeling, to proceed. Macrophages also secrete growth factors and other cytokines to promote collagen deposition, angiogenesis, and the creation of a new extracellular matrix. Finally, keratinocytes migrate over the wound to repopulate and close the wound\textsuperscript{26-28}.

\textbf{Figure 1.1:} Wound healing stages and immune cell interaction.
1.2.2 Innate and adaptive immune system in wound healing

The innate and adaptive immune systems work together to ensure proper protection against pathogens and infectious agents. Cells involved in the innate immune response include: neutrophils, macrophages, dendritic cells, mast cells, basophils, and natural killer cells. Following the innate immune response, the adaptive immune system activates and provides further protection. Upon phagocytosis, pathogens are intracellularly processed and presented on the cell surface of antigen presenting cells (APCs) in a major histocompatibility complex (MHC) class-restricted manner to T- and B-cells. Antigen-specific T-cells facilitate adaptive immunity and help establish long term protection through immunologic memory²⁶,²⁷.

1.3 Macrophage

Macrophages were first discovered by Metchnikoff, who characterized them by properties such as phagocytosis, motility, and biosynthetic capabilities³⁰. Macrophages are derived from hematopoietic myeloid progenitor cells (MPC) that are differentiated from circulating monocytes. Monocytes enter tissue from circulation and proceed to differentiate into tissue-specific macrophages. Macrophage activation results in the release of pro-inflammatory cytokines which includes: IL-1, -6, and TNF-α. These pro-inflammatory cytokines aid in host defense, via killing intracellular pathogens, stimulating wound healing, or enhancing immune regulation³¹-³³. Depending on the signals from their tissue
environment, macrophages can be polarized into two distinct phenotypes: classically activated macrophages (M1) and alternatively activated macrophages (M2)\textsuperscript{30-35}.

Alternatively activated macrophages, M2, do not produce pro-inflammatory cytokines. Instead, they serve as a source of anti-inflammatory cytokines, such as IL-10, and play a significant role in immune regulation\textsuperscript{30,36}.

Classically activated macrophages, also known as M1, are stimulated primarily through toll-like receptors (TLRs) and by phagocytosis of foreign antigens. Upon stimulation these immune effectors can produce nitric oxide (NO) and additionally pro-inflammatory cytokines such as TNF-\(\alpha\), IL-1\(\beta\), IL-6, and IL-12 to counter the first wave of pathogen insult. Persistent uncontrolled activation of macrophages, however, can lead to chronic inflammation and extensive tissue damage\textsuperscript{37-41}.

1.3.1 Macrophages are important in wound healing

Macrophages are the most prominent cells during cutaneous wound healing and are known to regulate the different stages because of their many functions in wounds, such as host defense, resolution of inflammation, removal of apoptotic cells, cell proliferation, and tissue restoration\textsuperscript{42, 43}. Depletion of macrophages impairs wound healing by preventing the reconstructive phase of the wound repair response and results in a chronic wound\textsuperscript{26, 27}. In addition to their role in wound repair, macrophages are also central mediators of other immune disorders such as inflammatory bowel disease\textsuperscript{33, 34, 38, 40, 44}. 
1.4 iNOS: a double edge sword in wound healing

The host response to tissue injury involves a variety of cell types and soluble mediators, like nitric oxide synthase (NOS). Three isoforms of NOS include endothelial nitric oxide (eNOS), neuronal nitric oxide (nNOS), and inducible nitric oxide (iNOS)\(^{45, 46}\). Produced predominately by macrophages, iNOS\(^ {43}\) catalyzes L-arginine to L-citrulline and nitric oxide (NO) which is an important mediator to ensure proper healing of wounds. This is evidence by iNOS-knockout mice and pharmacological iNOS inhibitors utilized in \textit{in vivo} murine wound healing models resulting in impairments of wound healing\(^ {40, 41}\). Moreover, transfecting these wound healing models with iNOS-cDNA or NO donors in these models reversed the effects and allowed wounds to properly heal\(^ {40, 41}\). These studies suggest that NO is important in wound healing.

In addition to increases in wound tissue, NO may also combine with superoxide (\(O_2^-\)), a free radical of the innate immune system available in wound tissue, to form peroxynitrite anion (\(\text{ONOO}^-\)), which is responsible for mediating cell toxicity (Figure 1.2)\(^ {45}\). An overproduction of iNOS further exacerbates skin erosions and damages the tissue. Utilizing inflammation or infection murine models to induce NO synthesis illustrate impairment of collagen production and delayed wound healing, which were restored with a pharmacological iNOS inhibitors or iNOS-knockout mice\(^ {41}\). These studies confirm that excessive amount of iNOS present in tissue exacerbate tissue destruction.
1.4.1 *NF-κB is the transcription factor for iNOS*

Even though inhibiting iNOS in inflammation or infection murine models has shown to be efficacious, pharmacologically iNOS inhibition has limited clinical use due to potential cytotoxicity and adverse effects on circulatory function. Therefore, another strategy to prevent overexpression of iNOS is by inhibiting the transcription factor of iNOS: nuclear factor kappa-light-chain enhancer of activated B cell (NF-κB).

NF-κB mediates transcription of proteins involved in cell survival, proliferation, inflammatory response, and anti-apoptotic factors which control genes involved in inflammatory diseases such as inflammatory bowel disease, arthritis, sepsis, and asthma\(^{47}\). NF-κB is normally transcriptionally silent due to suppression via inhibitor kappa light polypeptide gene enhancer in B-cells inhibitor, IκB, proteins in the cytoplasm. Upon activation, such as TLR signaling...
IκBα is ubiquitinated to release NF-κB which allows it to translocate into the nucleus and induce transcription (Figure 1.3)\textsuperscript{47}.

\textbf{Figure 1.3:} NF-κB activation pathway.

Inhibition of NF-κB has been extensively explored in inflammatory models, but it is not effective because of the side-effects of the inhibitors, which include mortality and cardiovascular diseases (CVD). Furthermore, inhibition of NF-κB in non-diseased tissue may result in multiple adverse effects since NF-κB is required for an effective immune response. Further research is required to determine a more effective path to inhibit or to dampen NF-κB activation.

\textit{1.4.2 Prevention of nitrogen mustard-induced detrimental effects}

NM exposure causes lung failure by an overexpression of iNOS via alveolar macrophages, which are macrophages of the lungs\textsuperscript{37, 48, 49}. Intervention
with iNOS inhibitors (i.e. N-acetyl cysteine, alfa-tocopherol, L-NAME, aminoguanidine, S-methylthiourea, and ebselen) is a strategy to mitigate upregulation of iNOS (Figure 1.4)\textsuperscript{50}. While studies show inhibition protects against tissue destruction and confirms that iNOS is the critical mediator of NM-induced toxicity, these inhibitors are limited due to potential cytotoxic effects.

![Chemical reaction diagram]

**Figure 1.4:** Prevention of iNOS expression post NM exposure using iNOS inhibitors.

## 1.5 Vitamin D\textsubscript{3}

Children with weak bones, bowed legs, and growth retardation, as well as, adults with pelvic bone deformity were diseases found in cities of both Northern Europe and United States\textsuperscript{51, 52}. This weakening and softening bone disorder is referred to as “rickets” in children and “osteomalacia” in adults. In the late 1800s, a cure was found in two different forms, which are sunlight exposure and cod liver oil; two mechanisms whereby the body’s natural stores of vitamin D\textsubscript{3} are increased\textsuperscript{53-55}. Therefore, vitamin D\textsubscript{3} became important for its roles in calcium
homeostasis and in bone metabolism. Within the past few years, vitamin D₃ deficiency, however, has been linked to chronic inflammatory diseases, chronic illnesses, common cancers, autoimmune diseases, infectious diseases, and cardiovascular disease⁸,⁵⁶-⁵⁹, drawing attention again to the potential role of vitamin D₃ in immune regulation.

1.5.1 Vitamin D₃ as an endocrine hormone

Sunlight and diet are the two main sources of vitamin D₃ (Figure 1.5)⁵¹. Upon exposure to sunlight, 7-dehydrocholesterol, a steroid in the skin gets converted to cholecalciferol (previtamin D₃), which diffuses into the blood stream and is bound by vitamin D₃ binding protein (DBP). The complex is then transported to the liver. Two hydroxylation steps are required to convert vitamin D₃ to its active hormone, 1,25(OH)₂D₃ (calcitriol). The first conversion occurs in the liver in which vitamin D₃ is converted to 25(OH)₂D₃. This stable metabolite is also often used to assess vitamin D₃ status levels in standard blood tests. The second hydroxylation occurs in the kidney where the active hormonal form, calcitriol, is generated. Excessive generation of calcitriol, however, can result in hypercalcaemia. The process in the kidney is the rate-limiting step for the conversion of optimal levels of calcitriol. Calcitriol is then transported throughout the body to cells where it binds to the vitamin D₃ receptor (VDR) and leads to specific gene expression, to immune response modulation and to cell differentiation, proliferation, and regulation of apoptosis⁵¹,⁶⁰.
1.5.2 Vitamin D₃ and immunity

In recent years, studies have shown that macrophages possess the necessary enzymes or receptors for direct uptake of 25(OH)D and are able to convert to 25(OH)D to calcitriol in an intracrine-like manner (Figure 1.6). 25(OH)D is taken up by macrophages by passive diffusion or by megalin (meg)-mediated uptake. Intracellular 25(OH)D is then translocated to mitochondrial 25-hydroxyvitamin D-1-α-hydroxylase (CYP27b1) for calcitriol formation. Calcitriol then binds to the VDR and forms a heterodimer complex that can regulate gene transcription. Interestingly, macrophages can convert higher levels of calcitriol intracellularly without overt systemic toxicity; a feature that is critical for anti-inflammatory effects.
1.5.3 Vitamin D₃: friend or foe

Vitamin D₃ has been well established to be anti-inflammatory and immunosuppressive. Supporting this claim, studies have shown vitamin D₃ supplementation prevents prostate cancer, rheumatoid arthritis, multiple sclerosis (MS), and even Alzheimer’s disease⁶³. Conversely, within the past decade, the debate on the use of vitamin D₃ to help treat patients with tuberculosis (TB) has still been ongoing⁶⁴. Vitamin D₃ deficiency has been associated with an increase in infections, and in pulmonary TB. Administration of vitamin D₃ supplements to patients with TB has shown to improve clinical outcomes⁶⁵-⁶⁷; however, the results have not been entirely consistent. Thus, more clinical trials are needed to test the optimal dose of
vitamin D₃. Furthermore, vitamin D₃ is multi-functional and more research is needed to determine the full spectrum of vitamin D₃'s functions.

1.5.4 Mechanism of action

Vitamin D₃ has been given as a supplement to patients with inflammatory diseases, but the mechanism is poorly understood. Studies have shown that vitamin D₃ up-regulates IkBα levels, which inhibits IkBα phosphorylation preventing NF-kB nuclear translocation and ultimately, preventing transcription of inflammatory mediators⁷,⁶¹,⁶⁸,⁶⁹.

The role of vitamin D₃ has been difficult to decipher since vitamin D₃ can directly influence over 200 genes. New DNA sequencing technology was utilized to produce a map of vitamin D₃ receptor binding across the genome, generating 2,770 potential binding sites⁷⁰. Interestingly, these sites were concentrated near a number of genes associated with susceptibility to autoimmune conditions such as MS, Crohn's disease, lupus, and rheumatoid arthritis, as well as cancers such as chronic lymphocytic leukemia and colorectal cancer⁶,⁵⁶,⁶⁰.
Chapter 2

Vitamin D₃ as an effective countermeasure to topical nitrogen mustard exposure
2.1 ABSTRACT

Mustard gas and mustard gas-related compounds have deleterious local and systemic cytotoxic effects upon exposure to skin and mucous membrane \(^{10-14}\). Historically, mustards have been exploited as chemical warfare agents causing severe tissue damage and bone marrow suppression. Further research noted that mustards, such as nitrogen mustard (NM), are alkylating agents that work by inducing DNA damage and disruption of cell division \(^4,17\). Consequently, mustard-mediated cytotoxicity found its use in cancer chemotherapy by targeting rapidly proliferating cancer cells. NM was effectively used as chemotherapy against blood cancers such as leukemia and lymphoma; however, its use has been limited by dose dependent toxicity \(^1,4\). Following exposure to vesicants, damaged epithelial cells (“first hit”) release cytokines and chemokines that recruit innate immune cells (i.e. neutrophils and macrophages) to the site of injury. Neutrophils, the first responders to injury, initiate bacteria clearance and wound healing. Upon neutrophil apoptosis chemotactic mediators are released which then exacerbates a secondary response to recruit hyper-inflammatory macrophages (“second hit”) which further destroy tissue resulting in severe delay of wound healing \(^41\). The prevention of the “first hit” may not be feasible due to the rapid toxic effects of NM exposed to the skin. Therefore, our strategy is to prevent the “second hit” or the persistent macrophage infiltration and release of pro-inflammatory mediators (i.e. inducible nitric oxide (iNOS) and tumor necrosis factor alpha (TNF-\(\alpha\))) that cause severely delayed wound healing. Previous studies have demonstrated that vitamin D\(_3\) plays a critical role in regulation of
inflammation. Therefore, vitamin D₃ has the potential to suppress the pro-inflammatory functions (i.e. iNOS of macrophages) without initiating overt systemic toxicity. **We hypothesize that administration of vitamin D₃ (25(OH)D) following NM exposure will prevent the recruitment of iNOS expressing hyper-inflammatory macrophages and therefore improve wound healing.**
2.1 INTRODUCTION

Vitamin D₃ has been traditionally important for mineral and skeletal homeostasis. Immunologically, vitamin D₃ deficiency is associated with chronic inflammatory diseases such as multiple sclerosis (MS), rheumatoid arthritis (RA), and psoriasis⁵²,⁵⁴,⁵⁶,⁵⁸ suggesting a critical role for vitamin D₃ in regulation of inflammation⁷,⁶¹,⁶⁸,⁶⁹.

Consistent with the literature and my preliminary data, vitamin D₃ suppresses iNOS levels preventing chronic inflammatory diseases⁶³. Hyper-inflammatory macrophages (macrophages expressing elevated levels of iNOS and TNF-α mRNA expression) were generated by a combination of statin/rosiglitazone followed by stimulation with lipopolysaccharide (LPS) using mouse bone marrow derived macrophages (BMDMs) in vitro (Figure 2.1). Statins, a class of drugs used to treat patients with high levels of cholesterol, inhibit the enzyme HMG-CoA reductase thereby reducing the production of cholesterol by the liver. Rosiglitazone or peroxisome proliferation-activated receptor gamma (PPAR-γ) is a nuclear hormone receptor that regulates fatty acid and glucose metabolism and is used to lower glucose levels by acting as an insulin sensitizer. Both of these drugs have anti-inflammatory effects alone; however, the combination of these drugs generates hyper-inflammatory macrophages by inducing elevated levels of iNOS and TNF-α mRNA expression.

Hyper-inflammatory macrophages using human monocyte derived macrophages (MDMs) were generated by utilizing the same inflammatory stimulus (statin/rosiglitazone/LPS combination) as mouse BMDMs. 25(OH)D and
calcitriol both showed potent suppression of TNF-α mRNA expression (Figure 2.2) and nitrite concentration (Figure 2.3) \textit{in vitro}. These results confirm that 25(OH)D and calcitriol have the ability to prevent the production of TNF-α and iNOS, molecules associated with hyper-inflammatory macrophages.

Figure 2.1: Generation of hyper-inflammatory macrophages with mouse BMDMs \textit{in vitro}. Mouse BMDMs were stimulated \textit{in vitro} with statin (10 μM) and rosiglitazone, PPAR-γ (5 μM) for 16 hours followed by 2 hours LPS (10ng/mL) stimulation to generate hyper-inflammatory macrophages (increased iNOS and TNF-α mRNA expression). (n=8; p<0.04 in all condition compared to statin/ppar-γ/lps treatment).
Figure 2.2: Vitamin D₃ inhibited TNF-α expression in human MDMs *in vitro*. Human MDMs were treated with calcitriol and 25(OH)D for 2 hours following 16 hours of inflammatory stimulus. Cells were harvested for RNA and analyzed for TNF-α expression. Inflammatory stimulus = Rosiglitazone (5 μM) / statin (10 μM) / LPS (10 ng/mL). N=9, *p=0.03; **p=0.01; ***p=0.009. 10 μM of either calcitriol or 25(OH)D.
Figure 2.3: Increased nitrite concentration inhibited by intervention of 25(OH)D or calcitriol in human MDMs.
Human MDMs were incubated for 16 hours with inflammatory stimulus with subsequent 2 hour incubation with either calcitriol or 25(OH)D prior to collection of supernatant. Nitrite concentration was analyzed by Griess reagent. Inflammatory stimulus = LPS (10 ng/mL) in presence of Rosiglitazone (5 μM) /statin (10 μM). N=6, *p=0.018; **p=0.04; ***p=0.02. 10 μM of calcitriol or 25(OH)D.

2.3 RESULTS

To determine whether 25(OH)D can counteract NM-induced inflammation, mice were given an intraperitoneal (i.p.) bolus of either 25(OH)D (50ng) or a specific pharmacological inhibitor of iNOS, 1400W (10mg/kg), one hour following topical NM exposure. Skin blisters and erosions occurred between 24 to 48 hours post NM exposure, however, intervention with 25(OH)D or 1400W histologically results in decreased inflammation, necrosis, edema (Figure 2.4),
and markedly suppressed iNOS and TNF-α mRNA expression compared to NM-treated mice (Figure 2.5).

Figure 2.4: Histopathological analysis of the NM exposed mice reveals inflammation and necrosis.

Dorsal skin biopsied from mice 48 hours following topical NM exposure were stained with H&E. In the skin treated with NM only, infiltration of cells in the dermis, full thickness necrosis with fat degeneration, and whole skin inflammation was observed. Mice that had been treated with either an i.p. injection of 1400W or 25(OH)D revealed an intact epidermis, dermis, and hair follicle morphology. Moreover, 25(OH)D treated mice have the most improved skin morphology (n=8).
Concurrent with the literature and our experiments, 1400W was able to prevent NM-induced inflammation and tissue destruction. To further confirm the importance of iNOS, NM-skin exposure was performed using nos2−/− mice. Histologically, nos2−/− wound biopsy taken 48 hours post NM exposure revealed inflammation with fat degeneration, but a normal epidermis and dermis (Figure 2.6) similar to that observed in 1400W or 25(OH)D treated skin. Furthermore, nos2−/− mice, as expected, have undetectable iNOS mRNA expression even following NM treatment. nos2−/− mice exposed to NM have a 10 fold increase in TNF-α mRNA level (Figure 2.7) suggesting that iNOS and TNF-α are independent of each other or TNF-α is overexpressing to compensate the loss of iNOS expression. These results confirms that iNOS plays a critical role in tissue destruction.
Figure 2.6: Histopathological analysis of skin biopsies from $nos^{-/-}$ mice treated with NM.

H&E analysis of dorsal skin biopsied from mice 48 hours following topical NM exposure. $nos2^{-/-}$ mice exposed to NM showed inflammation and mild fat degeneration but no observation of epidermis and dermis necrosis, revealing a healthier skin morphology compared to NM-treated skin. (n=6)
Figure 2.7: High TNF-α mRNA level in the skin of nos⁻/⁻ mice exposed to topical NM.

TNF-α mRNA expression were evaluated by qRT-PCR in NM-induced dorsal wounds biopsied from nos⁻/⁻ mice 48 hours post NM application. Data are presented as mean ± s.e.m. (n=6).

Macrophages are the primary cellular producers of iNOS⁴⁸, ⁴⁴, ⁴⁵, ⁷¹. Confocal microscopy illustrates an abundance of co-localized iNOS-expressing
macrophages (F4/80+) in the subcutaneous fat. In contrast, NM-treated mice with 25(OH)D intervention exhibit significantly diminished numbers of iNOS+ F4/80+ macrophages infiltrating the wound bed (Figure 2.8).

![Figure 2.8: 25(OH)D decreases iNOS-expressing macrophages following topical exposure to nitrogen mustard. Colocalization of F4/80+ (green) macrophages expressing iNOS (red) in NM-induced dorsal wounds biopsied from mice 48 hours following NM application by confocal microscopy; whereas, 25(OH)D intervention diminishes double positive cells. Arrows indicate F4/80+ iNOS+ cells. Scale = 100μM.](image)

To establish a role for iNOS-expressing macrophages in exacerbating tissue injury, mice were depleted of dermal macrophages by injecting liposomal clodronate at the site of injury (Figure 2.9). NM-exposed mice treated with clodronate liposomes demonstrate protection from skin erosion and fat necrosis.
(Figure 2.10) with concurrent decrease in iNOS and TNF-α mRNA expression (Figure 2.11). These observations are similar to those seen in mice treated with 25(OH)D. Furthermore, depletion of iNOS-expressing macrophages (F4/80⁺) (Figure 2.12), or antagonizing iNOS with either 1400W or 25(OH)D accelerated wound healing following NM exposure (Figure 2.13, 2.14). Together, these results support the hypothesis that macrophages are deleterious to the propagation of inflammation and subsequent tissue destruction, and show that 25(OH)D significantly arrests NM-induced tissue destruction by targeting macrophage-derived iNOS expression.

![Flow cytometric analysis of skin-derived F4/80⁺ cells five days following NM exposure in skin biopsies from mice receiving subcutaneous injections of PBS liposomes or clodronate liposomes following NM exposure.](image)

**Figure 2.9: Intradermal injection of clodronate liposomes depletes macrophages from mice.** Flow cytometric analysis of skin-derived F4/80⁺ cells five days following NM exposure in skin biopsies from mice receiving subcutaneous injections of PBS liposomes or clodronate liposomes following NM exposure.
Figure 2.10: Histopathological analysis of NM exposed mice with and without intradermal injections of clodronate liposomes. H&E analysis 48 hours following topical NM application of dorsal skin from mice injected intradermally with either clodronate or PBS liposomes (1 hour post NM exposure). Liposomal clodronate treatment revealed healthier skin morphology compared to PBS encapsulated liposomes. Scale = 100μM. (n=5)
Figure 2.11: Clodronate liposomes suppress iNOS & TNF-α mRNA expression back to control levels.
iNOS and TNF-α mRNA expression was determined by qRT-PCR using skin biopsies from NM-treated mice in the presence or absence of clodronate liposomes after 48 hours of NM exposure. Data presented as mean ± s.e.m; n=4.
Figure 2.12: Clodronate liposomes decreases F4/80⁺ iNOS⁺ cells in the skin.
Dorsal wounds biopsied from mice 48 hours following NM application were examined using confocal microscopy for change in F4/80/iNOS double positive cells with clodronate liposome treatment. Arrows indicate F4/80⁺ iNOS⁺ cells.
Figure 2.13: Intervention with 25(OH)D or 1400W accelerate wound healing and promote survival.
Topical NM was applied to the dorsal skin of a C57BL/6J mouse. 25(OH)D or 1400W was administered one hour after NM exposure. Photos were taken to monitor wound healing at indicated times.

Figure 2.14: Clodronate liposomes protect mice from severe wounds induced by nitrogen mustard.
Topical NM was applied to the dorsal skin of a mouse. Clodronate or PBS liposomes were intradermally injected around NM exposure one hour following NM application. Photos were taken to monitor wound healing at indicated times.
Chapter 3

Preventing adverse systemic effects and observed mortality post-NM exposure with vitamin D₃
3.1 ABSTRACT

Using a murine model of topical exposure to NM, we show a skin and bone marrow connection mediated by iNOS-expressing macrophages at the site of mustard exposure as critical mediators responsible for exacerbating tissue damage. Furthermore, these activated cells then exit the injured skin and traffic to the bone marrow where they contribute to myelosuppression, peripheral blood pancytopenia, and mortality. Inhibition of iNOS with a specific pharmacological inhibitor or with vitamin D₃, 25-hydroxyvitamin D (25(OH)D), known to suppress macrophage inflammation, protects the mice from local and systemic effects of NM. Additionally, local depletion of recruited macrophages accelerates healing of wounds inflicted by NM and rescued mice from bone marrow suppression and blood cytopenia. These findings suggest that 25(OH)D is an effective and practical countermeasure to prevent detrimental effects of NM exposure. Furthermore, once assumed to be solely mediated by the direct effects of NM, our observations establish a new critical role of the innate immune system in linking local tissue reaction with systemic pathologies.
3.2 INTRODUCTION

Exposure to mustard gas causes malfunction of organs (i.e. kidney, liver, lung, and spleen) and degeneration of the nervous system due to its lipophilic nature\textsuperscript{11, 15, 18}. Extensive research has shown that intravenous (i.v.) or intraperitoneal (i.p.) administration of mustards is less toxic compared to topical exposure to mustards. The mechanism, however, is unknown and has yet to be determined.

3.3 RESULTS

A longitudinal study of our model was designed to test the ability of 25(OH)D to ensure protection from further tissue destruction. NM-treated mice exhibit exaggerated skin wounds from days 6 to 9 (Figures 2.13, 3.1). In contrast, a single intervention treatment with either 25(OH)D or 1400W result in complete resolution of NM-induced skin injury by day 20 (Figure 3.1). Moreover, NM-treated mice looked severely ill, losing body weight progressively from 7.3±1.27\% by day 2 up to 37.3±0.390\% by day 4 (Figure 3.2), resulting in mortality or requiring euthanasia (Figure 3.3). A precipitous drop in body weight leading to death is evidence of systemic damage in addition to local cutaneous tissue destruction by topical NM exposure. However, administration of 25(OH)D rescued mice from mortality (Figure 3.1, 3.2) concurrent with maintenance of their body weight (Figure 3.2).
Figure 3.1: NM-treated mice with 25(OH)D or 1400W intervention promote survival and full healing of wounds. Topical NM was applied to the dorsal skin of a mouse with or without 25(OH)D or 1400W treatment. By day 20, mice treated with 25(OH)D or 1400W had full healing of wounds; whereas, NM-treated mice had persistent large wound size. (n=5)
Figure 3.2: NM exposure resulted in loss of body weight by 35%; which, 25(OH)D treatment maintains body weight.
Daily weight measurements of mice (vehicle, NM, NM + 25(OH)D treated) on days 0, 2, and 4 following topical NM exposure. Data presented as mean ± s.e.m. (n=6)

Figure 3.3: 25(OH)D rescues mice from NM-related toxicities.
Kaplan-Meier curve of mice treated with NM only or NM with a dose of 25(OH)D an hour following NM exposure. 100% mortality was observed in mice treated with NM only. (n=10)
Previous reports show that intravenous or intraperitoneal administration of NM affects the bone marrow, kidneys, lungs, and liver, in addition to splenic failure and degeneration of the central nervous system\textsuperscript{11, 15, 18}. However, in our skin injury model, we observe severe pathology in the bone marrow (Figure 3.4) with no histologic abnormalities in the visceral organs (Figure 3.5).

\textbf{Figure 3.4: Skin exposure to NM reduces bone marrow cellularity.}\nBone marrow cellularity was assessed using H&E analysis of sternums five days following topical NM application in mice administered 25(OH)D or 1400W i.p. at 50ng and 10 mg/kg, respectively for 1 time. (n=8)
Figure 3.5: No histological abnormalities of organs in NM-treated mice. H&E analysis of kidney, spleen, liver, brain, and lungs from mice five days post NM exposure.
Figure 3.6: Pancytopenia occurs in NM-exposed mice.
Peripheral blood smears (top) and complete blood count (bottom) of mice five days following NM exposure. NM-exposed mice have a 73.2% decrease of WBC and 50% decrease of HCT counts compared to control healthy mice. Intervention with 25(OH)D or 1400W restores WBC and HCT counts to normal ranges. Data presented as means ± s.e.m. N=9; *p<0.01; #p<0.004
In our model, detrimental effects of topical NM on the hematopoietic compartment include a depletion of bone marrow cellularity that is rescued by intervention with 25(OH)D or 1400W (Figure 3.6). Further supporting this observation are complete blood count (CBC) profiles which show that NM-treated mice have severe anemia and lymphopenia with a dramatic 20-point drop in the hematocrit (HCT) and 10-point drop of the white blood cell count (WBC) (Figure 3.6), respectively. Consistent with the preservation of cellularity seen in the bone marrow, intervention with 25(OH)D or 1400W (Figure 3.4) protected mice from blood cytopenias (Figure 3.6). These data strongly indicate that the NM-induced local inflammatory response driven by iNOS+ macrophages is associated with the observed systemic cytotoxicity and that a single dose of 25(OH)D can abrogate both local skin erosions and systemic toxicity.

The observation that 25(OH)D or 1400W confers protection against bone marrow suppression and mortality establishes a connection between marrow toxicity and iNOS in our NM-skin model. Since it is well established that iNOS induces bone marrow cell apoptosis\(^5\), \(^73\), we next sought to investigate the potential sources of iNOS. Examination of whole bone marrow cells isolated from NM-exposed mice reveal a 26-fold increase of iNOS expression as determined by qPCR, which was not observed with 25(OH)D treatment (Figure 3.7).
Figure 3.7: NM induces a significant increase iNOS expression in bone marrow five days post NM exposure, which 25(OH)D prevents. iNOS expression of bone marrow cells isolated from mice five days following NM exposure. Data represent the means ± s.e.m. (n=14)

This unexpected finding of increased iNOS levels in whole marrow isolates prompted further investigation to determine the cellular source. Baseline flow cytometric analysis of bone marrow cells reveals 3 distinct cell populations (Figure 3.8): erythroid (gate P2), granulocyte/neutrophils (gate P3) and macrophages (gate P4).
Flow cytometric analysis of bone marrow isolated from femurs of healthy control mice reveal three distinct populations in the FSC vs. SSC graph: erythroid (gate P2), granulocytes/lymphocytes (gate P3), and macrophages (gate P4).

In NM-treated mice, by day 5 (Figure 3.9), there is a significant decrease in the percentage of granulocytes/neutrophils (P3) with a concomitant increase in macrophages (P4). In contrast, the P2, P3, and P4 population percentages in mice treated with 25(OH)D or 1400W intervention maintain a bone marrow profile similar to baseline healthy control mice (Figure 3.9).
Figure 3.9: NM changes BM homeostasis, which is restored following either 25(OH)D treatment or iNOS inhibition.
Flow cytometric analysis of bone marrow isolated from femurs of mice five days following NM exposure. Flow histograms are representative of six experiments. (top) FSC vs SSC of one mouse per group. (bottom) N = 6; mean ± s.e.m.; * p<0.0004; # p<0.002
Figure 3.10: NM induces a significant increase in bone marrow F4/80+ macrophages by day five post NM exposure, which 25(OH)D prevents.
Flow cytometric analysis of F4/80+ macrophages from the P4 population of bone marrow from C57BL/6J mice five days after NM application. (top) FSC vs F4/80 of one mouse per group. (bottom) N=4; mean ± s.e.m.

Using the macrophage marker, F4/80+, we confirmed the increase of macrophages in the bone marrow within the P4 population (Figure 3.10) of NM-treated mice. This was not observed in mice with 25(OH)D or 1400W intervention (Figure 3.10). These results suggest that the observed cell depletion in the marrow may be a macrophage-derived iNOS-mediated effect. However,
the source of the increased macrophages in the bone marrow of the NM-treated mice remains unclear. This observation may represent accelerated myeloid differentiation of precursor cells within the marrow or an infiltration of the marrow by macrophages from a distant site. Hematopoiesis leading to cell maturation normally spans 10 or more days\textsuperscript{73, 74}. Our data shows an increase in mature F4/80+ macrophages in the bone marrow as early as 5 days post NM exposure (Figure 3.11), suggesting that the presence of functionally active iNOS-expressing macrophages are of extra-marrow origin.
Figure 3.11: NM changes BM homeostasis by day five post NM exposure. Bone marrow harvested days 1, 3, and 5 from mice treated with NM only or NM with 25(OH)D treatment. Cell populations on day 1 and 3 had no variation; however, by day 5, there is a decrease in the P3 population with an increase in the P4 population of NM-treated mice compared to control and 25(OH)D treatment.

In this model, we have shown that NM exposure results in inflamed skin infiltrated by iNOS+ macrophages which may be a potential reservoir for activated macrophages. To confirm our hypothesis, Dil (dialkylcarbocyanine dye) encapsulated liposomes were injected subcutaneously around the site of NM application to allow phagocytosis by dermal macrophages. Five days follow NM exposure, confocal images of sections from the sternum (Figure 3.12) and flow cytometric data from femoral bone marrow (Figure 3.13) show abundant
iNOS expressing F4/80+ macrophages co-localizing with the skin Dil marker. Treatment with 25(OH)D strongly inhibited iNOS expression (Figure 3.12) and reduced dermal macrophage (Dil and F4/80) migration into the bone marrow (Figure 3.13).

Figure 3.12: Dermal iNOS-expressing macrophages in bone marrow increases following topical exposure to NM. Confocal microscopy colocalization of Dil (red), iNOS (blue), and F4/80+ (green) macrophages in the bone marrow (sternum) of control, NM-treated mice, and NM-treated mice with 25(OH)D intervention five days after NM application. Arrows indicate F4/80+ iNOS+ Dil+ cells.
Figure 3.13: Topical exposure to nitrogen mustard increases dermal Dil⁺/F4/80⁺ expressing macrophages in bone marrow.
Flow cytometry demonstrates an increase of Dil and F4/80⁺ macrophages in the bone marrow of NM-treated mice compared to control or 25(OH)D treated mice five days post NM exposure. (top) isotype of F4/80. (middle) F4/80 vs. Dil of one mouse per group. (bottom) N=5; means ± s.e.m. Intradermal injections of Dil an hour following NM exposure.
Using complimentary assays that allow for whole organ imaging, mice were subcutaneously injected with pHrodo\textsuperscript{75, 76}, a pH-dependent fluorescent dye, around the site of NM application. pHrodo \textit{E.coli} BioParticles conjugates allow phagocytic cells, such as macrophages, to phagocytose pHrodo and the lysosomes in macrophages will enzymatically activate the fluorescence due to the change of pH from neutral to acidic resulting in emission of a signal that can be traced using Maestro imaging techniques. Five days following exposure to NM, Maestro imaging of whole sternum in NM-treated mice reveal enhanced fluorescence compared to 25(OH)D intervention and control (Figure 3.14). However, organs including, liver, kidneys, lungs, spleen, and brain did not reveal any striking fluorescence signal (Figure 3.15). The dramatic intensity of fluorescence differences between these groups is in the sternum which demonstrates dermal macrophages must have migrated to the bone marrow.
Figure 3.14: Topical exposure to NM increases dermal macrophage infiltration to the bone marrow.
Maestro imaging reveals the highest fluorescence in the sternum five days post topical NM exposure compared to control or 25(OH)D intervention.

Figure 3.15: Minimal difference in organs reveal by Maestro imaging.
Maestro imaging does not reveal significant pHrodo fluorescent changes in organs five days post topical NM exposure.
These data confirmed that the increase of iNOS-expressing macrophages in the bone marrow of NM-treated mice is migrating to the bone marrow from an extra-marrow site, specifically inflamed injured skin.

To establish a role for dermal activated macrophages in mediating marrow suppression and systemic toxicity in NM-treated mice, liposome-encapsulated clodronate was injected into the inflamed skin as previously described (Figure 2.9). Depleting skin macrophages had a profound effect on the bone marrow parallel to NM-treated mice with 25(OH)D treatment. The injection of clodronate liposomes in the skin not only prevented the influx of dermal iNOS-expressing (Figure 3.16) hyper-inflammatory F4/80+ macrophages (Figure 3.17) into the bone marrow (Figure 3.18), but also rescued mice from a) a precipitous weight loss (Figure 3.19) b), peripheral blood pancytopenia (Figure 3.20), and lastly c) bone marrow suppression (Figure 3.21).
Figure 3.16: Prevention of iNOS mRNA expression upregulation in the bone marrow using clodronate liposomes.
iNOS mRNA expression in bone marrow cells isolated from mice five days following NM exposure. Data presented as means ± s.e.m. n=14
Figure 3.17: NM induced a significant increase in bone marrow F4/80+ macrophages by day five post NM exposure which clodronate liposomes prevent. Flow cytometric analysis of F4/80+ macrophages in the P4 population in bone marrow from mice five days after NM application (top – representative of one mouse per group) (bottom – N=8).
Figure 3.18: Clodronate liposomes decreases iNOS expressing macrophages in bone marrow following topical exposure to NM. Confocal microscopy shows no evident iNOS and F4/80 double positive cells in the bone marrow following clodronate liposome treatment (sternum).

Figure 3.19: Clodronate liposomes prevents weight loss. Daily weight measurements of mice (vehicle, NM with PBS liposomes injection, NM with clodronate liposomes injection) on days 0, 2, and 4 following topical NM exposure. Data presented as mean ± s.e.m. n=6
Figure 3.20: Prevention of pancytopenia by clodronate liposome treatment.
Day 5 Complete blood count analyses of mice. Data presented as means ± s.e.m. N=8; *p<0.01; #p<0.004

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<th>NM + Clodronate Liposomes</th>
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<td>WBC (x10^3/μL)</td>
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<td>GRAN (x10^3/μL)</td>
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Figure 3.21: Preservation of bone marrow cellularity with clodronate liposome treatment. H&E analysis of sternums five days following NM application from mice administered PBS or clodronate liposomes.

These data reveal a critical role for dermally-activated macrophages in the marrow and support the hypothesis of a novel cellular trafficking pattern, whereby
dermal pathogenic macrophages exit the injured skin wound and migrate into bone marrow to exacerbate disease pathology.

Figure 3.22: Diagram of proposed topical NM skin contact model.
Topical exposure to NM result in epithelial cell death causing a release of cytokines and chemokines that recruit macrophages to infiltrate the affected area. Macrophages expressing high levels of iNOS possibly migrate to the marrow resulting in myelosuppression, pancytopenia, and death. Intervention with 25(OH)D (or iNOS inhibitor) prevents iNOS-expressing macrophages from NM-induced toxicity. To confirm, depletion of iNOS-expressing macrophages at the site of NM application reverse the effects of marrow suppresion and pancytopenia.
Utilizing our NM-mouse skin contact model (Figure 3.22), we are the first to report that the effect of topical NM exposure extends beyond local tissue injury to result in significant systemic damage, facilitated largely by activated macrophages and its mediators. Furthermore, we identify a new cellular trafficking mechanism whereby iNOS-expressing macrophages exit injured tissue and migrate to the bone marrow causing myelosuppression, peripheral blood pancytopenia, and mortality. Treatment with vitamin D₃ prevents overall tissue destruction and promotes survival of NM-treated mice by inhibiting activated iNOS-expressing macrophages at the site of exposure. Vitamin D₃ in the precursor form is safe, well tolerated, and translationally an ideal candidate to be developed as a countermeasure for mustard-induced injury.
Chapter 4

Rescuing mice from NM-induced toxicity in BALB/c mice with vitamin D$_3$
4.1 INTRODUCTION

In research, different strains of mice are chosen depending on the question at hand. Some mice are genetically mutated to knock-out a specific gene or may have been mutated to mimic a particular human disease. The most common control strains in use are: C57BL/6J and BALB/c. Even though these are widely used “wildtype” mice, there are some minor differences.

C57BL/6J mice have a T-helper cell type 1 (Th1)-skewed response; whereas the BALB/c mice response is skewed to Th-2. Th-1 responses induce cell-mediated immunity and phagocyte-dependent inflammation, which produce IFN-γ, TGF-β, IL-277. Th-2, on the other hand, induce antibody responses and eosinophil accumulation but inhibit several functions of phagocytic cells, which produce IL-4, -5, -6, -10, -1377.

Given that our NM-skin contact model is driven by iNOS, an acute inflammatory response, we hypothesize that observed phenomena is not specific to C57BL/6J but will generate similar observations using other strains such as BALB/c.

4.2 RESULTS

To test whether our NM-skin contact model is not strain-specific, we reproduced our study using BALB/c mice to examine local and systemic effects. As seen in C57BL/6J mice, the topical NM-induced wound appeared within 24 hours. Mice with 25(OH)D treatment, however, had delayed wound development (Figure 4.1). 48 hours post NM exposure, skin biopsy was obtained to examine
the pathology of the skin and evaluate iNOS expression. As expected, NM-treated mice had full thickness necrosis (Figure 4.2) and elevated iNOS and TNF-α mRNA expression compared to control mice (Figure 4.3). Intervention with 25(OH)D prevented necrosis of epidermis and dermis (Figure 4.2) and inhibited iNOS and TNF-α mRNA levels (Figure 4.3). In summary, treatment with vitamin D₃ prevents overall tissue destruction via attenuated inflammation.

![Image of wound formation and treatment with 25(OH)D over time.](image)

**Figure 4.1: Intervention with 25(OH)D delayed wound formation in BALB/c mice.** Topical NM was applied to the dorsal skin of a BALB/c mouse. 25(OH)D was administered one hour after NM exposure. Photos were taken to monitor wound development.
Figure 4.2: Histopathological analysis of the NM exposed mice shows inflammation and necrosis in the skin of BALB/c mice.

H&E analysis of dorsal skin biopsied from mice 48 hours following topical NM exposure. In the NM-treated skin, full thickness necrosis was observed. 25(OH)D rescued mice from skin degeneration.
Figure 4.3: High iNOS & TNF-α mRNA levels in the skin of NM-treated mice is effectively suppressed by 25(OH)D to baseline levels in BALB/c mice. iNOS and TNF-α mRNA expression changes were evaluated by qRT-PCR in NM-induced dorsal wounds biopsied from mice 48 hours after NM application. Data are presented as mean ± s.e.m. (n=5; p<0.05).
To determine whether NM-treated BALB/c mice have similar effects longitudinally (5 days post NM exposure); weights of mice were measured daily. Body weights exhibited a slight drop in NM-exposed mice; however, intervention with 25(OH)D protected from weight loss (Figure 4.4). A decrease in body weight suggests systemic effects from topical NM exposure. Similar to our previous data, the hematopoietic compartment was affected with a decrease in the WBC and HCT count in NM-treated mice compared to either control or mice treated with 25(OH)D (Figure 4.5). To further confirm the depletion of cells in the blood, sternum of these mice were sectioned and stained with H&E to reveal a depletion of bone marrow cellularity comparable to C57BL/6J mice in NM-exposed groups; however, intervention with 25(OH)D protected mice from myelosuppression (Figure 4.6). Flow cytometric data of bone marrow isolates confirmed a similar decrease in the P3 (lymphocyte/granulocyte) population with a concomitant increase in the P4 (macrophage) population (Figure 4.7). Thus, 25(OH)D effectively rescued mice from NM-induced injuries locally and systemically. The protective effect of 25(OH)D in NM-exposed BALB/c mice emphasizes the universality of the model and suggests that vitamin D₃ has potential to attenuate a hyperactive host immune response.
Figure 4.4: NM exposure resulted in loss of body weight in BALB/c mice. Daily weight measurements of mice (vehicle, NM, NM + vitamin D treated) on days 0, 2, and 4 following topical NM exposure. Data presented as mean ± s.e.m. n=6

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NM</th>
<th>NM+25(OH)D</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (x10^3/uL)</td>
<td>12.73 ± 2.99</td>
<td>10.23 ± 4.42</td>
<td>12.13 ± 1.12</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>47.96 ± 2.24</td>
<td>39.20 ± 3.43</td>
<td>42.97 ± 3.34</td>
</tr>
</tbody>
</table>

Figure 4.5: Peripheral blood smear and complete blood count illustrates pancytopenia in NM exposed BALB/c mice. Peripheral blood smears and complete blood count of mice five days following NM exposure. N=5
Figure 4.6: Skin exposure to NM reduces bone marrow cellularity in BALB/c mice. Bone marrow cellularity was assessed using H&E analysis of sternums five days following topical NM application in mice administered 25(OH)D i.p. at 50ng for 1 time.
Figure 4.7: NM changes BM homeostasis, which is restored following 25(OH)D treatment in BALB/c mice. Flow cytometric analysis of bone marrow isolated from femurs of mice five days following NM exposure. (top – FSC vs SSC of one mouse per group) (bottom – N=5; means ± s.e.m.).
Chapter 5

Rescuing pigs from NM-induced toxicity with vitamin $D_3$
5.1 INTRODUCTION

To date, dogs, mice, and guinea pigs have been the animal models of choice for the study of NM-mediated toxicity\textsuperscript{10, 78-80}. It is known that pig skin is used extensively in medical research due to its nearly identical properties and anatomic similarity to human skin. Additionally, in our mouse studies, we are limited to an intraperitoneal injection of vitamin D\textsubscript{3}, but vitamin D\textsubscript{3} is typically administered orally to humans, which is not an option in mice. Thus, as a continuation of our mouse studies, a more relevant model (pig) was used to determine the most effective oral dose of vitamin D\textsubscript{3} needed to counteract the severe effects of topical NM exposure.

5.2 PROCEDURE

Pigs were acclimated in the animal resource center (ARC) at CWRU for a week before they were subjected to the complex procedure outlined below (Figure 5.1). First, pigs are anesthetized for at least one hour by administering 6-8 mg/kg Telazol intramuscularly followed by inhalation of 1-5\% isoflurane. Once pigs were sedated, hair on the mid-line of the dorsal region between the center-line and shoulder blades was removed using electric clippers. Next, 26 mg/kg of NM diluted in PBS and petroleum jelly were applied on two 12 cm\textsuperscript{2} region on the mid-line of the dorsal region between the center-line and shoulder blades (Figure 5.2). Four hours following NM application, 100,000 IU of vitamin D\textsubscript{3} was
administered orally. Intra-procedure monitoring included taking the temperature, checking respiratory rate and pattern, checking palpebral reflex and applying ophthalmic lubricant every 15 minutes. Once the two 12 cm\(^2\) regions of the affected area were dry, a tegaderm patch was placed over the two 12 cm\(^2\) regions and wrapped with gauze. Pigs were observed hourly to ensure that full recovery from anesthesia was achieved.

Digital photographs were taken of the two 12 cm\(^2\) NM regions daily up to 5 days. For biopsies, pigs were anesthetized and an 8 mm biopsy was taken from the center of the two 12 cm\(^2\) regions.

Five days following NM application, 50 mLs of blood was collected from ear vein or cranial vena cava or external jugular vein of the sedated pig. Finally, pigs were euthanized and bone marrow, spleen, liver, lungs, urine, and kidneys are obtained for further analyses.

**Figure 5.1:** Timeline of pig study.
5.3 RESULTS

Vitamin D₃ capsules (100,000 IU) were orally administered to pigs 4 hours post NM exposure. To ensure vitamin D₃ was ingested, blood serum level for 25(OH)D was analyzed. As seen in table 5.1, pigs that were exposed to NM only had a constant blood serum level of 25(OH)D. In contrast, vitamin D₃ serum level rose from 37 ng/mL to 82 ng/mL in pigs that received 100,000IU of vitamin D₃, confirming that vitamin D₃ was ingested and was in circulation.

Table 5.1: Vitamin D₃ serum levels in pigs.
Serum of pig with vitamin D₃ intervention confirmed that the pig ingested vitamin D pills. n=2

<table>
<thead>
<tr>
<th></th>
<th>NM (average)</th>
<th>NM + VD (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D0</td>
<td>D2</td>
</tr>
<tr>
<td>vit d</td>
<td>32</td>
<td>33</td>
</tr>
</tbody>
</table>

Daily observations of wound development between the two experimental groups showed no striking differences (Figure 5.2). However, histologically, NM-treated pigs revealed epidermal and dermal necrosis that resembles a third degree burn (Figure 5.3) and had a concurrent elevated AST level (Figure 5.4) associated with severe tissue damage or severe burns⁸¹. In contrast, NM-exposed pig administered vitamin D₃ showed full thickness and dermal necrosis, however, there were distinct evidence of epidermal resurfacing and regeneration five days post NM exposure (Figure 5.3). Moreover, AST levels were not
increase with vitamin D₃ intervention (Figure 5.4). To determine whether skin erosion is mediated by NO, serum from peripheral blood was examined for NO concentration. Serum analysis revealed that NM-treated pigs had an increasing concentration of NO; however, vitamin D₃ intervention decreased NO concentration by day 5 (Figure 5.5). These results suggest that vitamin D₃ intervenes with the delayed sustained levels of NO thereby attenuating host response mediated tissue damage.
Figure 5.2: Gross phenotypic appearance of dorsal skin following NM exposure. Topical NM was applied to the dorsal skin of a pig and vitamin D₃ was administered orally 4 hours post NM exposure. Gross pathological were observed between two groups.
Figure 5.3: Histopathological analysis of NM exposed pigs with vitamin D$_3$ intervention shows re-epithelialization of epidermis. Topical NM was applied to the dorsal skin and vitamin D$_3$ was administered orally 4 hours post NM exposure. By day 5, re-epithelialization (arrows) of the epidermis was evident in the pig receiving vitamin D$_3$ treatment.
Figure 5.4: Increased levels of AST in NM-exposed pig.

AST levels did not increase in pig that received vitamin D$_3$. In contrast, the pig that did not receive vitamin D$_3$ had a dramatic increase of AST level indicative of severe tissue damage or burn.
Figure 5.5: Intervention with vitamin D$_3$ suppresses NO concentration back to control levels.

NM-treated pig shows a rise in serum NO levels from day 0 to 2 to 5. Vitamin D$_3$ intervention suppresses NO back to baseline by day 5.

From our previous mouse studies, weight loss proved to be a good predictor for systemic toxicity. Examination of weights on days 0, 2, and 5 demonstrate a body weight loss of 2% in the pig that was treated with NM-only. In contrast, NM-exposed pig with vitamin D$_3$ treatment gained 2% of its body weight (Figure 5.6). Further analyses of cellular compartments revealed a slight decrease in lymphocyte and granulocytes (P3) and a slight increase in the macrophage (P4) population reminiscent of what was observed for mice that developed pancytopenia (Figure 5.7). However, bone marrow smears show unclear differences in cellularity which questions acute systemic toxicity in NM only pigs (Figure 5.8). Thus, further experiments are needed to investigate
whether topical exposure to NM in pig skin exaggerates the host immune response enough to exacerbate systemic effects of NM.

Figure 5.6: Loss in body weight by 2% in NM-exposed pig which is rescued by vitamin D\textsubscript{3} treatment.
Pigs of different treatments were weighed on days 0, 2, and 5 following topical NM exposure. NM-treated pig lose 2% of its body weight. However, vitamin D\textsubscript{3} treated gained 2% of its body weight. Data presented as mean ± s.e.m. n=2
Figure 5.7: Vitamin D$_3$ treatment protects pig from NM-induced BM homeostasis. Flow cytometric analysis of bone marrow of pigs five days following NM exposure. A decrease in the P3 population is observed with a concomittant increase in the P4 population.

Figure 5.8: Bone marrow smear of pigs with or without vitamin D$_3$ treatment following NM exposure. Bone marrow smears from both pigs show little difference.
5.4 FUTURE DIRECTIONS

Our study was terminated five days post NM exposure and was not able to visualize a distinctive change on the skin of the pigs in the different groups (NM-treated only and NM exposure with vitamin D₃ treatment). A longitudinal study is needed to examine whether the pig that received vitamin D₃ will survive and show improved wound healing. Additionally, extending our study could help understand whether similar systemic effects has occurred that was observed in mice experiment.

Studies have shown that an abundance amount of vitamin D₃ could results in complications; however, inadequacy of vitamin D₃ will not effectively counteract NM-induced toxicity. Therefore, different dose of vitamin D₃ is required to be tested to determine which dose is the most effective to mitigate the detrimental effects of topical NM exposure without resulting in complications.

In our study, vitamin D₃ was administered 4 hours post NM exposure. Another study should examine different times of vitamin D₃ administration. This will determine the window of which vitamin D₃ can be given and still prevent the detrimental effects of topical NM exposure.
Chapter 6

Discussion and Future Directions
Mustard gas and mustard gas-related compounds have deleterious local and systemic cytotoxic effects upon contact exposure\(^1\). Understanding the mechanism of NM-induced injury will allow for the development of future therapeutic strategies to counteract NM-induced injuries. Previous studies used pharmacological inhibitors of iNOS as countermeasures. Although their data showed efficacy, the concentrations of iNOS inhibitors needed were toxic and had adverse circulatory effects.

In our research, we developed a murine model of topical exposure to NM. Based on the data, we identified iNOS-expressing macrophages at the site of mustard exposure as critical mediators responsible for exacerbating local tissue damage. Furthermore, these activated cells were shown to have exited the injured skin and trafficked to the bone marrow, where they contributed to myelosuppression, peripheral blood pancytopenia, and mortality. Administration of a pharmacological iNOS inhibitor or with vitamin D\(_3\), 25(OH)D, known to suppress macrophage inflammation, protected the mice from local and systemic effects of NM. Additionally, local depletion of recruited macrophages accelerated healing of wounds inflicted by NM and rescued mice from bone marrow suppression and blood cytopenia.

Some questions, however, still remain. Firstly, our data illustrated that activated dermal macrophages exited the injured skin and appeared in the bone marrow; however, the migration pathway of these cells remains unclear, such as what caused the macrophages to go back to the bone marrow, which is not a well-established macrophage migration. In addition, do iNOS-expressing dermal
macrophages in the bone marrow have the capability to cause cells to undergo apoptosis leading to bone marrow suppression? Lastly, a non-invasive approach to determine the levels of NO will be useful in aiding the development of an antidote to counteract NM-induced toxicity.

Our hypothesis to our remaining questions is that mustard is a lipophilic agent that once absorbed through the skin would re-deposit into fatty tissues, including the bone marrow. The bone marrow contains rapidly dividing cells and the mustards will target and damage cells which then cause a release of chemotactic factor causing the innate immune cells to home back to the marrow.

Previous studies also demonstrated that IV administration of mustard was less toxic compared to IP or topical administration of mustard. In both IP and topical administration, epithelial surfaces are damaged stimulating inflammation. Separating these compartments or routes of administrations will allow us to tease out the mechanism. A potential approach to understand the mechanism is to administer a low dose of mustard intravenously into a mouse. This small dose will not cause organ failure nor will it cause bone marrow suppression. It will only allow mustard to re-deposit into the fatty organs, including the bone marrow. Simultaneously, a low dose of UVB will be applied on the back of the mouse. This UVB dose will activate iNOS macrophages to migrate to the skin. If our hypothesis is correct, then IV mustard alone and UVB alone will not cause myelosuppression. Only IV mustard and UVB when combined will lead to myelosuppression, confirming that mustard exposure causes epithelial cell death (“first hit”). A release of cytokine and chemokine will then recruit and activate the
innate immune cells (“second hit”), which will exit the injured site and migrate to the bone marrow causing myelosuppression (“third hit”).

Our data illustrated that activated dermal macrophages exited the injured skin and appeared in the bone marrow; however, the migration pathway remains unclear. Fluorescence tomography in vivo imaging (FMT) technology allows for non-invasive, whole body deep tissue imaging in small animal models and can also monitor and quantify in real time. This technology has been advantageous in oncology, inflammation, pulmonary, cardiovascular, and skeletal disease research. In our preliminary data, we utilized FMT technology on our NM-skin contact model to track the migration pattern of recruited iNOS-expressing macrophages at the site of NM-induced injury. A three-dimensional image of the animal, therefore, was generated to determine which organs were of interest by the fluorescence detected.

To observe the migration pattern of fluorescently-labeled macrophages, mice were subjected to our NM-skin contact model with an intradermal injection of DiR-encapsulated liposomes (200μL) around the site of NM exposure. Whole mouse in vivo imaging on days 1, 3, and 5 post-NM exposure were examined.

The fluorescence areas seen in Figure 6.1 were initial intradermal injections of DiR-encapsulated liposomes. The dyes indicate no cellular movement from days 1, 3, to 5 following NM application. FMT, thus, was not sensitive enough to track specific cellular movements.
Figure 6.1: FMT *in vivo* analysis of mice on days 1, 3, and 5 post-NM exposure. Fluorescence corresponds with where DiR encapsulated liposomes were injected. No migration of fluorescence was observed.

Other possible avenues can be explored to track the migration pattern of activated dermal macrophages. Kanagawa and Tomura developed transgenic mice expressing the Kaede protein. Kaede is a photoconvertible fluorescence protein that changes from green to red upon exposure to UV light (350-400nm). The photoconversion of intracellular Kaede has no effect on cellular function and is irreversible\textsuperscript{83-86}. By using Kaede mice in our NM-skin contact model, we can track the movement of cells with the photoconverted Kaede protein and monitor where the cells (macrophages) migrate following topical NM exposure *in vivo* using Maestro *in vivo* imaging and 2-photon microscopy. Furthermore, migration
of the macrophages can be confirmed using flow cytometry or confocal microscopy.

Another potential method is to inject subcutaneously in vitro activated hyper-inflammatory macrophages and fluorescently labeled (i.e. GFP, Dil/DiR, pHrodo, etc.) around the site of NM exposure. Again, fluorescently labeled macrophages can be used to track the migration pattern influenced by our NM-skin contact model. Then, Maestro in vivo imaging and 2-photon microscopy can examine the fluorescence.

To approach our second question of whether the dermally activated macrophages in the bone marrow have the capability to cause the cells in the bone marrow to undergo apoptosis leading to bone marrow suppression, we performed in vitro co-culturing. Healthy bone marrow cells from a control mouse were co-cultured with bone marrow cells obtained from a NM-exposed mouse (Figure 6.2). These cells were co-cultured for four hours and were then analyzed for apoptosis by flow cytometry using propidium iodide (PI) in correlation with annexin V staining. Annexin V (Anx) fluorescently binds to phospholipid phosphatidylserine (PS) that is in the inner cytosolic side of the cell membrane in healthy cells and that is exposed in a cell undergoing apoptosis; whereas, PI binds to the nucleic acid of dead cells.
Figure 6.2: Schematic drawing of a Boyden chamber experiment where healthy bone marrow and bone marrow from mice exposed to topical NM exposure were co-cultured for 2 hours.

From co-culture preliminary data, the PI^+Anx^+ staining in normal bone marrow cells co-cultured with NM-exposed bone marrow cells increased compared to the control, healthy bone marrow cells (Table 6.1).

Table 6.1: Percent of PI^+/Anx^+ of healthy bone marrow cells co-cultured with NM-treated bone marrow (N=7).

<table>
<thead>
<tr>
<th>Combined (average)</th>
<th>P3 (PI+ANX+) (%)</th>
<th>P4 (PI+ANX+) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>media: fresh BM</td>
<td>2.64</td>
<td>5.90</td>
</tr>
<tr>
<td>fresh BM: fresh BM</td>
<td>2.44</td>
<td>6.80</td>
</tr>
<tr>
<td>mustard + NM BM: fresh BM</td>
<td>5.39</td>
<td>10.54</td>
</tr>
</tbody>
</table>

In a more applicable approach, the determination of NO levels will be useful in developing an antidote to counteract NM-induced toxicity. iNOS was analyzed by many techniques; however, a non-invasive, real-time read out of iNOS production has not yet been discovered. Current technologies include qRT-PCR, confocal microscopy, needle probe, and enzyme-linked immunosorbent assay (ELISA), in practice. While these current technologies are reliable, they are invasive and the iNOS levels cannot be determined quickly.
Infrared (IR) spectroscopy is an emerging technology that is sensitive to different types of bonds and generates a signature wavelength for each functional group or bond. Utilizing IR spectroscopy in our NM-skin contact model, we detected the NO (nitric oxide) bond and identified how much NO was produced. This method obtained a reliable, non-invasive read out in clinical trials to detect levels of NO in a wound.

We scanned a section of OCT-embedded skin section with IR spectra. Our bond of interest is NO with a signature peak at 1540 cm\(^{-1}\) (Table 6.2). Mouse skin treated with topical NM revealed an NO peak at 1540 cm\(^{-1}\). Mouse skin treated with a vehicle (PBS only) or 25(OH)D, however, had no NO peak detected (Figure 6.3). This finding is consistent with our previous results using other technologies: flow cytometry, confocal microscopy, and qRT-PCR.

**Table 6.2:** 1540 cm\(^{-1}\) is the absorption peak associated with nitro compounds.

<table>
<thead>
<tr>
<th>Bond</th>
<th>Type of bond</th>
<th>Specific type of bond</th>
<th>Absorption peak (cm(^{-1}))</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-O</td>
<td>nitro compounds</td>
<td>aliphatic</td>
<td>1540</td>
<td>stronger</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1380</td>
<td>weaker</td>
</tr>
</tbody>
</table>
Three skin sections of mice (vehicle, NM, and NM with 25(OH)D treatment) were scanned using IR spectra. The notable 1540 cm\(^{-1}\) peak was evident only in the NM treated skin.

To confirm if this NO peak is reliable, we used skin sections of iNOS knockout (KO) mice treated with a topical NM application. The NO peak should be non-existent since these mice have been genetically altered to prevent any iNOS expression; therefore, NO should not be expressed. IR spectra images of skin from iNOS-KO mice treated with NM, however, exposed peaks at 1540 cm\(^{-1}\) (Figure 6.4). Additional literature examination revealed that 1540 cm\(^{-1}\) has the same signature peak as proteins (Table 6.3).

Figure 6.3: Topical NM exposed skin reveals NO peak. Three skin sections of mice (vehicle, NM, and NM with 25(OH)D treatment) were scanned using IR spectra. The notable 1540 cm\(^{-1}\) peak was evident only in the NM treated skin.
Figure 6.4: *nos*−/− mice treated with topical NM reveal NO peak.

Table 6.3: 1540 cm−1 is the absorption peak that is associated with proteins.

<table>
<thead>
<tr>
<th>Proteins</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1650</td>
<td>Amide C=O stretch (amide I band)</td>
</tr>
<tr>
<td>1540</td>
<td>N-H bending (2° - amide) II band</td>
</tr>
<tr>
<td>1250</td>
<td>C-N stretching</td>
</tr>
<tr>
<td>1100</td>
<td>CH₂ rocking</td>
</tr>
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</table>

Another non-invasive and a more sensitive technology to detect changes in the skin is Raman spectroscopy. Previous studies used Raman spectroscopy with human skin *in vivo* to detect hydration of the skin. Additionally, raman spectroscopy had been used *in vitro* to detect live and dead cells. From these studies, raman spectroscopy had the potential to test skin treated with NM real-time.

Collectively, the observations and results presented in this dissertation demonstrate a novel macrophage migration pathway and a potential antidote to counteract NM-induced toxicity.
Chapter 7

Materials and Methods
**Mice**

Pathogen-free, 6-8 weeks old female C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, Maine). All animals received standard laboratory diet. All animal studies have been approved by Case Western Reserve Institutional Animal Care and Committee (IACUC).

**Nitrogen Mustard**

Nitrogen mustard \((\text{CICH}_2\text{CH}_2)\text{NCH}_3\times\text{HCl}\) was obtained from Sigma-Aldrich Chemical Company, Inc. (St. Louis, MO). In preparation for wounding, mice were anesthetized by intraperitoneal injection of avertin and the dorsal fur was removed with clippers and depilating cream. 24 hours later, nitrogen mustard (20uL of a 2.0% solution in 1X PBS [Hyclone; Logan, UT]) was applied by micropipette over an area approximately 0.8 cm in diameter on the dorsal of each mouse.

**Vitamin D**

25(OH)D (Sigma-Aldrich) was reconstituted in ethanol and then further diluted in mineral oil (Sigma-Aldrich) for use. 50ng of 25(OH)D was then injected intraperitoneal using a 27 gauge needle (Beckton Dickinson Bioscience; Franklin Lakes, NJ) one hour following nitrogen mustard exposure.
**1400W**

Hydrochloride (1400W) (Sigma-Aldrich) was reconstituted in ethanol and then further diluted in 1X PBS (Hyclone) prior to performing experiments. Mice were injected intraperitoneally using a 27 gauge needle (BD Bioscience) with 10 mg/kg of 1400W one hour following nitrogen mustard exposure.

**Measurements**

Wounds were measured length-wise and width-wise with a digital caliper every day following nitrogen mustard exposure until the wound healed. Weight was measured every other day using a Model CS 200 scale (Ohaus Corporation; Pine Brook, NJ).

**qPCR**

RNA was isolated using Trizol (Invitrogen; Carlsbad, CA) following the manufacturer's instructions. RNA, DNA and proteins were separated with chloroform (ACROS Organics; Pittsburgh, PA) and RNA was precipitated using isopropyl alcohol (Fisher Scientific; Pittsburgh, PA). RNA was then washed with 75% ethanol (Fisher Scientific), air-dried and resuspended in RNAse free water (Fisher Scientific). RNA quality and concentration was measured by Nanodrop (Wilmington, DE) and 100ng of RNA was used as a template to detect TNF-α, iNOS and 18s RNA expression using TaqMan Gene Expression Assays and the Taqman RNA-to-C_\text{T}_1-Step Kit (Applied Biosystems; Carlsbad, CA). Samples were analyzed using the Step-One System (Applied Biosystems) and cycle time,
temperature, and number were based on Applied Biosystems recommendations. Fold change in expression was calculated using the ΔΔCt method using 18s RNA as the normalization gene.

**Blood Smear**

A drop of blood was collected on a glass slide by tail snip. The drop of blood was smeared using a microscope slide (Fisher Scientific), fixed in 100% methanol (Fisher Scientific), and stained with Wright-Giemsa to observe cell types.

**Bone marrow staining**

Mice were sacrificed by cervical dislocation. Sternums were removed and fixed overnight in 10% formalin diluted in PBS (Fisher Scientific). Sternums were embedded in paraffin, sectioned (8um thick) and stained with haematoxylin and eosin.

**Complete blood count**

20uL of blood was obtained in an EDTA coated capillary tube by tail snip. Complete blood counts were determined using a HemaTrue machine (Heska; Loveland, CO) from the MPPC (Mouse Physiology Phenotyping Center) at Case Western Reserve University.
F4/80 and iNOS colocalization in skin

The designated 8 mm dorsal skin sections to which vehicle or nitrogen mustard was applied were excised, embedded in Optimum Cutting Temperature (OCT) media (Sakura; Torrance, CA), flash frozen in liquid nitrogen and stored at -80°C until use. Blocks were serially sectioned 8 µm thick and mounted 2 to a slide. Tissue sections were fixed for 10 min at RT in ice cold acetone (Fisher Scientific) and then rehydrated in 1X PBS (Hyclone) for 5 min at RT. Sections were then permeabilized with 0.3% saponin (Sigma-Aldrich) diluted in 1% BSA (Sigma-Aldrich) in 1X PBS for 30 min at RT. After permeabilization, the tissue sections were washed with PBS and then blocked with 10% goat serum (Invitrogen) for 1 h at RT. The tissue sections were washed 3x for 2 min each wash with 1X PBS to ensure removal of excess goat serum. On any given slide, one tissue section was incubated for 1 h at RT with 10 µg/mL Alexa Flour 488 conjugated anti-F4/80 antibody (eBioscience, San Diego, CA) diluted 1:100 in 10% goat serum and the other tissue section was incubated with 10 µg/mL Alexa Flour 488 conjugated rat IgG2a isotype (eBioscience) diluted in 10% goat serum. The tissue sections were then washed 3x for 5 min each wash with 1X PBS. The section incubated with anti-F4/80 was then incubated for 1 h at RT with 10 µg/mL rabbit anti-iNOS (Millipore; Billerica, MA) and the other tissue section was incubated with 10 µg/mL rabbit IgG (Invitrogen). Tissue sections were then washed 3x for 5 min each wash with 1X PBS and then incubated for 1 h at RT with an Alexa Fluor 647 conjugated goat anti-rabbit secondary Ab (Invitrogen) diluted 1:2000 in 1X PBS. Following incubation with the secondary Ab, tissue sections were washed 3x for
5 min each wash with 1X PBS. Sections were then mounted with fluoromount containing the nuclear counter stain DAPI (Abcam; Cambridge, MA) and imaged using a Ultra VIEW VoX spinning disk confocal system (PerkinElmer, Waltham, MA, USA) which is mounted on a Leica DMI6000B microscope (Leica Microsystems, Inc., Bannockburn, IL).

Flow Cytometry

Femurs were isolated by removing skin from the mouse leg exposing the muscle. The hind-leg was removed by cutting above the hip joint. The muscle was removed to expose the femur, the femur was cut below the hip joint and above the knee joint and bone marrow cells were isolated by flushing femurs with DMEM (Hyclone) using a 27 gauge needle (BD Bioscience) attached to a 5 mL syringe (BD Bioscience) and pelleted by centrifuging for 7 min at 1500 rpm. Cells were resuspended with flow buffer containing 5mM EDTA (Sigma-Aldrich) and 5% FBS (Gibco; Carlsbad, CA) in HBSS without phenol red (Hyclone), 100 μL aliquots were pipetted into polystyrene tubes (BD Biosciences) and cells were incubated with 4 μL of either Alexa Fluor 488 conjugated rat anti-F4/80 Ab (eBioscience) or Alexa Fluor 488 conjugated rat IgG2a isotype (eBioscience) for 15 min in the dark at RT. Cells were then washed with 500 μL flow buffer by centrifugation for 7 min at 1500 rpm and then resuspended with 300 μL flow buffer. Cells were analyzed with an Accuri C6 flow cytometer (BD Biosciences) and fluorescence was detected using the FL1 channel.
Bone Marrow H&E

Sternums were removed after the mice were sacrificed and fixed in 4% paraformaldehyde overnight. Sternums were then embedded in paraffin and sectioned 8 μm thick. Sections were then dehydrated in xylene for 5 min, stained with hematoxylin for 5 min followed by eosin for 2 min and then washed with distilled water. Sections were mounted with paramount. The 8mm dorsal area was excised, fixed in 10% formalin in 1X PBS (Leica Microsystems; Richmond, IL) overnight, embedded in paraffin and sectioned longitudinally 8μM thick. Sections were mounted to a microscope slide and stained with hematoxylin and eosin.

Liposomes

Liposomes comprised of phosphatidyl choline and cholesterol were suspended in 1X PBS (pH 7.4) were obtained clodronateliposomes.org (Amsterdam, Netherlands). Prior to injection, liposomes were allowed to reach RT. Using a 30 gauge needle (BD Bioscience), mice received four 50 μL subcutaneous injections of either PBS loaded or clodronate loaded liposomes 1 h after nitrogen mustard application around the designated 8 mm dorsal region to which nitrogen mustard was applied. To label liposomes with the dialkylcarbocyanine dye (Dil) (Invitrogen), 10 μL Dil was added for each 1 mL of liposome and incubated in the dark for 15 min at RT. Liposomes were then centrifuged in 4°C for 10 min at 20,000 g. Supernatants were aspirated, liposomes resuspended in the original volume of PBS and centrifuged for 10 min at 20,000xg in 4°C. Supernatants
were aspirated and Dil labeled liposomes were resuspended in the original volume of PBS for use.

**Nitrogen Mustard applications on pigs**

Pigs were anesthetized for at least one hour by first administering 6-8 mg/kg Telazol intra-muscularly followed by inhalation of 1-5% isoflourane. Once pigs are sedated, hair on the mid-line of the dorsal region between the center-line and shoulder blades was removed using electric clippers and 26 mg/kg of NM diluted in PBS or PBS alone was applied to a 6 cm region on the mid-line of the dorsal region between the center-line and shoulder blades. Once the 6 cm regions were dry, a tegaderm patch was placed over the 6 cm region and wrapped with gauze.

**Maestro Imaging**

Mice were placed on a 2018S Teklad Global 18% protein rodent diet for 10 days. Nitrogen mustard was applied topically and an hour following application, 200uL of pHrodo conjugated with *E.coli* was injected around the area of exposure. Five days following nitrogen mustard exposure, mice were sacrificed and organs were harvested to be analyzed using Maestro (Perkin Elmer).

**FMT Imaging**

Mice were placed on a 2018S Teklad Global 18% protein rodent diet for 10 days. Nitrogen mustard was applied topically and an hour following application, 200uL
of DiR encapsulated liposomes was injected around the area of exposure. Mice were analyzed on the FMT on days 1, 2, and 5.

**Biopsy (pigs)**

Forty-eight hours following NM application, pigs were anesthetized by administering 6-8 mg/kg Telazol intra-muscularly. Once sedated, an 8mm biopsy was taken from the center of two 12cm² region. Following this procedure, the two 12cm² region was stitched and covered with a new tegaderm patch wrapped with gauze.

**Infrared Spectroscopy**

The designated 8 mm dorsal skin sections to which vehicle or nitrogen mustard was applied were excised, embedded in Optimum Cutting Temperature (OCT) media (Sakura; Torrance, CA), flash frozen in liquid nitrogen and stored at -80°C until use. Blocks were serially sectioned 8 µm thick and mounted 2 to a gold-plated slide. Before reading the sections on the IR spectroscopy, gold slides were thawed and air-dried.

**Statistical Analysis**

2 sided unpaired student T-tests were used to determine all statistical significance. Data are shown as mean ± s.e.m, and p-values ≤ 0.05 were considered statistically significant.
Chapter 8

References


