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Doxorubicin-Induced Cardiotoxicity Is Mediated By Neutrophils Through Release Of Neutrophil Elastase

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DOXORUBICIN-INDUCED CARDIOTOXICITY IS MEDIATED BY NEUTROPHILS THROUGH RELEASE OF NEUTROPHIL ELASTASE

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DOXORUBICIN-INDUCED CARDIOTOXICITY IS MEDIATED BY NEUTROPHILS THROUGH RELEASE OF NEUTROPHIL ELASTASE

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Doxorubicin (Dox) is one of the most effective chemotherapy agents that is used for the treatment of childhood cancer. Unfortunately Dox treatment can cause damage to the heart. Indeed, childhood cancer survivors are at a higher risk of developing a cardiovascular disease at an earlier age. The mechanisms by which Dox causes acute and late cardiotoxicity are not completely understood. One understudied area in Dox-induced cardiotoxicity is the contribution of inflammation and innate immune cells, in particular neutrophils. Recognizing that neutrophils have been implicated in a number of heart diseases, we evaluated the role of neutrophils in Dox-induced cardiotoxicity. Here, using echocardiography, flow cytometry and immunofluorescence staining, we demonstrated increased infiltration of neutrophils that correlated with decreased heart function (as defined by a decrease in ejection fraction and fractional shortening), disruption of vascular structures and increased collagen deposition in the heart after Dox treatment. Depleting neutrophils protected the heart from Dox-induced cardiotoxicity and changes in vascular structure both in the acute (24 hours after therapy) and late stages (12 weeks after therapy). Furthermore, our data using neutrophil elastase (NE) knock-out mice and the NE inhibitor AZD9668 suggest that neutrophils induce cardiac damage by releasing NE and that inhibiting NE can prevent both acute and late Dox-induced cardiotoxicity. Taken together, this data indicates the contribution of neutrophils and NE in Doxorubicin-induced cardiotoxicity. This is the first demonstration of the importance of neutrophils and NE in heart damage caused by Dox and suggests a potential new therapeutic intervention.

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Chapter One: Introduction

This chapter is based upon “Bhagat A, Kleinerman ES. Anthracycline-Induced Cardiotoxicity: Causes, Mechanisms, and Prevention. Adv Exp Med Biol. 020;1257:181-192.”

Introduction to Anthracyclines

Anthracyclines are among the most effective chemotherapy agents used to treat pediatric, adolescent, and adult patients with osteosarcoma [1]. In addition to osteosarcoma and other sarcomas, anthracycline-containing regimens are used to treat lymphoma, leukemia, and breast cancer, with 50–60% of childhood cancer patients receiving an anthracycline-containing regimen during treatment [1]. Some of the commonly used anthracyclines are doxorubicin, mitoxantrone, epirubicin, idarubicin, and daunorubicin. The structure of an anthracycline is composed of a tetracyclic ring that is attached to a sugar. There are also quinone and hydroquinone moieties present on adjacent rings, which permit the gain and loss of electrons.

Doxorubicin (Dox) is one of the more widely used chemotherapy agents in the anthracycline family with respect to osteosarcoma. It, along with daunorubicin, was the first to be used in clinical practice. Daunorubicin was identified first from the bacterium Streptomyces peucetius. Dox, a derivative of daunorubicin, was identified in the 1960s and found to be a more effective antitumor agent. With regard to osteosarcoma, there is a correlation with dose intensity and patient survival. However, there is also a correlation between the dose of Dox and late comorbidities which results in compromised quality of life[1]. One of the most frequently seen late effects in

survivors of childhood cancer is cardiac disease. Sarcoma survivors are at a significantly higher risk of being diagnosed with some form of cardiovascular event compared to community controls. This is secondary to the high dose of Dox used to treat these patients (450 mg/m^2 vs 300 mg/m^2 for breast cancer patients) [1]. These include vascular disease, cardiac dysfunction, myocardial infarction, arrhythmias, dyslipidemia and essential hypertension. It is unclear whether the mechanism(s) responsible for anthracycline-induced cardiotoxicity are the same as those that are responsible for killing tumor cells.

In this chapter, we will review the identified mechanisms of action of anthracyclines, and Dox in particular, since it is one of the major chemotherapy agents used in the treatment of osteosarcoma. We will also summarize the cardiomyopathies that have been documented in survivors treated with Dox and what has been described in terms of monitoring patients for heart disease. This will include investigations looking for early biomarkers to identify patients and survivors at risk. Finally, we will summarize interventions aimed at decreasing anthracycline-induced cardiotoxicity.

Mechanism of Antitumor Action

There are three proposed mechanisms by which Dox acts as an antitumor agent [2]. These include (i) intercalation into DNA strands, (ii) DNA damage by topoisomerase suppression, and (iii) generation of free radical species and the subsequent cellular damage including lipid peroxidation of cell membranes.

Intercalation into DNA strands

Dox has the capacity to inhibit DNA biosynthesis by DNA intercalation and independent of inhibition of DNA polymerase activity. Dox intercalates into sites containing adjacent GC base pairs. Additionally, the drug has an affinity for these sites mainly due to hydrogen bond formation between Dox and guanine. This leads to the formation of Dox-DNA adducts that can activate DNA damage responses and induce cell death. This discovery has led to a few different approaches to increase the antitumor efficacy of Dox. One such approach has been to combine with compounds that release formaldehyde upon hydrolysis including pivaloyloxymethyl butyrate (AN-9), butyroyloxymethyl-diethyl phosphate (AN-7), and hexamethylenetetramine (HMTA) [3]. In some instances, this combination was effective in increasing the tumor-killing capacity of Dox. This is mediated by the stabilization of the covalent bond between Dox and DNA by formaldehyde, resulting in an increase in the formation of DNA adduct levels. However, contradictory data makes it difficult to establish the DNA adduct formation as the major mechanism of Dox-mediated tumor cell killing [3].

DNA damage by topoisomerase suppression

DNA topoisomerases are enzymes that play essential roles in the unwinding and rewinding of the DNA helix strands during replication, transcription, and recombination. The need for topoisomerase arises due to the double-helical nature of the DNA strand. In order to access information stored in DNA, the two strands of the helix must be separated temporarily. There are two main types of topoisomerase depending on whether there is a single- or double-stranded break in the DNA helix. Type I isomerase causes a single-stranded break, while Type II cuts both strands of

the DNA helix [4]. For the purposes of this discussion, Type II topoisomerase, which consists of Top2α and Top2β, will be discussed. In malignant cells that are proliferating rapidly, Top2α is more highly expressed. Top2α works in an adenosine-dependent fashion by recognizing a DNA substrate (G segment) and causing a double-stranded break which opens one of the DNA duplex. This helps facilitate the capture and entry of a second DNA piece termed the T segment through the opening in the G segment. This helps unwind the DNA strand and is followed by re-ligation of the double-stranded break in the G segment. Failure in this process results in DNA lesions that lead to mitotic and apoptotic cell death [5].

Dox targets Top2α which is highly expressed in osteosarcoma cells [6]. Dox intercalates into DNA and prevents Top2 from binding with DNA. This leads to the suppression of the Top2-DNA cleavage complex formation with subsequent transcriptional arrest, which then leads to DNA damage and ultimately cell death.

Generation of free radical species

Anthracylines including Dox induce the generation of free radicals [7]. Reactive oxygen species and reactive nitrogen species are collectively termed free radicals, molecules with one or more unpaired electrons in their outer shells. Free radicals include hydroxyl (OH•), superoxide (O2•–), nitric oxide (NO•), nitrogen dioxide (NO2•), peroxyl (ROO•), and lipid peroxyl (LOO•) [8]. Under homeostatic conditions, these free radicals act as an integral part of the host defense system and aid in maturation of cellular structures. However, when produced in excess, these free radicals give rise to oxidative stress due to an imbalance between formation and the elimination of the free radical species. This can ultimately lead to damage to cell membranes by a

process called lipid peroxidation [9,10]. Dox has a quinone structure which allows for the drug to act as an electron acceptor which is mediated by other oxoreductive enzymes such as cytochrome P450 reductase, NADH dehydrogenase, and xanthine oxidase. This addition of the free electron facilitates the conversion of the quinone to a semi-quinone free radical. Once these free radicals are generated, they then induce free-radical injury to DNA. Unlike the DNA damage associated with inhibition of topoisomerase II, this damage is not associated with proteins[11]. This DNA damage can be prevented by superoxide dismutase, catalase, and glutathione peroxidase. For this reason, it has been observed that free radicals generated by Dox toxicity result in the alterations of glutathione levels which in turn have an impact on cell sensitivity to Dox [11]. However, studies conducted have reported mixed findings as to whether free radical generation is one of the main mechanisms by which Dox can cause damage to tumor cells.

Cardiotoxicity

Cardiovascular disease and heart failure are the most common late effects which compromise quality of life and the long-term survival for sarcoma survivors [11]. Dox-induced cardiotoxicity can be debilitating and an often-deadly consequence of successful tumor treatment. The acute damage of the juvenile heart caused by Dox makes the adult heart more vulnerable to stresses over time, putting the heart at risk for ischemic damage, and predisposing to late-onset cardiomyopathies at a much earlier age. Thus, a minor ischemic event that would cause minimal or no damage in a healthy individual would result in more significant damage in a heart previously damaged by Dox. Cardiotoxicity is defined as the inability of the heart to pump blood

through the body effectively. In 2014, there were 14.5 million cancer survivors [1]. This number is expected to increase to 18 million by 2020, indicating that this late effect will be seen in increasing numbers. One risk factor associated with the Dox-induced cardiotoxicity is age of the patients, with children under 4 years and adults over 65 years of age being at a higher risk of developing cardiotoxicity. Furthermore, Lipshultz SE et al. have also reported that females had more severe cardiotoxicity with more compromised contractility [1]. It is estimated that 60% of pediatric patients receive an anthracycline-containing treatment regimen and 10% of these are expected to develop symptomatic cardiomyopathy up to 15 years after completing therapy [1]. Another study reported the incidence of subclinical and overt cardiotoxicity to be 17.9% and 6.3%, respectively, in cancer patients treated with anthracyclines after 9 years of follow-up [12]. Thus, cardiotoxicity can develop after a significant amount of time has passed after completion of treatment indicating that long-term cardiac monitoring of survivors is essential. One factor that contributes to the development of the cardiotoxicity is the dose of Dox used in the cancer treatment. In patients who received more than 500 mg/m2 of anthracyclines, a 63% prevalence of left ventricular dysfunction after 10 years of follow-up was reported, in contrast to an 18% prevalence in those who received less than 500 mg/m2 [13]. Another study reported that in patients who received a cumulative dose of 400 mg/m2, there was a 5% risk of developing heart failure which increased to 25% at 700 mg/m2 [2]. This is indicative of the fact that the risk of cardiotoxicity correlates with increased drug dose. However, another separate study looking at the histopathological changes in endomyocardial biopsy specimens from patients concluded that there is no particularly safe dose of Dox [2]. Unfortunately, the

exact mechanism of Dox-induced cardiotoxicity is unknown but there are several proposed molecular mechanisms.

Definition and detection of cardiotoxicity

Cardiotoxicity is defined by a number of different parameters. These include (1) reduction of left ventricular ejection fraction (LVEF), either global or specific in the interventricular septum; (2) symptoms or signs associated with heart failure (HF); (3) reduction in LVEF from baseline to lesser 55% in the presence of signs or symptoms of HF; or (4) a reduction in LVEF greater than or equal to 10% or an LVEF lesser than 55% without signs or symptoms of HF [14]. Left ventricular ejection fraction is defined as the central measure of left ventricular systolic function and is the fraction of volume ejected during the contraction phase(systole) of blood circulation in relation to the volume of blood in the ventricle at the end of the dilution phase (diastole). Normal LVEF for males is 52–72%, while for females, it is 54–74%. Less than 52% LVEF is considered abnormal and suggests compromised heart function [15].

There are several different methods to detect anthracycline-induced cardiotoxicity. One of the most successful methods is an endomyocardial biopsy. However, this technique has several limitations: first, it is invasive and second, with regard to the quality of the sample and whether the sample obtained by the biopsy contains damaged myocardium. It is for these reasons that noninvasive methods are preferred. One of the most widely used methods in this regard is echocardiography. Using this technique, the left ventricular ejection fraction, which is an indicator of cardiac systolic function, can be quantified. A study conducted on 1664 patients who were treated with anthracyclines as part of their breast cancer treatment showed that

the absolute value of pretreatment LVEF was indicative of a later occurrence of heart failure. Despite its effectiveness in adult patients, there have seen some discrepancies regarding its predictive value in children treated with anthracyclines [16].

Some of the disadvantages of standard 2D echocardiography are the following: (i) the quality of images obtained determines the accuracy of the LVEF measurement, (ii) ventricular foreshortening can contribute toward lack of accuracy in measured LVEF, and (iii) use of mathematical models and geometrical assumptions to calculate LV volumes [16]. As a result, there have been further enhancements made to the echocardiography technique including use of contrast agents to help highlight the endocardial border and improve tracing of the end-systolic and end-diastolic volumes. Additionally, 3D echocardiography has helped with reducing analysis time and interobserver variability [16].

Monitoring Cardiotoxicity and Heart Function

Biomarkers to identify acute cardiotoxicity and predict late cardiac effects: Early detection of at-risk patients for cardiotoxicity combined with early intervention could help decrease the occurrence of heart failure in survivors. To address this need, many studies have been conducted to try to determine serum and plasma biomarkers that are relevant to cardiotoxicity and predicting which patients are at risk for developing cardiotoxicity and heart failure [17]. Unfortunately, such suitable biomarkers have yet to be identified that both document acute heart damage and correlate with the development of heart failure. However, what is established is that elevated levels of

troponin and natriuretic peptides are two biomarkers that are associated with acute coronary syndrome and heart failure, respectively [17]. We will now discuss these with regard to identifying anthracycline-induced cardiotoxicity, monitoring for heart failure, and/or predicting patients at risk for late cardiotoxicity.

Cardiac troponins

Troponins are proteins that are found in skeletal, cardiac, and smooth muscles [17,18]. Troponin T (TnT) and troponin I (TnI) are both exclusively found within cardiac myocytes. Upon damage to cardiomyocytes, troponins T and I are released into circulation. Elevated troponin levels indicate cardiac damage and LV dysfunction [17,18]. Investigations looking at cardiac troponins with relevance to cardiotoxicity have concentrated on early onset heart damage. What has been observed is that early troponin elevation preceded changes in LVEF. In a study of 703 patients with breast cancer and lymphoma, it was seen that TnI elevation measured within 72 h as well as 1 month after chemotherapy administration had significantly greater risk of developing cardiotoxicity over a mean follow-up of 20 months [17]. Another study looked at changes in TnI and LVEF after cycles of high-dose anthracycline-containing chemotherapy in 204 patients. It was found that patients who had elevated TnI had significant reduction in their LVEF 7 months after completion of treatment [18]. From this standpoint, it suggests that looking at troponin levels at an acute stage of cardiotoxicity may be useful. However, other studies conducted found no correlation between troponin level and the development of late heart failure due to the use of anthracycline. One such study looked at 150 childhood and 53 adult cancer survivors with hematologic malignancies and breast cancer, respectively [19]. It was found that there was no

detectable elevation of TnT or TnI after a 2-year and 1-year follow-up confirming that there is a lack of correlation between troponin and LV dysfunction, particularly in childhood cancer survivors.

Natriuretic peptides

These are peptide hormones that include atrial natriuretic peptides (ANP) and brain natriuretic peptides (BNP). These peptides promote natriuresis and help with the protection of the heart from mechanical stress and volume overload. Published literature illustrated an early association between anthracycline-induced cardiotoxicity and brain natriuretic peptides. In one study, 71 breast cancer patients received 6 cycles of liposome-encapsulated Dox (40–50 mg/m2), docetaxel (50 mg/m2), and epirubicin (90 mg/m2) in combination with fluorouracil and cyclophosphamide. Upon completion of the treatment cycle, it was observed that BNP levels were elevated and that the elevation 24 h after treatment was associated with reduction in LVEF. However, other studies failed to show a link between elevated natriuretic peptides and late-onset cardiotoxicity. The reason for this discrepancy could be that elderly individuals and females have higher than normal natriuretic peptide levels. Additionally, compromised renal function can increase natriuretic peptide levels. Finally, cancer itself may increase BNP levels through inflammation. Indeed, patients with metastatic disease were found to have higher levels of BNP than those without metastasis[19].

Myeloperoxidase

Another potential biomarker is myeloperoxidase, a pro-inflammatory enzyme that is expressed by neutrophils. Myeloperoxidase is an indicator of oxidative stress and is induced following damage to the myocardium by reactive oxygen species generation and is part of the inflammatory response. In one study conducted on 78 breast cancer patients treated with Dox as part of their chemotherapy regimen, patients with increased MPO levels along with elevations in TnI over a 15-month time period had a 46.5% increased risk for developing cardiotoxicity [19]. However, further studies are needed to confirm this finding.

MicroRNAs

Several studies have also explored microRNAs as viable biomarkers for anthracycline-induced cardiotoxicity. These are small non-coding RNA molecules that aid in regulation of gene expression. Some of the common cardiac miRNAs under investigation include miR-1, miR-133, miR-208, and miR-499. In myocardial infarction models in both humans and animals, miR-208 and miR-499 are found to be elevated. These two microRNAs are also specifically found in cardiac myocytes. In a study of 33 children with anthracycline exposure, it was observed that there was elevation in plasma levels of miR-29b and miR-499, and this correlated with rise in troponin levels and increase in dose of anthracyclines. In a separate study of breast cancer patients who were treated with Dox, there was an increase in miR-1 that was associated with decline in LVEF. The monitoring of miRNAs therefore may provide a potential new biomarker for assessing and monitoring early- and late-onset cardiotoxicity, as

microRNAs are present in all body fluids, have a long half-life, and are relatively stable under extreme temperatures and pH [17].

Mechanisms of Anthracycline-Induced Cardiotoxicity

The exact mechanism(s) by which Dox induces cardiotoxicity is poorly understood. This section summarizes several of the proposed mechanisms.

Oxidative stress

The most frequently proposed mechanism for Dox-induced cardiotoxicity is the generation of reactive oxygen species followed by lipid peroxidation [20] . Some of the common reactive oxygen species generated include superoxide(O_2^-), hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2). A low level of oxidant species is necessary for normal transduction process; however, if these levels exceed the threshold level, this can be damaging to cells. The myocardium is especially vulnerable as there are lower levels of the antioxidant enzymes peroxidase, catalase, and superoxide dismutase present in the heart. Peroxidase helps catalyze oxidation of substrates and uses hydrogen peroxide as the electron acceptor. This helps with elimination of the toxicity of hydrogen peroxide and oxidizes phenols, amines, and hydrocarbon oxidation products. Catalase helps with the decomposition of H_2O_2 and removal of H_2O_2, thereby protecting cells from H_2O_2 poisoning. Due to a lack of these enzymes, there is an accumulation of H_2O_2 which leads to damage [20].

One of the major subcellular targets of Dox is the mitochondria [21]. The number of mitochondria in cardiomyocytes is increased by 35–40%. In the mitochondria, ROS-producing enzymes transform the quinone moiety present in Dox to a semi-quinone

through a one electron reduction. Semiquinones can then be converted to a superoxide anion by reaction with oxygen. These superoxide anions can then be transferred to ROS or reactive nitrogen species via the redox cycle. However, high levels of these superoxide anions can produce highly reactive and toxic hydroxyl radicals during a reaction catalyzed by iron called the Fenton reaction [21].

As mentioned earlier, a lack of antioxidant enzymes in the myocardium means the heart is more vulnerable to oxidative stress. Furthermore, with a higher number of mitochondria in the cardiomyocytes, this leads to a higher production of ROS once Dox binds to the mitochondria and initiates the production of superoxide anions which in turn can produce toxic hydroxyl radicals and in turn higher oxidative stress.

Cardiolipin, a phospholipid, is a component within inner mitochondrial membrane which Dox has a high affinity for [22]. As a result, an irreversible complex is formed between Dox and cardiolipin that accumulates on the inner mitochondrial membrane. This complex can then lead to mitochondrial dysfunction through oxidation of enzymes that are catalyzed by cardiolipin. This creates a disturbance of the electron transport chain in mitochondria as proteins, including cytochrome C oxidase, cytochrome C reductase, and NADH dehydrogenase, are oxidized which subsequently leads to energy reduction and apoptosis of cells as 90% of the ATP utilized by cardiomyocytes is produced by the mitochondria [21].

One other proposed mechanism of oxidative stress is the “ROS and Iron” hypothesis[23]. As mentioned earlier, iron catalyzes the Fenton reaction that is needed to convert the superoxide anions to hydroxyl radicals. Dox can form a complex with iron which can potentially lead to excessive ROS production causing apoptosis of

cardiac cells. This accumulation of iron in mitochondria is facilitated by members of the ABC protein family particularly ABC protein B8(ABC8). ABC8 has a role in mitochondrial iron homeostasis and helps with facilitating mitochondrial iron export. One study conducted showed that Dox downregulated the ABC8 protein and mRNA levels which in turn affected the export of iron from the mitochondria leading to excessive accumulation of iron in the mitochondria which is toxic to the cells. By contrast, levels of another protein, mitoferrin 2, were found unchanged. Mitoferrin 2 is involved in mitochondrial import of iron. The alteration of iron export while maintaining levels of iron import leads to excessive iron and to a disturbed state of iron homeostasis in mitochondria [23]. Another mechanism through which iron overload is caused is through the interaction of Dox with iron-transporting and iron-binding proteins including IRP (iron regulatory protein). As mentioned earlier, Dox can form a complex with iron, and this complex reduces the amount of free iron [24]. This free iron is then unable to bind to IRP. This leads to inactive IRPs which then bind with iron-responsive elements (IREs) which in turn leads to a disruption of proteins related to iron metabolism. This disruption leads to a decrease in synthesis of ferritin and upregulation of transferrin receptor (TfR) that leads to increase in iron levels which disturbs the iron homeostasis [24].

Nicotinamide adenosine dinucleotide phosphate is another enzyme that helps with generation of free radicals by the redox cycle [20]. These are a group of plasma membrane-associated enzymes that serve as a source of ROS. Similar to what has been discussed earlier, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) helps with conversion of quinone moiety to a semi-quinone radical

that can react with oxygen to generate hydrogen peroxide. NOXs can be activated by many stimuli including TNF-a and are pivotal in cardiac remodeling [20]. Furthermore, Nox 2 was found to be the main mediator of NOX-derived ROS.

Nitric oxide is also a major source of Dox-induced oxidative stress. In normal homeostatic conditions, nitric oxide is a vasodilator that mediates heart contractions. An elevated level of NO is observed in Dox-induced cardiotoxicity due to isoforms of NOS, namely, endothelial NOS, inducible NOS, and neuronal NOS [25]. Dox captures electrons from NADPH by directly binding the reductase domain of eNOS. This helps with superoxide formation. The role of eNOS was confirmed in a study conducted on eNOS knockout mice that displayed low levels of ROS and preserved myocardial function after exposure to Dox. Basal production of NO is needed to modulate cardiomyocyte contractility and blood flow distribution [25]. However, higher levels of NO production via INOS are associated with dilated cardiomyopathy and congestive heart failure[7].

There are multiple sources of oxidative stress in the myocardium. Reactive oxygen species that generates the oxidative stress overwhelms cardiomyocyte enzymatic defenses and alters gene expression through interaction with regulatory proteins. ROS also can affect function of G proteins via lipid peroxidation. Despite this overwhelming connection between oxidative stress and Dox-induced cardiotoxicity, the experimental evidence that treating this oxidative stress will reduce Dox-induced cardiotoxicity has not been conclusive.

Cardiomyocyte apoptosis

Oxidative stress can activate apoptotic signaling that leads to cardiomyocyte apoptosis. In this scenario, both extrinsic and intrinsic pathways are involved. However, apoptosis can be induced via an oxidative stress-independent manner. The B-cell lymphoma 2 (Bcl 2)-Bcl-2-like protein 4 (bax) ratio is important in apoptosis and involves heat shock proteins [25]. These proteins act as molecular chaperones and stabilize other proteins involved in anti-apoptotic signaling by preventing dephosphorylation, ubiquitination, and degradation. The heat shock proteins that are important in having a role in the cardiac microenvironment include heat shock protein 27 (Hsp27), heat shock protein 10 (Hsp10), and heat shock protein 60 (Hsp60). In a study conducted by Liu et al., they found that overexpression of Hsp27 prevented Dox-induced apoptosis and myocardial dysfunction [26]. In another study, overexpression of Hsp10 and Hsp60 was found to shift toward an anti-apoptotic pathway due to increased posttranslational modification of Bcl-2 protein[26]. Hsp20 aids in maintenance of Akt phosphorylation which is one of the main cell survival pathways. Heat shock proteins also act as ligands for Toll-like receptors (TLR) after being secreted into the bloodstream [26]. In addition to its effect on modification of Bcl-2, Hsp60 interacts with TLR-2, while Hsp70 interacts with TLR-4 [20]. The exact role of Toll-like receptors in Dox-induced cardiotoxicity is not well-defined as yet; however, studies conducted indicate that apoptosis is initiated by TLR-2- and TLR-4-mediated signaling through pro-inflammatory NFκB post Dox treatment [26].

Dox also influences caspase activity. In particular, caspase-3 activation is associated with Dox administration in vivo. In one study, suppression of caspase

activity in cardiomyocytes was achieved through the administration of NO donor S-nitrosyl-N-acetyl-penicillamine. In another study, blocking of volume-sensitive chloride channels prevented Dox-induced caspase-3-dependent apoptosis [27]. It has also been observed that Dox elevated the expression of death receptors such as tumor necrosis factor receptor 1 (TNFR1), fas cell surface (Fas) death receptor, DR4, and DR5 in cardiomyocyte. This elevated expression leads to activation of caspase cascade [20].

Calcium dysregulation

Control of calcium levels is important in cardiomyocytes as calcium aids in regulating contractile activity[26]. Intracellular calcium levels are increased in Dox-induced cardiotoxicity. The ROS and H2O2 that are generated can alter normal calcium homeostasis in several different muscle types including the heart by disrupting normal sarcoplasmic reticulum. By inhibiting the Ca2+ ATPase pump and reducing the expression levels of SERCA2a MRNA, the free radicals can impair Ca2+ metabolism [20]. Dox can also induce the release of calcium from sarcoplasmic reticulum by promoting the opening of the calcium channels. Dox has also been shown to inhibit the sodium-calcium exchanger channel in the sarcolemma[26].

Caspase-12 activates apoptotic pathways, and its activation is dependent on calpain dysregulation which sends out signals of distress from sarcoplasmic reticulum [26]. Calpains are proteases that are activated by calcium. Much of the intracellular calcium in cardiomyocytes is present in the sarcoplasmic reticulum. Oxidative stress can cause calcium leakage, calpain activation, and caspase-12 cleavage. Calpains have been found to degrade titin which is a large protein and a key component of cardiac sarcomere which helps in maintaining cardiac contractility [26]. Hence,

prevention of calpain activity helps maintain contractility after Dox exposure [20]. Dox has also been found to enhance the sensitivity of mitochondria to intracellular calcium [26]. In a study conducted in rats, it was found that mitochondria of cells in Dox-treated heart had a decreased ability to retain calcium [26].

Another possible mechanism by which Dox can affect calcium intracellular concentrations is through regulation of its metabolism. Dox can generate a toxic metabolite, namely, DOXol, which inhibits the sodium-calcium exchanger channel [28]. DOXol can also disrupt the sodium gradient that is needed for calcium to flow into sarcolemma of a cardiomyocyte. This leads to an imbalance in energetics of the myocardium and diminished systolic function. DOXol accumulation can thus contribute significantly to dysregulation of calcium homeostasis leading to myocardial damage [29].

Immune system

Studies have shown that elevated levels of IL-1β, IL-6, and TNFα, all pro-inflammatory markers, are elevated following Dox therapy [30]. As discussed above, Dox can activate the NFκB pathway which enhances inflammatory mediators as mentioned above along with vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP2) [30]. Phosphorylation of IκB is mediated by Iκκ and is an important step in NF-Kb activation. In one study, it was observed that there was a significant increase in Iκκα expression in heart following Dox treatment and that this increase was observed after only 24 h of a single dose [30]. This indicates that Dox treatment can induce a quick activation of transcription factors.

Toll-like receptors (TLRs) have also been shown to be involved in Dox-induced cardiotoxicity [26]. Toll-like receptors are part of a family of pattern recognition receptors that act to detect danger signals including pathogens, oxidative stress among many. The role of TLRs in cardiovascular diseases will be described in greater detail in chapter 2.

Prevention of Dox-Induced Cardiotoxicity

Dexrazoxane

Dexrazoxane is the only FDA-approved cardioprotective agent for anthracycline-induced cardiotoxicity[31]. Dexrazoxane is an adjunctive agent that can act as a free radical scavenger. It is converted into a ring-opening chelating agent and can replace iron in the Dox Fe3+ complex and combine with iron. In this manner, dexrazoxane interferes with iron ion-mediated free radical production, weakening the cardiotoxic immune effector cells and blocking the inactivation of respiratory enzymes by iron complexes. Additionally, dexrazoxane can chelate iron and prevent ROS through non-enzymatic mechanism of Dox.

Many studies have been done to determine the efficacy of dexrazoxane in preventing Dox-induced cardiotoxicity. In a group of 200 children with acute lymphoblastic leukemia (ALL) who had received a cumulative Dox dose of 300 mg/m2, blinded troponin T measurements were taken at different time points before, during, and after Dox infusion [11]. These children were randomized to receive Dox plus dexrazoxane or Dox alone. At the end of treatment, it was found that 20% of children who had been given both Dox and dexrazoxane had elevated TnT levels as opposed

to 47% who only received Dox [11]. Furthermore, in a 5-year follow-up, left ventricular fractional shortening was found to be lower in children who were treated with Dox alone as opposed to those who were treated with both Dox and dexrazoxane. In another study conducted by Cheng et al. in BALB/c mice with and without tumors, it was found that mice treated with Dox plus dexrazoxane showed normal heart tissues morphologically with no characteristic inflammation or tissue injury [11].

However, a major concern with regard to use of dexrazoxane as a cardioprotectant is the potential risk of secondary malignancy [11]. It is for this reason that currently the Food and Drugs Administration (FDA) has approved the use of dexrazoxane only in women with metastatic breast cancer who received cumulative doses of 300 mg/m2 [32]. There have been studies conducted however to disprove these findings. In one large study, in pediatric patients with acute lymphoblastic leukemia, it was observed that for children who received dexrazoxane, the occurrence of a secondary malignancy was a rare event. In the study conducted for 553 pediatric patients with ALL, only 1 developed acute myeloid leukemia in a median follow-up period of 5 years. In another group, there was no significant difference reported in incidence of secondary malignancy in pediatric ALL patients (n = 173) who received dexrazoxane as compared to placebo (n = 150) [6]. This seems to suggest that the risk for developing secondary malignancy using dexrazoxane is low, but the FDA recommendation is that this cardioprotectant not be used for pediatric patients until further studies are done.

ACE inhibitors and beta-blockers

Angiotensin-converting enzymes (ACE) are a mainstay in the treatment of heart failure [20]. Angiotensin is a peptide hormone that is involved in regulating blood pressure. Angiotensin-converting enzyme is part of the renin-angiotensin system that controls blood pressure by converting the angiotensin I to an active vasoconstrictor angiotensin II. Therefore, ACE can cause an increase in blood pressure by causing vessels to constrict. Hence ACE inhibitors, including enalapril, zofenopril, and lisinopril, have been used to treat heart failure [20]. These drugs can act as an antioxidant as it was observed that administration of enalapril helped attenuate Dox-induced cardiac dysfunction by preserving mitochondrial respiratory efficiency and reducing free radical generation. However, for long-term use, the effectiveness of these drugs in childhood cancer survivors diminishes after 6–10 years [20]. Furthermore, ACE inhibitors could also have adverse side effects, and hence use of these agents for cardioprotection needs to be monitored.

Beta-blockers, including carvedilol, have been shown to preserve left ventricular function after Dox treatment in patients as compared to placebo [26]. Beta-blockers work by blocking adrenaline and reducing blood pressure. Additionally, early addition of β-blockers and angiotensin-converting enzymes has been shown to improve myocardial contractility in Dox-induced cardiotoxicity [26]. However, Georgakopoulos et al. demonstrated that metoprolol, a β-blocker without antioxidative properties, was not able to provide cardioprotection in lymphoma patients treated with Dox [11].

Other cardioprotectants

Antioxidants including resveratrol have been used in acute Dox treatment to significantly decrease ROS generation which in turn improved glutathione, superoxide dismutase, and catalase activity [26]. This helps with improving cardiac function. Erythropoietin (EPO) is a cytokine that stimulated the production of red blood cells in the bone marrow. EPO can act as a cardioprotective agent against Dox-induced apoptosis[26]. Another drug sildenafil, a phosphodiesterase 5 inhibitor, has been used to attenuate cardiomyocyte apoptosis and preserve the mitochondrial membrane potential to maintain myofibril integrity and prevent left ventricular dysfunction in a mouse model of Dox-induced cardiotoxicity [26]. Pretreatment with sildenafil maintained mitochondrial integrity by augmenting cellular mechanisms mediated by NO/cyclic GMP.

Another cardioprotective agent is monoHER which is the main constituent of flavonoids Venoruton [26]. In an in vivo and ex vivo mouse model, pretreatment with monoHER protected against Dox-induced cardiotoxicity and additionally did not interfere with the antitumor effect of Dox. However, more studies are needed to demonstrate the efficacy of this agent. There is also new evidence that cardiacα1-adrenergic receptors can protect from Dox-induced cardiotoxicity by protecting cardiomyocytes [26]. Stimulation of these receptor agonists, including phenylephrine and dabuzalgron, reduces apoptosis and interstitial fibrosis and in turn decreases myocardial dysfunction caused by Dox [26]. This effect was associated with anti-apoptotic proteins of the Bcl2 family and preserving mitochondrial function.

Exercise intervention

Aerobic exercise has been shown to have cardioprotective effects and is recommended by the American Heart Association to promote cardiac health and in particular for cancer survivors of all ages [33]. Recently, in a study by Wang et al., it was observed that aerobic exercise was an effective intervention in mitigating acute cardiac side effects in juvenile mice treated with Dox [33]. The Dox-induced reduction in ejection fraction and fractional shortening were found to be prevented by exercise in mice that had been treated with Dox. Additionally, it was found that Dox caused a reduction in body weight and the heart weight: tibia length ratio in tumor-bearing mice that were treated with Dox alone. However, the exercise intervention administered during therapy helped mitigate this weight loss and prevented the reduction in heart weight: tibia length ratio. The exact molecular mechanism(s) by which exercise had this cardioprotective effect in mice treated with Dox is yet to be determined. Additionally, the cardioprotective benefits of exercise were also seen when the exercise was initiated after Dox therapy [33,34].

Targeting the Immune system

Recent studies have begun to focus on the contribution of inflammation and an inflammatory response to cardiotoxicity, particularly the role of innate immune cells and the cytokines these cells produced. A more in-depth understanding of these parameters has the potential to identify mechanisms and interventions such as blocking cytokine/chemokine production, to reduce the impact of Dox-induced cardiotoxicity.

Chapter Two: The Innate Immune System in Cardiovascular Diseases

Inflammation in the heart

Inflammation that occurs due to trauma or chemically induced injury and in the absence of pathogens is termed 'sterile inflammation'. In the case of cardiac injury/damage, sterile inflammation forms the foundation of the first phase of cardiac remodeling and involves the production of chemokines and cytokines and the recruitment of innate immune cells such as neutrophils and macrophages [35]. During the inflammation phase, the pathogenic source of infection danger signals is detected by certain pattern recognition receptors (PRRs) include Toll-like receptors (TLRs), nucleotide-binding and oligomerization domain (NOD) like receptors and C-type lectin receptors. In a similar manner, when there is tissue damage, endogenous molecules related to the injury are released as damage associated molecular patterns (DAMPs). These DAMPs can also be detected by PRRs. Once these danger signals are detected, an inflammatory cascade is activated. This cascade includes the release of cytokines/chemokines, the activation of inflammatory pathways, and the subsequent recruitment of immune cells from circulation and bone marrow. These processes are meant to clear damaged tissue and other necrotic cells to move to the next phase of repair.i.e. deposition of new extracellular matrix (ECM). At first this is a necessary process to maintain homeostasis and modulate repair. However, unchecked activation leads to chronic inflammation and is detrimental.

Cardiac remodeling post ischemic injury occurs in three phases: inflammation, granulation and maturation. The inflammatory phase is characterized by an increase in pro-inflammatory cytokines and chemokines, such as interleukin 1 (IL-1), IL-6, IL-8,

TNFα, granulocyte colony-stimulating factor (G-CSF), GM-CSF, CXCL-1. This leads to the recruitment of neutrophils and subsequently macrophages, two essential components of the myeloid system [36]. The granulation phase involves ECM turnover and differentiation of cardiac fibroblasts and the final maturation phase includes deposition of new ECM including collagen. Excessive deposition of collagen however can lead to fibrosis, and subsequently heart failure. Cardiac fibrosis is a characteristic of several conditions, such as cardiomyopathy, myocardial infarction (MI), pressure overload, and the aging process[37].

Pattern Recognition Receptors

Toll-like Receptors

In patients with MI, endogenous DAMPs are released from damaged cells and detected by TLRs and other PRRs, which initiate a signaling cascade. These DAMPs include: heat shock proteins, S100 proteins, uric acid, high mobility group box protein 1 (HMGB1), and endogenous nucleic acids. Additionally, components of the ECM, such as hyaluronan, heparan sulfate and proteoglycans can also act as DAMPs.[35] TLRs comprise 3 structural domains: an extracellular C-terminal leucine-rich repeat domain, a central transmembrane domain, and a cytoplasmic domain. TLR signaling occurs via two main pathways: the MyD88-dependent and TRIF-dependent pathways.

In the MyD88-dependent pathway, there is first a recruitment of a Toll/IL-1 receptor domain containing adaptor protein that initiates TLR4/2 related signaling. This leads to activation of the downstream IL-1 receptor-associated kinase 4(IRAK4), induction of IRAK1 phosphorylation, and recruitment of TNF-receptor-associated factor-6 (TRAF6). Phosphorylated TRAF6 forms a complex with transforming growth

factor β-activated kinase (TAK- 1), TAK1-binding protein-1(TAB1), and TAB2. This complex then interacts with ubiquitin ligases to activate the TAK1. Next, activated TAK1 phosphorylates the IKK complex and p38 kinases. The IKK complex will then phosphorylate I-κB. I-κB's subsequent ubiquitylation and degradation leads to NF-κβ translocation to the nucleus, which in turn leads to pro-inflammatory cytokine production.[38]

TLRs also signal through the TRIF-dependent pathway which involves signaling via TRIF- related adaptor molecule (TRAM) and TRIF. This signaling activates TANK Binding Kinase-1 (TBK1) and the eventual activation of interferon- regulatory factor-3 (IRF3). TRIF-dependent signaling can also occur through NF-κβ via TRAF6 recruitment as described above.[39]

Inflammasome

Activation of the NF-κβ pathway can lead to the expression of inflammasome components. In particular, the NLRP3 inflammasome has been shown to be induced in cardiovascular diseases. This cytosolic protein consists of three domains: a C-terminal leucine-rich repeat domain, a NOD also called a NACHT domain, and an N-terminal pyrin domain. NLRP3 binds to an adaptor protein, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC). ASC binds to pro-caspase 1 and activates it. The active caspase 1 cleaves its substrates including pro-IL-1β and pro-IL-18. This leads to extracellular release of mature IL-1β and IL-18. These two cytokines have been implicated in several cardiovascular diseases (CVDs) including atherosclerosis, hypertension, myocardial infarction.[40,41] Inflammasomes also play a role during granulopoiesis aiding in myeloid lineage specification.

Specifically, caspase-1 cleaves transcription factor GATA1, thus increasing the production of neutrophils. Furthermore. IL-1β activates a PU.1- dependent gene program that stimulates granulocyte lineage differentiation.[42]

TLRs in the heart

TLRs have been shown to play an important role in immune signaling in the heart after damage. In the heart, TLR2, 3, 4 and 6 have been identified in cardiomyocytes while TLR1 through 6 have been found in smooth muscle and endothelial cells [43]. In particular TLR2 and TLR4 are important in inflammation of the heart. TLR4-deficient mice have a weakened inflammatory response characterized by reduced levels of NF-κβ and pro-inflammatory cytokines TNFα and IL1β, smaller infarctions, and fewer cardiac infiltrations[44]. TLR2 activation in the heart has been shown to upregulate pro-inflammatory cytokines. At first this is beneficial as it leads to mitochondrial stabilization; however, sustained TLR signaling leads to activation and recruitment of leukocytes to the cardiac microenvironment, which in turn leads to tissue destruction[37].

Inflammatory cytokines released in the cardiac microenvironment following damage

Inflammatory cytokines and chemokines play an important role in the pathogenesis of myocardial dysfunction and cardiac remodeling. These pleiotropic, multifunctional cytokines are upregulated in response to myocardial injury in patients following MI.[45] The early inflammation phase of remodeling is characterized by release of TNFα, IL-1α, IL-1β, and IL-18. The next phase is characterized by release of anti-inflammatory cytokine such as TGFβ.

Pro-inflammatory cytokines

High TNFα levels have consistently been documented in experimental models of heart failure and in patients. This cytokine exerts a negative inotropic action on cardiomyocytes by disturbing the calcium homeostasis, triggering apoptosis by activating cell death pathways.[45] In fibroblasts, the balance between matrix metalloproteinases (MMPs) and their inhibitors is affected by TNFα, leading to extracellular (ECM) destruction.[46] In the microvasculature, TNFα modulates cyclooxygenase-2, which in turn leads to increased expression of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). This causes pro-inflammatory immune cells to migrate to and remain in the cardiac microcirculation, leading to tissue injury and cardiac dysfunction.[47]

IL-1 has two ligands, IL-1α and IL-1β, both of which have high sequence homology. Infarcted hearts have been shown to have increased levels of IL-1 family proteins. It has been observed that IL-1 has a pro-apoptotic and hypertrophic effect on cardiomyocytes, which can depress cardiac contractility. IL-1, along with TNFα, causes cardiomyocyte apoptosis via pathways involving nitric oxide (NO) and by upregulating Bax, Bak, and caspase-3. In addition, IL-1 has been shown to promote expression of cardiac fibroblast MMP-3, MMP-8, MMP-9 while downregulating expression of their inhibitors TIMP-2 and TIMP-4 which could lead to degradation of ECM and is detrimental to heart tissue.[48]

Anti-inflammatory cytokines

TGFβ is crucial in cardiac fibrosis and remodeling. TGFβ works by binding to Ser/Thr kinase receptors- TGFβ receptors type I (TGFβRI) and type II (TGFβRII) on

the surface of cells. Macrophages that infiltrate following myocardial injury have been observed to release TGFβ in significant quantities. Once released, TGFβ induces expression of genes that promote and increase ECM production which in turn suppresses MMP expression and enhances cardiac repair. Additionally, TGFβ binds to its type I and II receptors and initiates SMAD signaling. This signaling sets into motion the transformation of fibroblasts to myofibroblasts and eventually ECM deposition in a cyclical manner. TGFβ can also contribute to fibroblast differentiation by combining with other pro-inflammatory cytokines to promote endothelial to mesenchymal differentiation.[49]

Neutrophils in Heart Damage

Introduction to Neutrophils

Neutrophils are the most abundant granulocytic leukocytes in human peripheral blood (50-70%). These cells are an essential part of the innate immune system and help with host defense and inflammation resolution. They are among the shortest-lived cells and require continuous replacement from the bone marrow. Neutrophils are generated by a process known as granulopoiesis. This starts with a granulomonocytic progenitor that goes through maturation stages from myeloblast to promyelocyte to myelocyte and then a mature neutrophil. These stages are regulated by the cytokine G-CSF, which also promotes the circulation of neutrophils by disturbing the CXCR4-CXCL12 interaction that helps keep neutrophils in the bone marrow. G-CSF causes release of neutrophils by reducing expression of CXCL12 on stromal cells in the bone marrow and CXCR4 on the neutrophils.[50] Once tissue damage occurs, and

inflammatory stimuli and chemokines are released, neutrophils home into these signals by leaving the circulation and migrating to the site of damage.

Neutrophil Elastase

Neutrophil elastase (NE) is a 29 kDa serine protease from the chymotrypsin family found in the primary granules of neutrophils. NE activity depends upon a catalytic triad comprising aspartate, histidine, and serine residues that are separated in primary granules but come together at the enzyme's active site in the tertiary structure. Primarily, NE is active in neutrophils' response against bacteria, but it has also been known to cause ECM destruction. In the granules, an acidic milieu protects the cells from proteolytic activity. Upon activation of neutrophils, fusion between granules and cytoplasmic phagosomes occurs causing alkalosis which further triggers degranulation. NE is liberated into extracellular space in both free and membrane bound forms.[51]

NE can target ECM, cell surface ligands, proteins and adhesion molecules. NE can degrade matrix proteins, including collagen, fibronectin, proteoglycans, heparin and fibrin. NE also has the ability to evade tissue anti-proteases in the extracellular environment.[52]

Neutrophils in CVDs

Neutrophils play a detrimental role in acute MI and neutrophil count correlates with infarct size, , and heart failure development.[53] Neutrophils have also been shown to be involved in atherosclerosis, thrombosis, and acute coronary syndrome.

Atherosclerosis is one of the most common causes of MI leading to death. Neutrophils have been shown to promote the initiation of atherosclerosis by dysregulating vascular endothelial cells. Also, increased neutrophil counts have been observed in human atheroma specimens.[53] In animal models with atherosclerotic plaques, activated macrophages produce chemokines to attract neutrophils. The neutrophil transmigration is mediated by oxidized low-density lipoproteins via an increase in endothelial contractility and upregulation of ICAM-1.[54] Depletion of neutrophils reduced atherogenesis. In atherosclerotic lesions, neutrophils release CAMP and AZU1, which recruit inflammatory monocytes and help upregulate the expression of adhesion molecules on the surface of endothelial cells, regulating the endothelial cell layer permeability.[42,55] Myeloperoxidase released by neutrophils catalyze the conversion of hydrogen peroxide to hypochlorous acid, which produces toxic chloramines that can alter lipoprotein function. Neutrophils also release reactive oxygen species (ROS), which contribute to plaque vulnerability by oxidizing LDL and recruiting additional neutrophils.[56] Further, study suggests that neutrophils co-localizes with TLR-2 expressing atherosclerotic plaques on endothelial cells. TLR-2 activation on endothelial cells leads to cell stress and apoptosis. This effect is substantially enhanced by adhesion of neutrophils to endothelial cells. This in turn correlates with an increased number of luminal apoptotic endothelial cells in atherosclerotic lesions.[42]

In MI, neutrophils can exacerbate damage and increase infarct size. Neutrophils infiltrate at the ischemic border zone and release ROS, which then leads to acute inflammation and cardiomyocyte apoptosis, leading to destruction of cardiac tissue

and ultimately impaired heart function. Additionally, release of myeloperoxidase into extracellular space causes the generation of cytotoxic aldehydes, oxidative stress, and activation of enzymes that degrade the ECM, leading to impairment in remodeling. During acute MI, neutrophils release Ca^{2+} binding proteins S100A8/A9 that primes the NLRP3 inflammasome to release IL-1β, which stimulates granulopoiesis in the bone marrow. This leads to accumulation of neutrophils in the infarcted heart, adverse remodeling, and heart failure.[57] Anti-inflammatory strategies have focused on inhibiting cardiac neutrophil recruitment. Brahma related gene 1(BRG1) is crucial in mediating neutrophils' adhesion to the endothelium infiltration of the infarct.[58] When BRG1 was deleted, reduced infarct size, less fibrosis, and recovery of cardiac function were reported. Additionally, obstructing the interaction between CC motif chemokine 5 (CCL5) and CXC motif chemokine receptor 4 (CXCR4) in MI shrank infarct size, conserved heart function and was related to reduced presence of neutrophils and the formation of neutrophil extracellular traps.[59] A study conducted in a mouse model of MI found that starting 3 days after MI, two populations of neutrophils were found in the heart: $SiglecF^{hi}$ and a $SiglecF^{low}$. $SiglecF^{low}$ represented young blood neutrophils while $SiglecF^{hi}$ shared characteristics of aged neutrophils (i.e low CD62L, high CXCR4, expression of specific transcripts, enrichment of ribosomal protein encoding genes). The $SiglecF^{hi}$ population was also seen to express high surface ICAM1 expression. As a result, these subsets have different functional capacities. The $SiglecF^{hi}$ population was shown to have higher phagocytic capacity and higher ROS production than the $SiglecF^{low}$ population and this was detrimental to cardiac remodeling.[60]

Neutrophil elastase in CVDs

As discussed previously, NE is in the primary granules of neutrophils and is released during degranulation. Continuous NE secretion can cause excessive tissue destruction. NE has also been implicated in arthritis, respiratory, and cardiovascular diseases. Patients with acute MI have elevated level of NE in plasma. NE degrades elastin, collagen, and fibrinogen, which can lead to damage following MI. Additionally, NE induces IL-6 release which leads to impairment of cardiac contractility.[61] Investigations in NE $^{-/-}$ mice confirmed that NE was responsible for promoting excessive inflammation and activating proinflammatory cytokines that contributed to the cardiac damage following MI.[62] NE enhanced myocardial injury by suppressing the PI3K/AKT pathway. Insulin driven AKT signaling is critical in preventing apoptosis.[63] NE can enter intracellular space and mediate degradation of the insulin receptor substrate protein, which in turn prevents AKT activation and protection against apoptosis. In NE$^{-/-}$ mice, AKT signaling was activated and cardiomyocytes were protected from apoptosis. In this study a pharmacological inhibitor of NE, silvestat, was found to improve survival and cardiac function after MI.[62]

In a study conducted in patients with type II diabetes and MI, there were elevated levels of NE and neutrophil in atherosclerotic plaques. Additionally, an association between high NE and MI severity was observed.[64] This study showed that higher NE was detected in detached emboli of atherosclerotic plaques, contributing to plaque instability.[64] NE has also been found to mediate a chronic inflammatory state by activation of pro-form of TNF and IL-1β, PAR2, and phospholipase C, leading to translocation of NF-κβ and activation of that signaling pathway.[65,66] NE can also lead

to pro-apoptotic signaling by endothelial cells via ERK, JNK, and p38 MAPK which can lead to apoptosis of cells.[67]

Macrophages in CVD:

While neutrophils form an essential part of the early innate immune response in heart damage the role of macrophages too needs to be discussed in adult mouse heart. Resident cardiac macrophages form 6-8% of the non-cardiomyocyte population. The origin of these macrophages is thought to be diverse: primitive yolk sac-derived, fetal monocyte-derived and adult monocyte-derived. Studies have shown that neonatal mouse heart contains one macrophage population ($MHCII^{low}$ $CCR2^{-}$) and one monocyte ($MHCII^{low}$ $CCR2^{+}$) subset, the adult heart contains two different resident CMs subsets ($MHCII^{low}$ CCR^{-} and $MHCII^{high}$ $CCR2^{-}$). In steady state adult mouse heart contains embryonic resident $CCR2^{-}$ $MHCII^{low}$ macrophages with reparative function. These $CCR2^{-}$ macrophages are lost within infarcted myocardium, reduction by 60% almost 2 days after infarction [68].

While neutrophils are the predominant early immune cells in heart post damage, monocytes/macrophages are the predominant immune cell after (2-5d post injury). It has been observed that after MI monocytes and macrophages transit from a pro-inflammatory phase to an anti-inflammatory phase. The main function of these monocytes and macrophages is to remove debris, myocytes and apoptotic neutrophils to prepare for tissue regeneration. The heart becomes rapidly infiltrated with $Ly6C^{hi}$ monocytes that differentiate into M1(pro-inflammatory) macrophages. The numbers of these monocytes peak around day 3 after infarction [69]. These macrophages engulf cellular debris, degrade ECM and secrete pro-inflammatory cytokines such as TNF-α,

IL-1β and IL-6. However, it has been shown that M1-like macrophages contributed to myocardial injury by secreting pro-inflammatory exosomes and miRNAs which inhibited cardiac healing and angiogenesis. Additionally, macrophages have PRRs that can detect DAMPs. It has been observed that MI causes the release of danger signals such as double stranded DNA from dying cells that can be detected by macrophages. By day 4-7 after damage $Ly6C^{low}$ monocytes were recruited to the infarcted myocardium. These $Ly6C^{low}$ monocytes give rise to M2 macrophages which secrete IL-10 to suppress inflammation and initiate ECM remodeling and angiogenesis [70,71].

This leads to activation of transcription factor IRF3 (interferon regulatory factor 3) and subsequent induction of type I interferon production that is detrimental to the heart. In fact, reduced post MI IRF3-dependent signaling reduced cardiac inflammation and improved cardiac function and survival [72].

In observational studies in mice and humans it has been seen that there is a correlation between classical monocytosis and degree of LV dysfunction after MI. In a mouse model with a chronically expanded pool of circulating $Ly6C^{hi}$ monocytes there was an increased infiltration of these monocytes and reduced EF following coronary artery ligation [73]. In a study in patients with ST-segment elevation there was a negative correlation between peripheral blood monocytosis and ventricular function [74]. Another study showed corroboration between high levels of classical monocytes($CD14^{+}CD62L^{+}$) and greater infarct size and systolic LV dysfunction at 4 month follow-up after ST-segment elevation MI [75,76].

Macrophage recruitment via stress signals released by cardiomyocytes have also been found to be a source for myofibroblasts. When macrophages release TGFβ this provides a signal to the activated fibroblasts to trans-differentiate. TGFβ signaling occurs within fibroblasts through phosphorylation of SMAD2/3 [77]. Myofibroblasts maintain the structural integrity of myocardium by remodeling ECM scaffold. They are characterized by an increase in α-SMA (smooth muscle actin). Activated myofibroblasts secreted elevated levels of collagens and other ECM proteins to prevent wall rupture and myocardial dysfunction. Excess deposition of these ECM proteins and collagens leads to ventricular stiffness and dysfunction [68]. This process is called fibrosis. Other cell types such as endothelial cells have also been described as a source for myofibroblasts via endothelial-mesenchymal transition. Pericytes also contribute to the fibrotic process by converting to activated myofibroblasts [78].

Immune system in Dox-induced cardiotoxicity

Although studies have demonstrated the critical role of the innate immune system in heart, the role of innate immunity system in Dox-induced cardiotoxicity has not been assessed in great detail. The limited studies done in this field have identified some key components of the innate immune system that contributed to acute Dox-induced heart damage. Here we discuss the role of immune cells and cytokines in context of Dox-induced cardiotoxicity

Cytokines and chemokines

Several cytokines and chemokines have been targeted with respect to Dox-induced cardiotoxicity.

In one study IFN-γ was implicated in Dox-induced cardiotoxicity.[79] IFN-γ was shown to reprogram lipid metabolism and sensitize cardiomyocytes to cardiotoxicity which worsened heart function. Cardiomyocytes need fatty acids to develop respiratory capacity and impeding oxidation will interfere with that process. AMPK signaling enhances fatty acid oxidation and helps regulate the respiratory capacity of cardiomyocytes. It was observed that with Dox treatment, IFN- γ interfered with AMPK signaling by the suppression of the AMPK/ACC axis in a p38-dependent pathway which enhanced the Dox-induced cardiotoxicity.[80] Importantly, antibody treatment against IFN-γ improved the heart function in mice. This demonstrated that inhibiting IFN-γ could mitigate new as well as previously established Dox-induced cardiotoxicity. The investigators also found that IFN-γ inhibition had no effect on the therapeutic efficacy of Dox in mice with tumors.[81]

A study in breast cancer patients receiving Dox found that the plasma levels of cytokines CCL27 and MIF were elevated after two cycles of Dox.[82] CCL27 is associated with homing of T lymphocytes to sites of inflammation while MIF is a crucial cytokine involved in acute and chronic inflammatory response. MIF has been found to play a role in maintaining cardiac homeostasis and found to be elevated in MI, atherosclerosis, and other disorders.[83] MIF could play a role in protecting against cardiotoxicity by attenuating the loss of autophagy and ATP availability in the heart leading to maintenance of cardiac homeostasis.[84] CCL23, also called macrophage inflammatory protein 3, was also found to be elevated after each cycle. This cytokine has a suppressive effect on hematopoietic progenitor cells. Previous studies have shown an association between high levels of CCL23 and coronary atherosclerosis.[82]

Another study, conducted in $HER2^+$ breast cancer patients receiving anthracycline revealed a significant increase in CXCL10 levels from baseline to post-anthracycline and post-trastuzumab treatment. This increase correlated with a decline in global longitudinal strain.[79] CXCL10 has several roles, including serving as a chemoattractant for monocytes, macrophages, T cells, and NK cells and promoting T-cell adhesion to endothelial cells, thereby leading to significant infiltration of these immune cells during cardiac remodeling.[85]

When aortas of mice treated with Doxorubicin were studied, there was a higher concentration of pro-inflammatory mediators such as IL-1β, IL-2, IL-6 and TNFα. TNFα levels had the highest elevation which was associated with intrinsic wall stiffness that was prevented by inhibition of TNFα.[86]

A study conducted in breast cancer patients who received Dox found that compared to baseline the levels of IL-10 were significantly increased 7 days after therapy completion in patients with cardiotoxicity. Increased levels of plasma NT-proBNP (a marker for cardiac injury) correlated with the increased IL-10 levels in patients with cardiotoxicity. IL-10 levels also were positively correlated with IL-1β in the patients with cardiotoxicity, even though IL-10 is an immunosuppressive cytokine.[87]

TLRs

TLRs have also been found to be important as part of the innate immune response to Dox-induced cardiotoxicity.[88] TLR5 was found to be significantly elevated in hearts of mice treated with Dox. TLR5 deficiency led to reduced NOX2 levels in particular. NOX2 is an isoform of NADPH oxidase, a primary source of ROS in the heart. This was important as the investigators demonstrated that TLR5 activated NOX2 through

Syk phosphorylation. [88] TLR5 deficiency attenuated this effect. Dox was found to activate the p38 signaling pathway, which led to apoptosis of cardiomyocytes. This p38 pathway was NOX2 dependent and hence activated by ROS. This pathway was also inhibited in TLR5 deficient mice.[89, 88] TLR5 deficiency led to lower TNFα and IL-1β mRNA levels and NF-κβ translocation was also inhibited in these mice and this led to improvements in heart function and less myofibrillar disruption in mice treated with Dox..[88]

In a study conducted in mice with TLR9 deficiency that had been treated with Dox it was found that cardiac function, myocardial fibrosis and markers for myocardial damage were all reduced as compared to mice treated with Dox alone. TUNEL staining further revealed that in TLR9 KO mice with Dox treatment there was a significantly reduced number of apoptotic cardiomyocytes and reduced ROS production compared with wild-type (WT) mice with Dox treatment. Furthermore, it was found that TLR9 promoted the oxidative stress and apoptosis through p38 MAPK-dependent autophagy leading to death of cardiac cells.[90]

Another TLR whose relationship to Dox-induced cardiotoxicity has been studied in mice is TLR2. TLR2- KO mice showed less NF-κβ activation, along with a lower production of pro-inflammatory cytokines (TNFα and IL-6), compared with WT mice. The TLR2-KO mice had higher survival rates than WT mice after Dox treatment. Furthermore, fewer TUNEL-positive cells were found in the myocardium, and caspase-3 activation was suppressed in the TLR2 KO mice with Dox treatment.[91] In a study measuring inflammatory biomarkers in patients with heart failure, expression levels of TLR2 increased in patients in both the Dox group without heart failure and

the Dox plus heart failure group.[92] In another study evaluating the anti-inflammatory role of LCZ696 (sacubitril/valsartan), an angiotensin receptor neprilysin inhibitor that is used to reduce the risk of cardiovascular death for patients with heart failure, with respect to TLR2 deficiency, it was found that administration of the drug improved heart function and prevented cardiac fibrosis after Dox treatment. In addition, LCZ696 also prevented high TNFα expression. In TLR2-KO mice, similar results were observed, suggesting a connection between drug action and TLR2. Further studies found that LCZ696 attenuated the formation of the TLR2-MyD88 complex and this in turn alleviated the negative effects of Dox, as Dox promotes the formation of the TLR2-MyD88 complex.[93]

TLR4, a receptor of endotoxin, has also been shown to contribute to cardiac inflammation in Dox-induced cardiotoxicity. TLR4-KO mice had improved LV function and a reduction in cardiac ET-1 which contributes to heart failure. Additionally, when lipid peroxidation and nitrotyrosine were examined as markers of oxidative stress in TLR4-KO mice treated with Dox, there was significantly reduced oxidative stress. This study in an animal model of ischemia/reperfusion also suggested that TLR4 contributed to the development of oxidative stress. Furthermore, it was observed that infiltration of lymphocytes, monocytes, and macrophages was reduced in the TLR4-KO mice treated with Dox compared to Dox-treated control mice. [94] Upregulation of the pro-apoptotic protein Bax, was observed in WT Dox-treated mice which was not seen in the TLR4-KO mice. These findings were confirmed by TUNEL assay where reduced apoptotic cells were seen in the TLR4-KO group. This study also found a significant upregulation of Bcl-2, an anti-apoptotic protein, in TLR4-KO mice with Dox

treatment as compared to TLR4-KO mice. In a mouse study, downregulation of the GATA-4 pathway was seen in Dox-induced cardiomyopathy, down regulation of this pathway is known to promote Dox-induced cardiotoxicity. However, in TLR4-KO mice this down-regulation did not occur, nor did the disease.[94] Another finding that supports the importance of TLR4 in Dox-induced inflammation was that TLR4 expression was increased in macrophages following Dox treatment. When TLR4 was suppressed or depleted by injecting TAK-242 or using $TLR4^{lps\text{-}del}$ mice lower myofibrillar disruption as compared to Dox groups was observed.[95]

Signaling pathways

One signaling pathway that has been implicated in dox-induced cardiotoxicity is the cGAS-STING pathway. It has been observed that cGAS-STING dependent type I IFN signaling which is induced by DNA damage is responsible for driving inflammation in the cardiac microenvironment leading to pathological cardiac processes. In $Sting^{gt/gt}$ mice which had loss of cGAS-STING signaling, cardiac function was rescued even after a five-dose regimen of Dox. An inhibitor of CGAS-STING signaling, H-151, prevented the decline in ejection fraction and the ventricular dilation that occurs via the increase in LV end diastolic volume [79].

Innate Immune Cells

The role of innate immune cells, especially neutrophils, has been discussed about in great detail in CVDs. However, the role of these immune cells in Dox-induced cardiotoxicity needs to be looked at in greater detail.

Macrophages

In the case of macrophages in Dox-induced cardiotoxicity, most studies have focused on polarization of macrophages in the cardiac micro-environment. In a study conducted in mice evaluating the effects of Latifolin in Dox-induced cardiotoxicity it was found that Dox increased the expression of markers for M1 macrophages(pro-inflammatory) while latifolin, a new flavonoid, attenuated this effect by increasing the expression of M2(anti-inflammatory) markers. The increase in M1 markers in the Dox group was accompanied by increase in release of inflammatory cytokines such as IL-6, TNFα and IL-1β which latifolin was also able to prevent [96]. In a similar study conducted using glabridin, an isoflavone, it was found that glabridin modulated colonic macrophage populations skewing it more towards an M2 like phenotype. It was also observed that this modulation by gut microbiota prevented cardiomyocyte apoptosis [97].

In another study conducted to evaluate the effect of adoptive transfer of M2 macrophages in Dox-induced cardiotoxicity it was found that M2 macrophages improved left ventricular function post Dox treatment as compared to Dox treatment alone. Additionally, Dox caused increase in cardiomyocyte vacuoles and interstitial fibrosis and decrease in cardiomyocyte size that was counteracted by M2 macrophage transfer. Levels of c-caspase 3 and TUNEL positive cells were also increased in Dox treatment alone mice. However, in mice with M2 macrophages and Dox treatment this effect was reversed, and this was found to be independent of the AKT and ERK pathway. This study also indicated that mitochondria internalization into

cardiomyocytes in the presence of M2 macrophages alleviated the cell stress induced by Dox [98].

In a study conducted in mice with IL-22 knockout it was found that cardiac oxidative stress was reduced in these mice and phosphorylation of p38 was also decreased in cardiac macrophages. This decrease in p38 led to lower cardiomyocyte apoptosis. Further studies showed that IL-22 knockout reduced phosphorylation of p65 that maybe downstream of p38 and could regulate macrophage differentiation. The deletion of IL-22 also was found to alleviate the imbalance between M1 and M2 macrophages [99].

Invariant natural killer T cells

Invariant natural killer T-cells (iNKT) are a subset of T-lymphocytes that express properties of both T cells and natural killer cells. Studies have shown that iNKT cells modulate cardiac tissue inflammation. For example, treatment with alpha-galactosylceramide (αGC), an activator of iNKT cells, prevented damage after MI. Another study looked at these cells in the context of Dox-induced cardiotoxicity and found that heart function was normal in mice treated with Dox and αGC simultaneously but not in mice treated with Dox only. Additionally, an analysis for fibrosis found that there was low collagen deposition in the mice treated with Dox+ αGC compared to mice with treated with Dox only. A qPCR analysis revealed that M2 macrophage expression was higher in mice treated with Dox+ αGC mice than in mice with Dox only.[100]

Neutrophils

Neutrophils may also contribute to Dox-induced cardiotoxicity. In one study examining therapy-related clonal hematopoiesis following anti-tumor agents including Dox, cardiotoxicity was augmented by the infiltration and activation of neutrophils. In this study, an elevation of neutrophils was observed in cardiac tissue, which peaked at 7 days after treatment with single bolus of Dox. When mice were transplanted with Trp53 heterozygous mutant bone marrow cells to establish a model of clonal hematopoiesis, neutrophil recruitment was higher in heart tissue compared to mice transplanted with WT cells post Dox treatment. Furthermore, when these Trp53 heterozygous deficient neutrophils were analyzed for gene expression, these neutrophils were enriched for genes related to the inflammasome pathway (i.e. *Nlrp1b, Gbp5, Il18*) and chemokines (e.g. *Ccl25, Ccrl2 and Cxcl1*). When neutrophils were depleted in the mice with Trp53-deficient cells an amelioration of echocardiographic parameters including fractional shortening was observed after Dox treatment indicating that neutrophil involvement is crucial for the detrimental effects of Dox.[101] In another study conducted in breast cancer patients receiving anthracyclines high level of plasma neutrophil extracellular traps was seen to be associated with Dox-induced cardiotoxicity.[102]

However more studies need to be done to examine this crucial component of the innate immune system in Dox-induced cardiotoxicity as there is increasing evidence implicating neutrophils in Dox-induced cardiotoxicity.

Chapter Three: The role of neutrophils in Dox-induced cardiotoxicity

This chapter is based upon " Bhagat A, Shrestha P, Jeyabal P, Peng Z, Watowich SS, Kleinerman ES. Doxorubicin-induced cardiotoxicity is mediated by neutrophils through release of neutrophil elastase. Front Oncol. 2022 Aug 10;12:947604" as a part of CC-BY Creative Commons attribution license.

Introduction:

The mechanisms that contribute to Dox-induced acute and late cardiotoxicity are not completely understood. One understudied area is whether acute inflammation and the innate immune response contributes to Dox-induced cardiotoxicity. Other models of heart damage, such as myocardial infarction, showed that an inflammatory component was induced in response to damaged cardiomyocytes which included release of damage associated molecular patterns (DAMPs) that are detected by pattern recognition receptors (PRRs) [103]. Neutrophils have been shown to play an important role in cardiac healing by clearing debris and necrotic tissue and inducing the polarization of monocytes/macrophages to the M2 phenotype- a critical process to healing. However, neutrophil persistence can be detrimental facilitating myocardial damage [42].

Neutrophils are the most abundant leukocytes and are the first to be recruited to an inflammatory site. Neutrophils act by releasing reactive oxygen species, a demonstrated mechanism of Dox-induced cardiotoxicity, as well as serine proteases. One such serine protease is neutrophil elastase (NE), which is secreted extracellularly

from azurophilic granules upon activation [50]. NE plays a crucial role in neutrophil mediated killing of bacteria. However, NE is also involved in tissue destruction and inflammation. NE release may therefore contribute to the cardiac tissue damage following Dox therapy. Studies have shown that NE has the capacity to degrade elastin, collagen and fibrinogen, thereby contributing to damage after myocardial infarction (MI). It has also been shown that NE deficiency improved survival and cardiac function post-MI [62]. Neutrophils and NE-mediated tissue damage have been shown to play a role in arthritis and respiratory diseases [104,105]. However, whether neutrophils and NE contribute to Dox-induced acute cardiac damage has not been determined.

Here, we investigated whether neutrophils and NE contribute to cardiac damage and the acute and late cardiotoxicity that develops following Dox therapy.

Results:

Effect of Dox therapy on cardiac function and neutrophil infiltration:

Our juvenile mouse cardiotoxicity model [33,34] was used to investigate whether neutrophils contribute to Dox-induced cardiac damage. Echocardiograms showed a decrease in EF, FS and an increase in LVID(s) 24 h after Dox therapy (Figures 1a and 1b). Heart tissue analyzed at the time by flow cytometry showed that neutrophils ($CD11b^+Ly6G^+$ cells, gated from the $CD45^+$ population) were significantly elevated in the hearts of Dox-treated mice compared to controls (Figure 1c, $p < 0.01$). This finding was further validated by immunofluorescence staining using anti-Ly6G antibody

(Figure 1d). Having demonstrated that neutrophils are increased 24 h after Dox treatment, we next determined whether neutrophils persisted in the heart. Mice were treated with Dox twice a week for 2 weeks and then evaluated for cardiac neutrophil infiltration 11 weeks after the therapy. No differences were observed in neutrophil numbers ($CD11b^{+}Ly6G^{+}$) between the hearts of Dox-treated and control mice 11 weeks after therapy ($p=0.52$) (Fig 1e). These data indicate that Dox therapy induced an acute increase in cardiac neutrophils, which was not sustained.

CXCL1 is involved in neutrophil recruitment. To determine if Dox-induced the upregulation of CXCL-1 in the heart tissue, CXCL1 mRNA was quantified and found to be significantly upregulated in the cardiac tissue 24 h after Dox therapy (Figure 2, $p<0.05$).

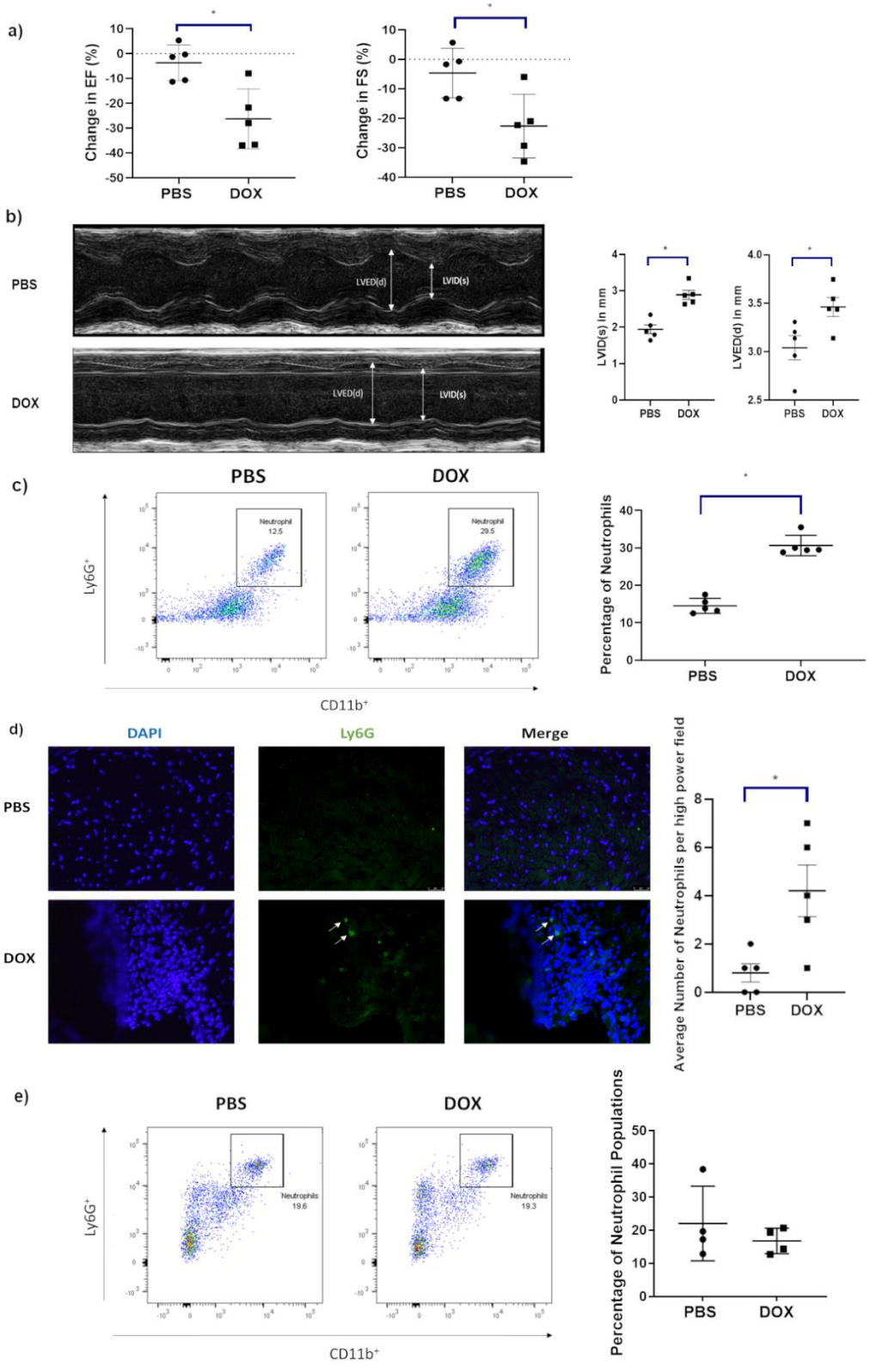
a)
Change in EF (%)
Change in FS (%)
PBS
DOX
b)
PBS
DOX
LVED(d)
LVID(s)
LVID(s) in mm
LVED(d) in mm
c)
PBS
DOX
Neutrophil
12.5
Neutrophil
29.5
Ly6G+
CD11b+
Percentage of Neutrophils
d)
DAPI
Ly6G
Merge
Average Number of Neutrophils per high power field
e)
Neutrophils
19.6
Neutrophils
19.3
Percentage of Neutrophil Populations

Figure 1: Effect of Doxorubicin (DOX) on heart function and neutrophil infiltration 24 h after therapy. a) Ejection fraction (EF) and fractional shortening (FS) in mice were quantified by echocardiography 24 h after treatment with DOX or control phosphate-buffered saline (PBS); b) Left ventricular internal dimension was measured in systole (LVID, s). Data are presented as mean ± SEM, n=5 each, $p<0.05$, a Mann-Whitney U-test was used to compare two groups.; c) Infiltration of neutrophils ($CD11b^+$ $Ly6G^+$ cells) in hearts of mice treated with DOX or control phosphate-buffered saline(PBS) was quantified by flow cytometry 24 h after therapy; d) Infiltration of neutrophils in hearts ($Ly6G^+$) was quantified by immunofluorescence staining 24 h after therapy; e) Infiltration of neutrophils ($CD11b^+$ $Ly6G^+$) in hearts was quantified by flow cytometry 11 weeks after therapy. Data are presented as mean ± SEM, n=5 each, *$p<0.05$, a Mann-Whitney U-test was used to compare two groups

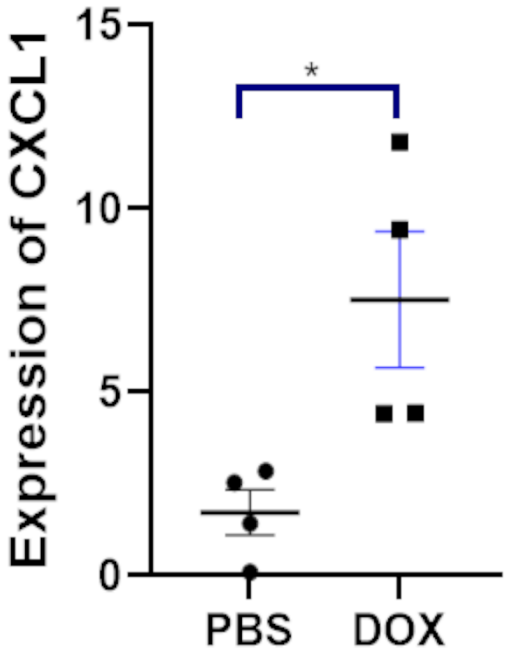

Figure 2: CXCL1 expression in cardiac tissue as quantified by qPCR 24 h after Dox treatment. Data are presented as mean ± SEM, n=4 each, $p<0.05$, a Mann-Whitney U-test was used to compare two groups.

Effect of neutrophil depletion on *acute* Dox-induced cardiotoxicity:

We next determined whether depleting neutrophils using an anti-Ly6G antibody prior to Dox therapy inhibited the Dox-induced decrease in cardiac function as defined by EF, FS and LVID(s). Using Balb/c mice successful depletion of neutrophils was confirmed by flow cytometry in peripheral blood mononuclear cells (PBMCs) 24 h after the antibody treatment (Figure 3a). Twenty-four hours after Dox therapy the EF, FS and LVID(s) changes described above were seen in the Dox treated mice but not in the neutrophil-depleted mice (Figure 3b, 3c). Control IgG antibody given prior to Dox did not prevent Dox-induced cardiotoxicity. The decreases in EF and FS and increase in LVID(s) in the group treated with Dox and IgG was similar to that of the group treated with Dox only. Increased neutrophils were detected in heart sections of mice treated with Dox alone and Dox plus IgG ($p<0.01$). In contrast, neutrophils were not increased in hearts from mice treated with the anti-Ly6G antibody compared to control hearts (Figure 3d).

We previously demonstrated that there were changes in the morphology of the cardiac vessels 24 hours after Dox therapy [33]. These included a decrease in $CD31^+$ and $NG2^+$ vessels, a decrease in the $NG2^+$ pericyte coverage of the cardiac vessels and a decrease in the number of open lumen vessels with a diameter over 100 µm which correlated with a decrease in cardiac blood flow. Similar to our previous report, here we show that the cardiac vessels from mice treated with Dox alone Or Dox plus IgG appeared more punctate and collapsed, a sign of decreased pericyte coverage. There was also a significant decrease in the number of vessels with open lumens

>100μm. These changes were not seen in the cardiac vessels from neutrophil depleted mice (Fig 3e). These data indicate that neutrophils contribute to damaging the cardiac vessels following Dox therapy, which leads to an acute effect on heart function. Depletion of neutrophils inhibited these Dox-induced structural and functional changes in heart.

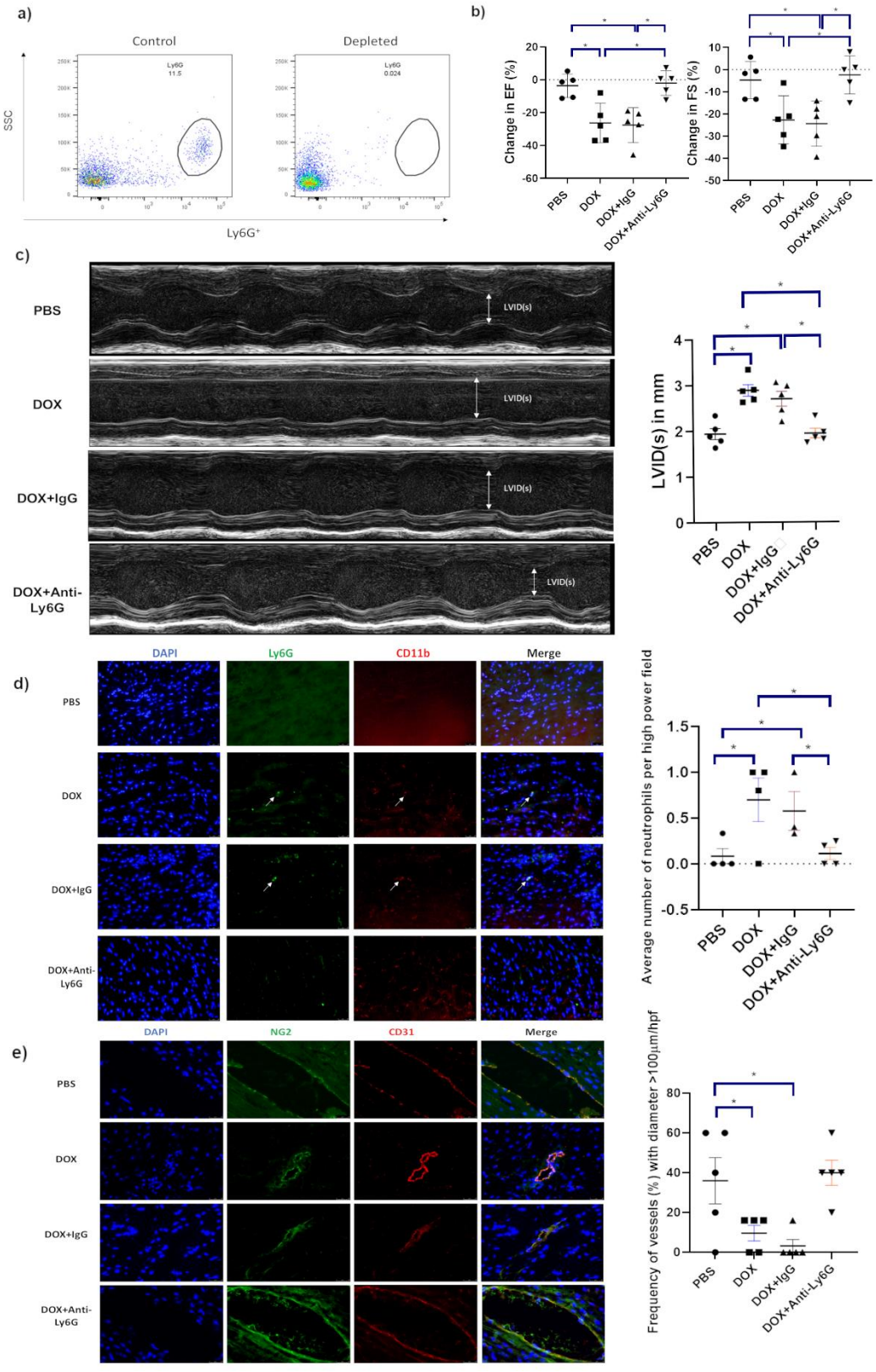
a)
Control
Depleted
SSC
Ly6G+
b)
Change in EF (%)
Change in FS (%)
PBS
DOX
DOX+IgG
DOX+Anti-Ly6G
c)
LVID(s)
LVID(s) in mm
d)
DAPI
Ly6G
CD11b
Merge
Average number of neutrophils per high power field
e)
NG2
CD31
Frequency of vessels (%) with diameter >100μm/hpf

Figure 3: Effect of neutrophil depletion on heart function and vascular morphology 24 h after therapy. a) Depletion of neutrophils was quantified by flow cytometry as described in Figure 1 b and c; b) In mice with control phosphate-buffered saline(PBS), doxorubicin(DOX), DOX plus control IgG, or DOX plus anti-Ly6G antibody, ejection fraction (EF), fractional shortening (FS) were quantified; c) Left ventricular internal dimension in systole (LVID(s)) were quantified; d) Representative images of neutrophils ($CD11b^+$ $Ly6G^+$) in the hearts of mice treated with control phosphate-buffered saline(PBS), doxorubicin(DOX), DOX plus control IgG, or DOX plus anti-Ly6G; e) Representative images of $NG2^+$ and $CD31^+$ vessels. Results are presented as mean ± SEM, n=5 each, *p<0.05, one-way ANOVA analysis with Tukey comparison was used to compare the groups.

Effect of neutrophil depletion on *late* Dox-induced cardiotoxicity:

To determine whether neutrophil depletion decreased late cardiotoxicity, neutrophils were depleted as described above and mice were treated with Dox. Heart function was then monitored for 10 weeks after therapy. There was a continuous decline in EF and FS in Dox-treated mice ($p<0.0001$). In contrast, no change was seen in the neutrophil depleted mice treated with Dox (Figure 4a, $p=0.3643$). Additionally, LVID was increased in Dox and Dox plus IgG-treated mice compared to control mice 10 weeks after therapy. This was not seen in the neutrophil-depleted mice (Figure 4b). Ten weeks after therapy, the compromised vascular structures remained in the hearts from Dox and Dox plus IgG-treated mice, as defined by a decrease in $CD31^+$ vessels and $NG2^+$ vessels. By contrast, there remained, no significant decrease or difference in $CD31^+$ or $NG2^+$ vessels in hearts from neutrophil-depleted mice (Figure 4c).

We previously showed that while no cardiac fibrosis was seen 24 h after Dox treatment, there was significant cardiac fibrosis (collagen formation) 10-12 weeks after therapy [33]. To determine whether neutrophil depletion impacted Dox-induced collagen formation, we performed Masson Trichrome staining 10 weeks after therapy. There was a significant increase in collagen in hearts from Dox-treated mice ($p= 0.0015$) but not in the hearts from neutrophil-depleted mice treated with Dox (Figure 4d, $p=0.07$).

Macrophages have been shown to contribute to cardiovascular diseases [106] . We therefore determined whether our neutrophil depletion protocol was specific for neutrophils with no impact on macrophage numbers in the heart 24 h following Dox therapy. $CD11b^+Ly6Chi^+$ and $CD11b^+F4/80^+$ populations were used to identify and

quantify pro-inflammatory monocytes and macrophages respectively. Monocyte and macrophage numbers were similar in the neutrophil- depleted and control mice treated with Dox (data not shown). This data indicates that early depletion of neutrophils prevented late stage cardiotoxicity from developing and also prevented excess collagen deposition. Additionally, we found that depletion of neutrophils did not affect the level of monocytes and macrophages in the cardiac microenvironment post Dox treatment.

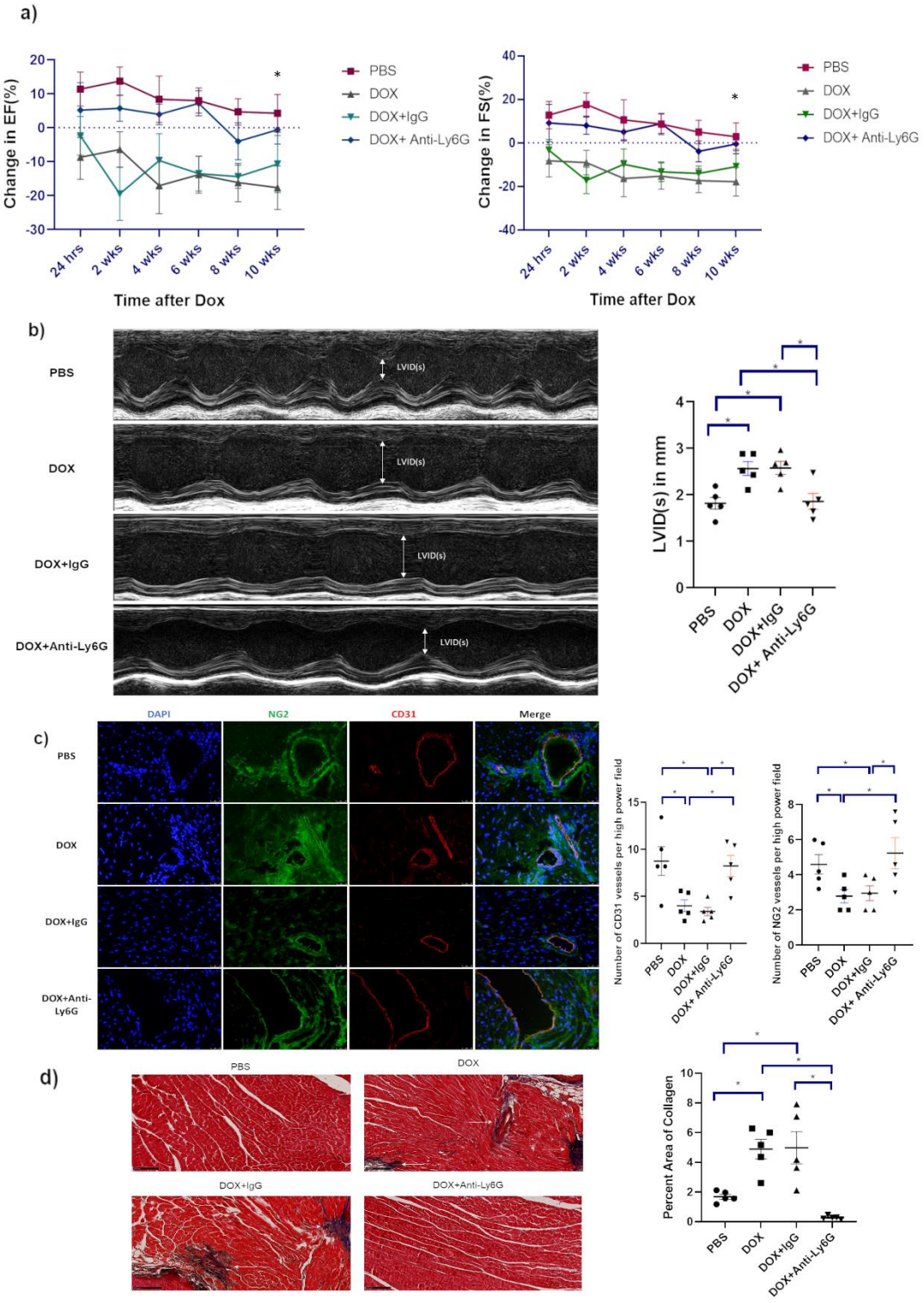
a)
Change in EF(%)
Time after Dox
PBS
DOX
DOX+IgG
DOX+ Anti-Ly6G
Change in FS(%)
24 hrs
2 wks
4 wks
6 wks
8 wks
10 wks
b)
LVID(s)
LVID(s) in mm
c)
DAPI
NG2
CD31
Merge
Number of CD31 vessels per high power field
Number of NG2 vessels per high power field
d)
Percent Area of Collagen

Figure 4: Effect of neutrophil depletion on heart function and vascular morphology 10 weeks after therapy. a) In mice treated with control phosphate-buffered saline(PBS), doxorubicin(DOX), DOX plus control IgG, or DOX plus anti-Ly6G, ejection fraction(EF) and fractional shortening(FS) were quantified by echocardiography and followed for 10 weeks after therapy; b) Left ventricular internal dimension in systole (LVID(s)) was also quantified 10 weeks after therapy; c) Representative images and numbers of $NG2^+$ and $CD31^+$ vessels in the hearts of mice treated with control phosphate-buffered saline (PBS), doxorubicin (DOX), DOX plus control IgG, or DOX plus anti-Ly6G 10 weeks after therapy; d) Collagen deposition in the hearts was determined using Masson trichrome staining 10 weeks after therapy completion. Data are presented as mean ± SEM, n = 5 each, $p < 0.05$, one-way ANOVA analysis with Tukey comparison was used to compare the groups.

Contribution of neutrophil elastase (NE) to acute and late Dox-induced cardiotoxicity:

Neutrophils release NE following activation. To investigate whether NE was elevated and contributed to Dox-induced cardiac damage, NE mRNA levels were quantified in the cardiac tissue of Dox treated and control mice 24 h after therapy. Higher NE mRNA levels were seen in the hearts from the Dox-treated mice (Fig 5a). $NE^{-/-}$ mice were used to evaluate the contribution of NE to Dox-induced acute and late cardiotoxicity. First to confirm that neutrophils from the $NE^{-/-}$ mice have similar migratory ability to neutrophils from wild type mice we performed an in-vitro trans-migration assay using WKYMVm, a chemoattractant for neutrophils. The migratory function of neutrophils from the $NE^{-/-}$ mice was not significantly different compared to neutrophils from wild type mice (Figure 6). Next, we confirmed that Dox therapy induced an increase in cardiac neutrophils 24 h after therapy in these $NE^{-/-}$ mice. Similar to wild type mice neutrophils were significantly elevated in Dox-treated $NE^{-/-}$ mice (Figure 5b, p=0.04). Therefore, the NE knockout did not inhibit neutrophil migration into the heart 24 h after Dox treatment. To There was a significant decrease in EF and FS in the Dox-treated wild-type mice but not in the $NE^{-/-}$treated with Dox. The EF and FS in Dox-treated $NE^{-/-}$ mice were similar to those of $NE^{-/-}$ control mice (Figure 5c). Blood vessel morphology (as determined by quantifying the number of open vessels with diameter >100μm) was also not significantly different in the $NE^{-/-}$ control and $NE^{-/-}$ Dox treated mice and there was no decrease in $CD31^{+}$ and $NG2^{+}$ vessels (Figure 5d, p=0.0115).

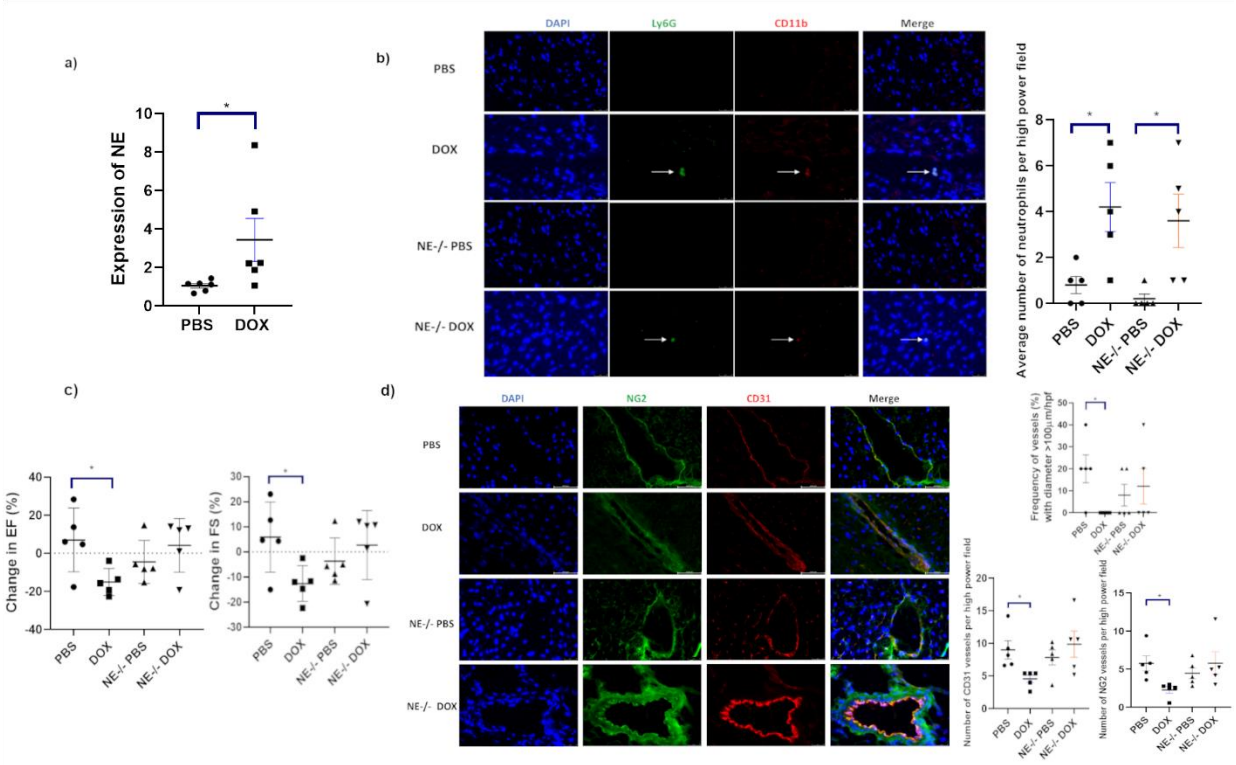

Figure 5: Effect of neutrophil elastase knockout ($NE^{-/-}$) on heart function 24 h after therapy. A) NE as measured by quantitative PCR in hearts of wild-type and $NE^{-/-}$ mice 24 h after treatment with control phosphate-buffered saline(PBS) or doxorubicin(DOX); b) Representative images of neutrophils ($CD11b^{+}$ $Ly6G^{+}$) 24 h after therapy; c) Ejection Fraction (EF) and fractional shortening (FS) were quantified by echocardiography 24 h after therapy; d) Effect of NE knockout on cardiac vessel morphology 24 h after therapy. Representative images, the number of $NG2^{+}$ and $CD31^{+}$ vessels, and the frequency (%) of vessels > 100µm/hpf 24h after therapy. Data are presented as mean ± SEM, n = 5 each, $p < 0.05$, one-way ANOVA analysis with Tukey comparison was used to compare the groups.

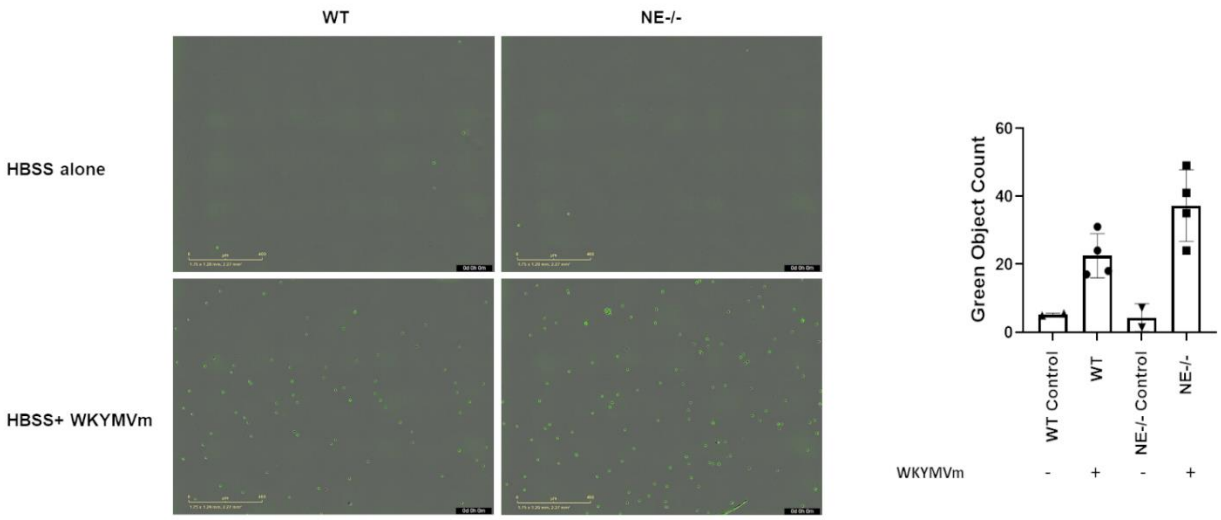

Figure 6: Neutrophil Transmigration following doxorubicin treatment was quantified by Incucyte in the presence and absence of a chemoattractant, a Mann-Whitney U-test was used to compare two groups.

To evaluate the role of NE in the late stage of Dox-induced cardiotoxicity mice were treated with Dox for 2 weeks, and cardiac function monitored for 12 weeks. There was a significant decrease in EF and FS in the Dox-treated wild type mice 24 h after therapy that persisted over 12 weeks. In contrast, no decrease in EF or FS was seen in the Dox treated $NE^{-/-}$ mice (Figure 7a). Cardiac vascular morphology was also unchanged in the Dox-treated $NE^{-/-}$ mice (Figure 7b). The number of open vessels with a diameter >100μm in the $NE^{-/-}$ control and $NE^{-/-}$ Dox treated mice were not significantly different (Figure 7b). This data indicated that inhibiting or blocking NE prevented both the acute and late Dox-induced cardiac damage.

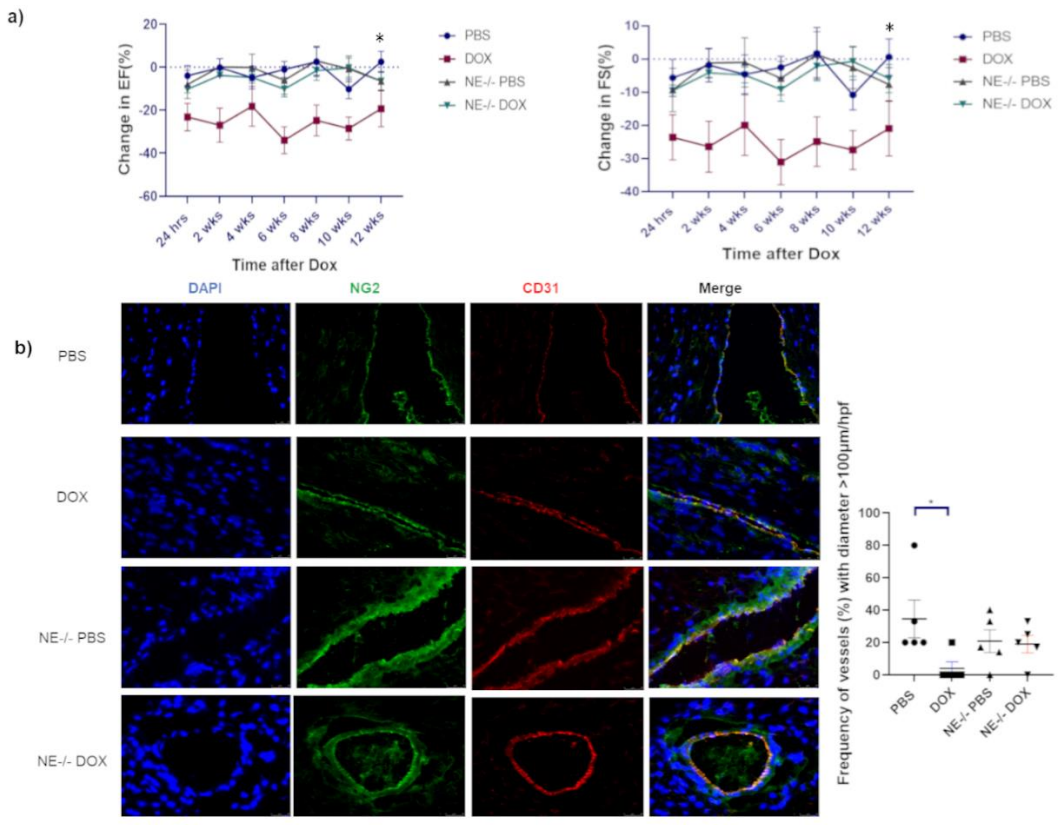

Figure 7: Effect of neutrophil elastase knockout ($NE^{-/-}$) on heart function and cardiac vessel morphology 12 weeks after therapy. a) Ejection Fraction (EF) and fractional shortening (FS) were quantified by echocardiography and followed for 12 weeks after treatment with control phosphate-buffered saline (PBS) or doxorubicin (DOX) in wild-type and $NE^{-/-}$ mice; b) Representative images of cardiac $NG2^{+}$ and $CD31^{+}$ vessels 12 weeks after therapy and frequency (%) vessels with >100µm/hpf. Data are presented as mean ± SEM, n = 5 each, $p < 0.05$, one-way ANOVA analysis with Tukey comparison was used to compare the groups.

Ogura et al showed that the NE caused cardiomyocyte apoptosis in myocardial infarction [107]. We wanted to investigate these findings in our context of Dox-induced cardiotoxicity. TUNEL staining was used to identify apoptotic cells. The number of TUNEL cells was significantly higher in Dox treated mice as compared to $NE^{-/-}$ mice (Figure 8). We also found that the TUNEL positive cells were specific to cardiomyocytes as evidenced by staining with cardiac troponin (Figure 9). Additionally, we quantified cleaved caspase-3 levels in Dox-treated control and $NE^{-/-}$ mice to investigate the effect of decreased NE on cardiomyocyte apoptosis after Dox treatment. Cleaved caspase-3 protein was significantly elevated in the control Dox treated hearts as compared to the $NE^{-/-}$ Dox treated hearts (Figure 10).

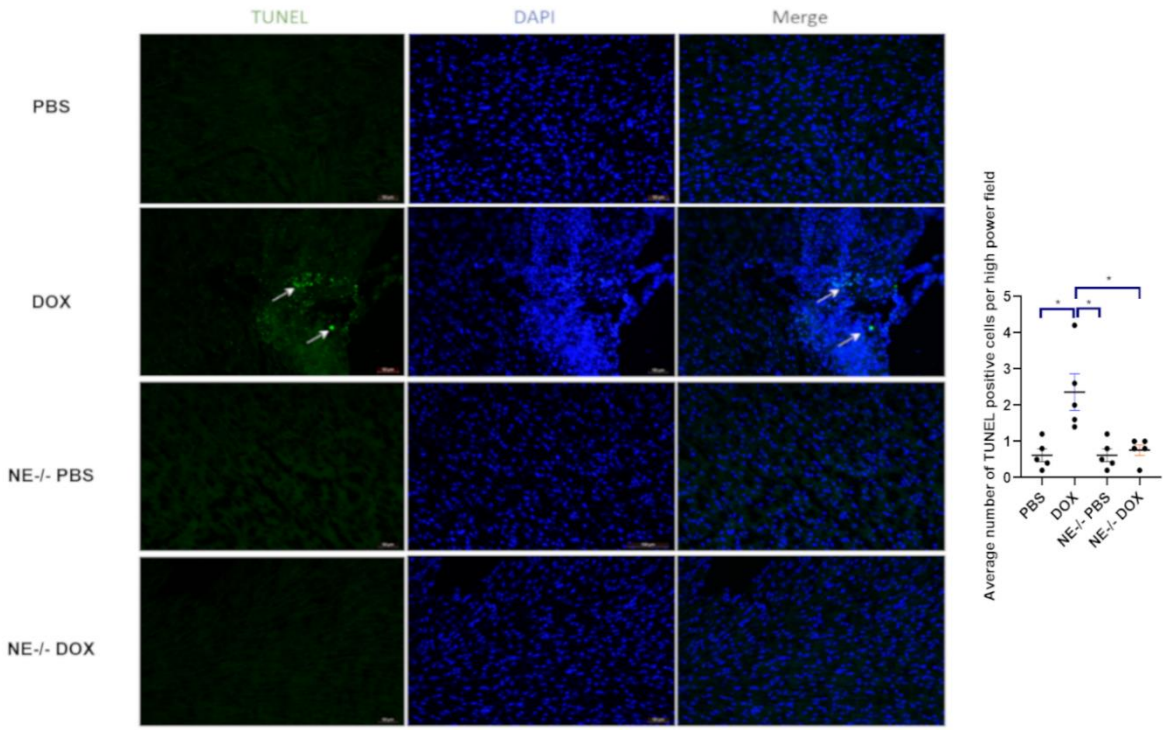

Figure 8: Effect of neutrophil elastase knockout ($NE^{-/-}$) on apoptosis in heart tissue following doxorubicin (Dox) treatment. Representative TUNEL staining images of heart sections 24 h after Dox treatment; Data are presented as mean ± SEM, n = 5 each, $p < 0.05$, one-way ANOVA analysis with Tukey comparison was used to compare the groups.

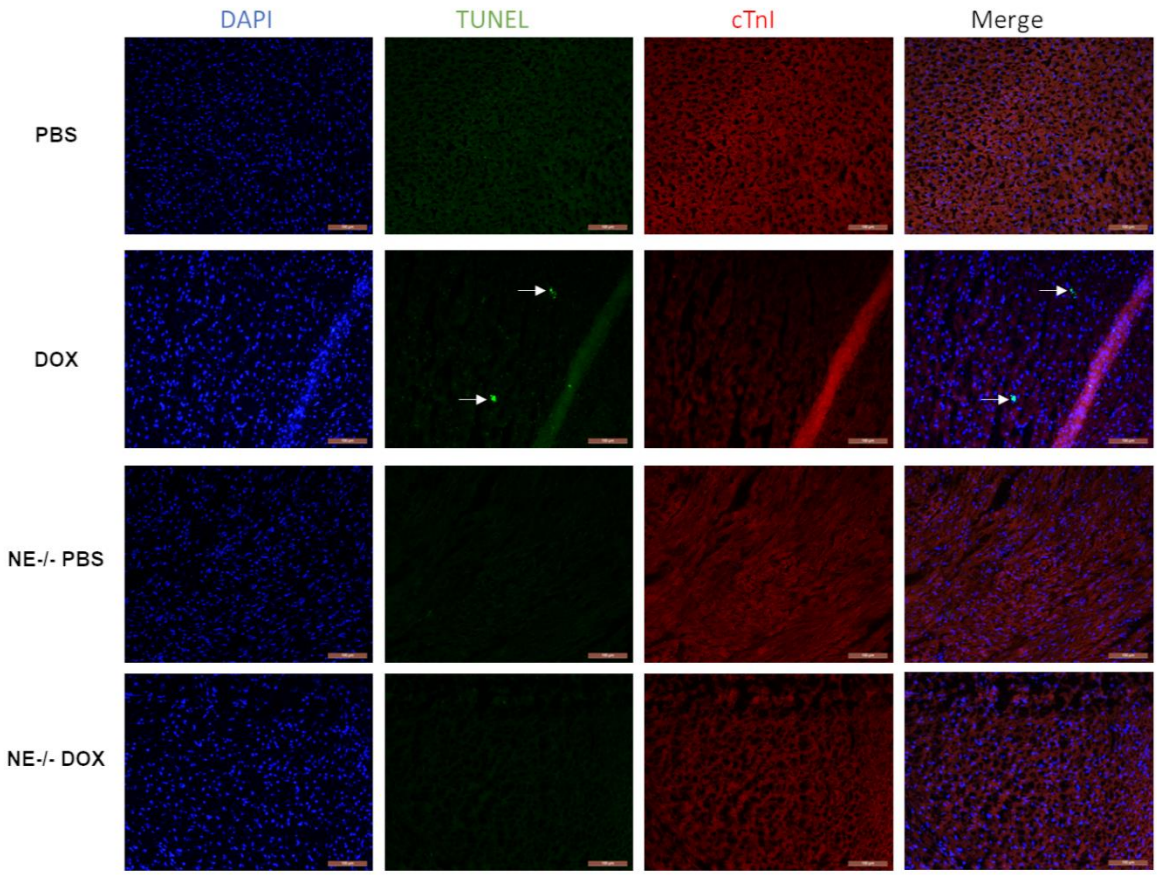

Figure 9: Representative TUNEL staining images of heart sections 24 h after Dox treatment counterstained with CnTI (Cardiac Troponin I).

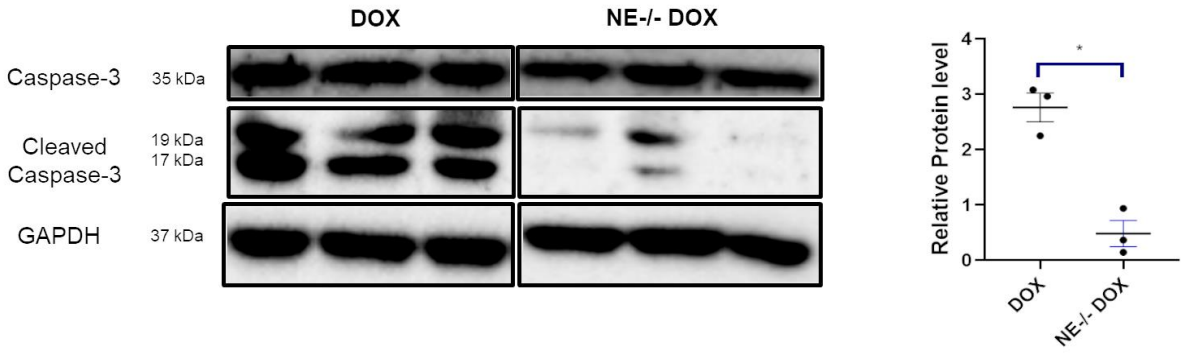

Figure 10: Representative images of Western blots for caspase-3, cleaved caspase-3 and GAPDH from hearts 24 h after treatment; the protein levels of cleaved caspase-3 were quantified using densitometry analysis and normalized to the levels of caspase-3; Data are presented as mean ± SEM, n=3 each, a Mann-Whitney U-test was used to compare two groups.

Having demonstrated that NE contributed to Dox-induced cardiotoxicity we investigated the therapeutic potential of an NE inhibitor, AZD9668. Here mice were treated with Dox, AZD9668 or both drugs concurrently. Decreased EF and FS were seen in the Dox-treated mice but not in mice that received Dox plus AZD9668 (Figure 11). This effect persisted for 12 weeks after Dox treatment had ended indicating that an NE inhibitor has therapeutic potential to prevent Dox-induced heart damage.

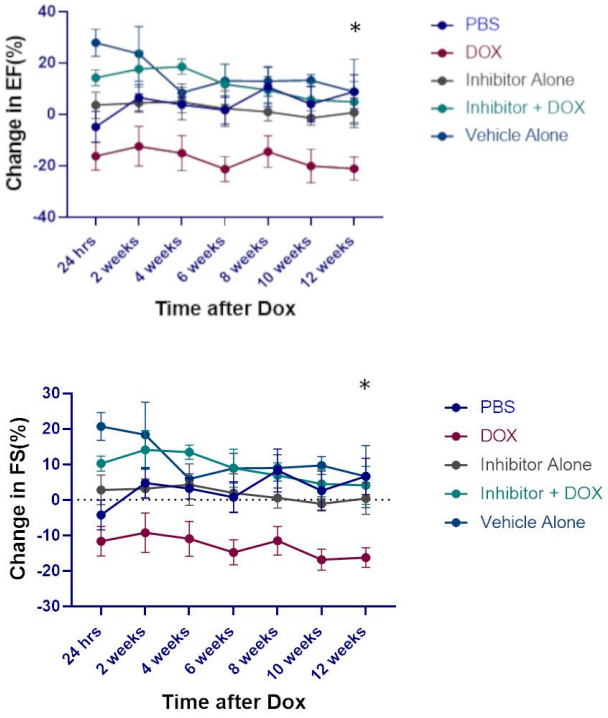

Figure 11: Effect of neutrophil elastase (NE) inhibitor on heart tissue following doxorubicin (DOX) treatment. Ejection fraction (EF) and fractional shortening (FS) were quantified by echocardiography up to 12 weeks after therapy. Data are presented as mean ± SEM, n = 5 each, $p < 0.05$, one-way ANOVA analysis with Tukey comparison was used to compare the groups.

Discussion:

Dox continues to be an integral part of treatment for pediatric and adolescent and young adult patients (AYA) with sarcoma due to the lack of new effective targeted therapies, including immunotherapies. Unfortunately, Dox causes acute damage to the heart resulting in late cardiac morbidities which range from changes in myocardial structure and function, to severe cardiomyopathy, valvular disease and congestive HF in long-term survivors. These cardiac morbidities compromise quality of life (QOL) and longevity and may result in a need for cardiac transplant [108,109]. Indeed, Dox-induced cardiotoxicity is the second leading cause of death in childhood and AYA cancer survivors [110,111]. Therefore, strategies to limit the acute heart damage caused by Dox are expected to result in lower cardiac morbidities, improved longevity and better QOL. Identifying effective interventions requires an understanding of the *multiple* mechanisms that contribute to Dox-induced cardiotoxicity [112] .

Here we demonstrated for the first time that Dox therapy induced the acute infiltration of neutrophils into the heart (as quantified by flow cytometry and immunofluorescence staining. This was accompanied by an acute decline in cardiac function, (defined by decreased in EF and FS and increased LVID), an alteration in cardiac vascular morphology, and a decrease in the number of cardiac vessels with open lumens. The increase in cardiac neutrophils did not persist. No differences in neutrophil numbers in the heart were seen 4-11 weeks after therapy in Dox-treated versus control mice. This is in accordance with previous studies demonstrating that neutrophil infiltration was short lived, peaked early but dissipated at later time points

[104]. Despite the normalization of neutrophil numbers, the decreased cardiac function persisted for 2-12 weeks after therapy.

The importance of neutrophils and NE in the acute Dox-induced cardiotoxicity process was confirmed using neutrophil-depletion techniques and NE-knockout mice. We confirmed that neutrophil depletion using anti-Ly6G was successful and that this resulted in no increase in cardiac neutrophils 24h following Dox therapy. Our data showed that Dox-induced acute cardiotoxicity was not seen in either neutrophil-depleted mice or $NE^{-/-}$ mice. Under these conditions EF, FS, LVID(s) and vascular morphology were unchanged 24 h after Dox therapy. We also confirmed that the increase in neutrophil infiltration following Dox therapy in the $NE^{-/-}$ mice was not significantly different than that seen in wild-type mice, indicating that the absence of NE did not interfere with the neutrophils' ability to migrate into the heart. Similar to our results, Hirche et al showed that during infection the neutrophil recruitment to sites of inflammation were not hindered in elastase knockout mice [113]. Their data showed no differences in the ability of WT and $NE^{-/-}$ neutrophils to migrate to sites of inflammation. This is in contrast to the findings of Voisin et al where impaired neutrophil invasion into the heart tissue was seen following ischemia/reperfusion injury [114]. The difference between our findings may be due to the fact that we are investigating Dox-induced cardiac damage which is not the same as damage induced by ischemia.

We have previously shown that Dox therapy acutely affected cardiac vascular structure, morphology and function as defined by decreased pericytes, collapsed

cardiac vessels, and a decrease in the number of vessels with open lumens, resulting in decreased cardiac diastolic and systolic blood flow [33,34] . Neutrophil-depleted and $NE^{-/-}$ mice showed none of these acute changes in vascular structure 24h after Dox therapy. We interpret this to mean that neutrophil depletion and the absence of NE prevented acute Dox-Induced vascular damage.

Cardiotoxicity is known to develop in survivors many years after treatment. We therefore investigated whether neutrophil depletion and NE knockout prevented the late Dox-induced cardiotoxicity. When we evaluated heart function 10 weeks after treatment was completed, we observed significant decreases in EF and FS, and an increase in LVID(s) in the Dox-treated mice. However, as demonstrated in the acute cardiotoxicity experiments, there was no change in cardiac function in the neutrophil depleted mice. The vascular changes also were not observed in the neutrophil-depleted mice. In addition, there was significant collagen deposition consistent with the development of cardiac fibrosis in the hearts of the Dox-treated control mice but not the neutrophil-depleted mice treated with Dox.

Taken together, these data further confirm that neutrophils contribute to the acute heart damage caused by Dox. This acute damage had a prolonged effect on the heart. Inhibiting this acute phase prevented the development of late-stage cardiotoxicity. Our data suggest that neutrophils contribute to both acute and late Dox-induced cardiotoxicity. Neutrophil depletion had no effect on macrophage infiltration following Dox therapy. We also demonstrated in the heart tissue that after Dox treatment Dox there was increased expression of CXCL1, a cytokine involved in neutrophil

recruitment. These results suggest a direct mechanism by which Dox induces neutrophil infiltration into the heart.

Previous reports have also shown that neutrophils are recruited to the heart following cardiac injury such as myocardial infarction. Here damaged cardiomyocytes act as DAMPs that are detected by PRRs which release cytokines such as CXCL1 that helps recruit neutrophils [103]. Furthermore, when we performed TUNEL assay on wild-type and $NE^{-/-}$ mice following Dox treatment we observed a significant number of apoptotic cells in hearts from the wild-type mice but not the $NE^{-/-}$ mice. Taken together, these results indicate that neutrophils through the release of NE contribute to cardiotoxicity and that inhibiting NE prevents this cardiotoxicity. To test the therapeutic potential of targeting NE we administered the NE inhibitor AZD9668 during Dox treatment. We found that the EF and FS did not change in mice treated with AZD9668 and Dox and more importantly that this protection persisted for up to 12 weeks after the Dox treatment had been completed. These results suggest that targeting NE during Dox therapy may decrease acute Dox-induced cardiac damage.

In this study we focused on NE as we have observed higher expression levels of this enzyme in hearts with Dox. However, the role of other serine proteases that neutrophils release upon degranulation such as cathepsin G and proteinase 3 [115], may also contribute to Dox-induced cardiotoxicity and should be investigated. Additionally, we briefly monitored levels of another myeloid cell population, monocytes/macrophages, in hearts of Dox-treated mice after neutrophil depletion. Traditionally, macrophages participate in phagocytosis, chemotaxis, secretion and

antigen presentation for immune defense and tissue healing [116]. The plastic nature of these cells has rendered their exact function in the cardiac microenvironment post heart damage unclear [117,118]. Hence, a deeper look into the role of macrophages with further characterization of macrophages that are characterized into either M1(classically activated pro-inflammatory) or M2 (alternately activated anti-inflammatory) [119,120] in Dox-induced heart damage needs to be done.

In summary our results show that neutrophils contribute to Dox-induced cardiac damage, through the release of NE leading to vascular damage and decreased heart function that persist many weeks after therapy completion. This is the first study of its kind to demonstrate that neutrophils and neutrophil elastase are involved in Dox-induced cardiotoxicity. In our study we made clear that Dox was causing cardiac cell death. This cardiac cell death in turn induced the upregulation of chemotactic cytokines such as CXCL-1 as part of the inflammatory process that is triggered following cardiotoxicity. This upregulation then led to neutrophil migration into the damaged heart tissue contributing to additional damage amplifying cardiotoxicity. It is not our contention that Dox does not control or induce NE directly. Rather that the tissue damage *induced* by Dox leads to the initiation of the inflammatory process that involves neutrophils. When neutrophils migrate into the damaged tissue, NE is released, and it is this *released* NE that causes further cardiac damage. Our results showing that a NE inhibitor mitigates Dox-induced cardiotoxicity supports this hypothesis. In pre-clinical models in rats, NE inhibitors ablated the ischemia-induced myocardial damage and coronary endothelial dysfunction [61]. Furthermore in clinical trials, NE inhibitors have been used to treat cystic fibrosis and chronic obstructive

pulmonary disease and have been found to be safe and well tolerated and effective in curbing the excess inflammatory response [121,122]. AZD9668 in particular, has been used in clinical trials for patients with chronic obstructive airway disease and was found to have no significant toxicity while showing promising therapeutic potential in early phase studies [123]. In our cardiotoxicity mouse model, AZD9668 was well tolerated and effective in inhibiting Dox-induced cardiac damage and in preserving heart function after Dox therapy. This is a significant finding with translational potential to decrease the incidence and degree of cardiomyopathies in CCS, which in turn will impact both QOL and patient longevity, as cardiac disease is the second leading cause of death in these individuals [124]. Our data supports consideration for the inclusion of a NE inhibitor with Dox with the goal of preventing Dox-induced acute and late cardiotoxicity in survivors. NE inhibitors may decrease the acute inflammatory response induced by Dox preventing cardiomyocyte apoptosis and the late fibrosis that develops.

Conclusion:

In conclusion we found that neutrophils play an important role in Dox-induced cardiotoxicity through release of neutrophil elastase. Our finds suggest a new treatment approach for mitigating this damage during Dox treatment.

Chapter Four: Global Discussion

Introduction

We have demonstrated the importance of neutrophils in Doxorubicin (Dox)-induced cardiotoxicity and a possible therapeutic intervention targeting neutrophils. However, there are several other questions that arise from this. The roles of macrophages need to be examined in greater detail. Monocytes/macrophages act in tandem with neutrophils when it comes to immune responses to tissue injury. We have generated some preliminary data regarding macrophages in Dox-induced cardiotoxicity that will be discussed below.

Furthermore, we have shown that release of neutrophil elastase and its blockage has an ameliorative effect. Probing the other serine proteases released from the azurophilic granules will provide a more thorough understanding of how neutrophils contribute to Dox-induced cardiotoxicity. Additionally, neutrophils also release reactive oxygen species (ROS) as a mechanism for microbial killing and formation of neutrophil extracellular traps (NETs). This could be another avenue for exploration. Additionally, blocking neutrophil elastase in a tumor bearing mouse model would be important to determine whether AZD9668 affects efficacy of anti-tumor action of Dox.

While cardiomyocytes form the main component of heart there are other cells that play important roles in development and response to tissue injury. One such cell population is cardiac fibroblasts. Previous studies have identified this population to be vital in the third phase of cardiac remodeling post injury. However, there are other

studies that have found them to be crucial mediators of innate immune response in heart. The role of cardiac fibroblasts in Dox-induced cardiotoxicity hasn't been examined in great detail. This would be a promising target to look at in future experiments to further delineate the innate immune response in heart.

Neutrophil Elastase inhibition in a tumor bearing mouse model

In our study we examined the effects of a pharmacological inhibitor of NE on Dox-induced cardiotoxicity in a non-tumor bearing mouse model. In a future study this would need to be studied in a tumor bearing mouse model to examine if the NE inhibitor has any effect on the efficacy of Dox in its anti-tumor action. Our hypothesis is that AZD9668 would have no effect on Dox's anti-tumor action due to differences in mechanism of action of both drugs. Dox acts on tumors by i) intercalation of DNA strands, ii) topoisomerase suppression and iii) generation of free radical species. In contrast AZD9668 acts by binding the active site of enzyme and in particular the catalytic triad of S195, D57 and H102(serine, aspartate and histidine) [125].

Macrophages

Our results showed that neutrophils play an important role in Dox-induced cardiotoxicity. While these cells are one of the first to infiltrate into infected or damaged tissue, macrophages also have been shown to play a key role in the acute innate immune response. Neutrophils have been shown to peak on day 1 post myocardial infarction [126], however monocytes aggregate over days. This accumulation occurs in two waves: first $Ly6C^{hi}$ monocytes (days 1–4 post-MI) and then $Ly6C^{lo}$ monocytes (days 4–7) [126].

Ly6C^{hi} monocytes give rise to cardiac macrophages which proliferate and replenish cardiac resident macrophages lost after myocardial infarction (MI). Cardiac macrophages release inflammatory mediators promoting local inflammation such as proteases, reactive oxygen species and inflammatory cytokines like TNFα. These macrophages are needed for debris clearance and infarct healing. Ly6C^{hi} monocytes while being crucial to infarct healing can also be detrimental due to excess production of inflammatory mediators [73]. In the second reparative phase that takes place 3 days after MI the Ly6C^{hi} monocytes differentiate to reparative macrophages that produce TGFβ, VEGF and IL-10. The release of these factors induces collagen production by myofibroblasts [127]. Excessive collagen deposition however is detrimental and can lead to fibrosis and compromised heart function due to stiffening of cardiac muscle.

In mice undergoing depletion of macrophages by chlodronate liposomes post myocardial injury it was observed that there was a reduced removal of necrotic cells, impaired neovascularization and increase in infarct size. This in turn led to increased mortality in macrophage depleted mice [128].

ROS

From literature it is known that Dox induces ROS production which can result in tissue damage. NADPH oxidase, cytochrome P-450 reductase and xanthine oxidase transform Dox in the form of quinone to semiquinone via one electron reduction of the quinone moiety in ring C within mitochodria [129]. The semiquinone regenerates back to parental quinone by reacting with oxygen generating superoxide anion further converting to other ROS species. It is this redox recycling that amplifies production of free radical species. Endothelial nitric oxide synthase also affects Dox-induced ROS

production [130]. Furthermore, the amount of antioxidant enzymes in cardiac tissue such as superoxide dismutase and catalase is lower than in other organs making the heart more susceptible to damage from ROS [131]. Dox also binds to cardiolipin, a phospholipid component of heart mitochodrial inner membrane [132]. Dox is also retained in the cardiac cells as doxorubicinol. In cancer patients receiving Dox treatment there is a decrease in glutathione suggesting a reduction in antioxidant status [133].

ROS has also been implicated in modulation of several cellular hypertrophic pathways including tyrosine kinases (Src and focal adhesion kinase), protein kinase C (PKC), mitogen activated protein kinases (MAPK; ERK1/2, p38, and JNK), calcineurin, PI3K/Akt, and NF-κB [134]. ROS was also seen to activate apoptosis signal-regulating kinase 1, this stimulates p38 and JNK MAPK and NF-κB pathways [135].

Other serine proteases

In our study AZD9668 is specific for neutrophil elastase (NE), but does not inhibit proteinase 3 or cathepsin G. We demonstrate the importance of NE in Dox-induced cardiotoxicity; however, it would also be important to examine the roles of both serine proteases in Dox-induced cardiotoxicity.

Cathepsin G

Another serine protease from the azurophilic granules is cathepsin G. Similar to neutrophil elastase, cathepsin G has pro-inflammatory properties that play a role in degradation of extracellular matrix (ECM) components, as well as being a chemoattractant for leukocytes such as T-cells. It also plays a role in tissue

remodeling. Additionally, Cathepsin G has been observed to activate the matrix metalloproteinase MMP-2 which can induce apoptosis [136]. Cathepsin G has also been found to mediate the regulation of the chemokine RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted) which are associated with the severity of coronary artery disease [137]. Additionally, cathepsin G activity also leads to the generation of angiotensin II which induces the expression of the monocyte chemoattractant protein-1 (MCP-1) that triggers a profibrotic response by TGF-β1 leading to cardiac fibrosis. Cathepsin G through TGFβ1 formation has also been observed to initiate calcification of the aortic valve. In human stenotic aortic valve cathepsin G has been found to be significantly elevated and is associated with formation of atheroma of carotid artery [138].

Additional studies have shown that cathepsin G triggers a chemotactic response in absence of myocardial injury. Cathepsin G treated hearts showed increase ECM degradation. Cathepsin G also induced increase in activity of elastase and chymase which led to activation of cytokines/chemokines that amplify leukocyte migration to heart at the time of injury. This induces a cycle with excess neutrophil degranulation and inhibition of tissue repair [139]. This same study also demonstrated the mechanisms by which cathepsin G provokes the pro-inflammatory response. By inducing cleavage of IL-1β and IL-18, cathepsin G activates STAT3 and NF-κB signaling pathways to initiate an inflammatory response [140]. Intracardiac administration of cathepsin G in rats led to early changes in left ventricular (LV) remodeling with a decrease in LV wall thickness. These rats also had significant ECM degradation and MMP activity. This study also found that treatment with cathepsin G resulted in more myocyte apoptosis

[139]. These studies have explored the role of cathepsin G with respect to heart diseases but not Dox-induced cardiotoxicity. Hence it would be important to study this serine protease in the context of Dox-induced cardiotoxicity.

Proteinase-3

Proteinase-3 is a 29 kDa serine proteinase that is present in azurophilic granules of neutrophils. It is also present in other specific granules, secretory vesicles and cell surface. Following translocation to the cell membrane this proteinase is secreted into extracellular medium by activated neutrophils [141,142]. Proteinase-3 is identified by a highly conserved catalytic triad (His57, Asp102 and Ser195; using chymotrypsinogen numbering) [143]. It is initially an inactive precursor that undergoes a two-stage post-translational modification and becomes active. Proteinase-3 can act in both an intracellular and extracellular manner. Proteinase-3 has many functions such as degrading ECM proteins including fibronectin, type IV collagen and laminin [144]. Additionally, it also has a defensive immune role by regulating a number of cellular processes and can cleave protein to antibacterial peptides and activate pro-inflammatory cytokines [145].

Several studies have shown a relationship between proteinase-3 and plaque stabilization in atherosclerotic cardiovascular disease. Proteinase-3 mediates a chronic inflammatory state by activation of TNF and IL1β [146], activation of proteinase-activated receptor 2 and phospholipase C that can lead to translocation of NF-κβ [147]. Proteinase-3 has been shown to activate pro-apoptotic signaling of endothelial cells through multiple pathways: ERK, KNK and p38 MAPK [64,67].

Cardiac Fibroblasts

Cardiac fibroblasts form a major part of the cell population in heart. Studies in rates showed that the heart comprised of 70% myocytes and 30% non-myocytes. Another study in murine hearts showed that 45% of cells are non-myocytes with a vast majority being fibroblasts [148]. In human hearts the numbers vary. Some reports found the proportion of fibroblasts to be in the 20-60% range [149]. The reason for this disparity in determining the percentage of fibroblasts is due to the lack of specific markers.

Some of the molecular markers that have been used to identify fibroblasts include: discoidin receptor 2(DDR2) which can label fibroblasts but not endothelium, smooth muscle or myocytes. However, not all fibroblasts are $DDR2^{+}$. Another marker used to label fibroblasts is Thymocyte 1 (CD90). However, this receptor is also expressed by immune cells, pericytes and endothelium. Vimentin, an intermediate filament is also used as a marker for fibroblasts, however it has also been shown to be expressed on endothelial cells. Fibroblast specific protein 1(FSP1) is considered a reliable fibroblast marker. However recent findings have shown that it also labels a subset of immune and endothelial cells [150]. For activated fibroblasts αSMA is the most commonly used marker for activated fibroblasts. Another marker for activated fibroblasts is periostin as it is expressed developmentally by cardiac fibroblasts but not by adult fibroblasts [151]. Platelet-derived growth factor receptor α(PDGFRα) was another marker that was used to identify fibroblasts as it appears to be involved in formation of CF from the epicardium [152].

The main function of cardiac fibroblasts is to synthesize the collagen rich ECM network, especially during myocardial remodeling post injury. When fibroblasts react

to injury, they transition from a quiescent state to an activated state called myofibroblasts. This transition post injury is shown to be mediated by multiple mechanisms including growth factor release in the myocardial environment such as the cytokines TNFα, TGFβ, PDGFs, cytokines like IL-1, IL-10 and renin angiotensin system (RAS) proteins. RAS are produced by macrophages and stimulate cardiac fibrosis by TGFβ signaling pathway. Incorporation of α-SMA into stress fibers is a characteristic of the myofibroblasts. TGFβ leads to promotion of α-SMA transcription in fibroblasts by the Smad3 signaling cascade. Active TGFβ can also activate downstream signaling pathways in the absence of Smad signaling. In fact both Smad-dependent and non-Smad pathways have been seen to cause up-regulation of α-SMA and ECM protein [153]. Myofibroblasts have two functions post injury such as that caused by MI: secreting ECM protein to replace damaged myocardium and to stimulate infarct contraction and produce factors to regulate inflammatory response [154]. Some of the ECM proteins that myofibroblasts express include but are not limited to Type I, III, IV, V and VI collagen, glycoproteins, proteoglycans including fibronectin, laminin and tenascin [155]. Excessive deposition of collagen causes development of cardiac fibrosis.

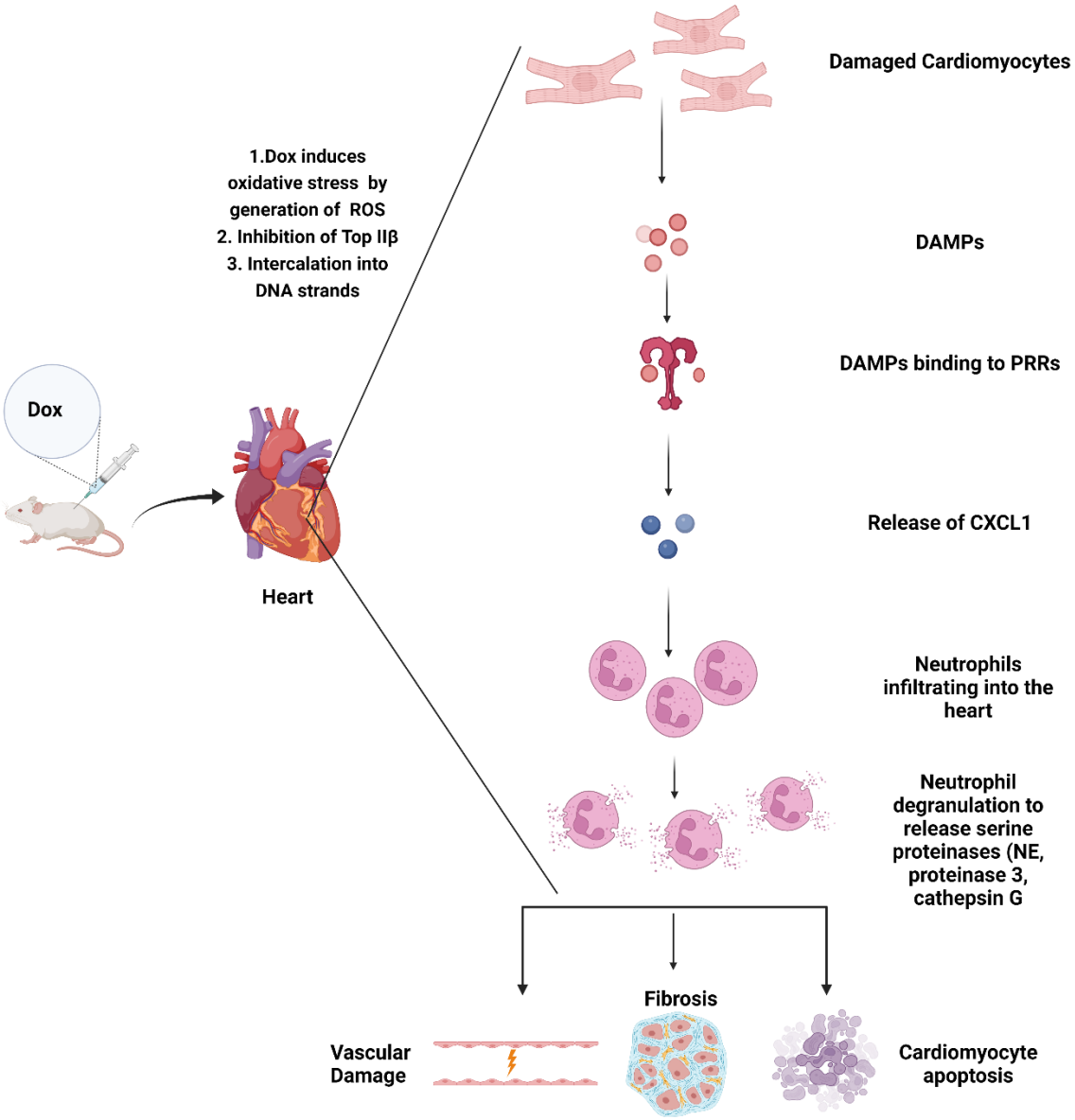

Figure 12 Graphical Summary

Chapter Five: Future Directions with preliminary results

As discussed in the previous chapter there are several future steps that will further our understanding of the innate immune system in Dox-induced cardiotoxicity. This chapter will report our findings from preliminary experiments done. Here we look into the role of macrophages, ROS and cardiac fibroblasts in Dox-induced cardiotoxicity.

Macrophages

The exact role of macrophages in Dox-induced cardiotoxicity is not clear. We observed an elevation in macrophage and monocytes in neutrophil depleted mice treated for two weeks with Doxorubicin. In order to further delineate the importance of macrophages in Dox-induced cardiotoxicity or mouse model we depleted macrophages prior to Dox therapy using chlodronate liposomes. We first confirmed depletion by quantifying and comparing macrophages ($CD11b^{+}$ $F4/80^{+}$) in heart tissue 48 hrs post depletion. We confirmed the depletion of macrophages in heart tissue of chlodronate liposome treated group. Dox treatment was then initiated in the control and macrophage depleted mice for 2 weeks. Depletion of macrophages was repeated every 3 days during the course of treatment. Twenty-four hours following completion of Dox treatment performed echocardiographic measurements to assess heart function to assess acute toxicity. Echocardiographic measurements were repeated at 2-week intervals, up to 8 weeks after Dox treatment to monitor heart function.

Similar to control mice there was a significant decrease in EF and FS in the macrophage depleted mice 24 h after Dox therapy. This decrease in heart function persisted and was still evident 8 weeks after Dox treatment (Figure 12). This is in

contrast to our results with neutrophil depletion where there was no evidence of Dox-induced cardiotoxicity either acutely or 12 weeks post therapy. This indicates that neutrophils are the critical cells that cause damage post Dox treatment. Delineating the exact role of macrophages in Dox-induced cardiotoxicity would be key to understanding the innate immune environment in heart post Dox treatment.

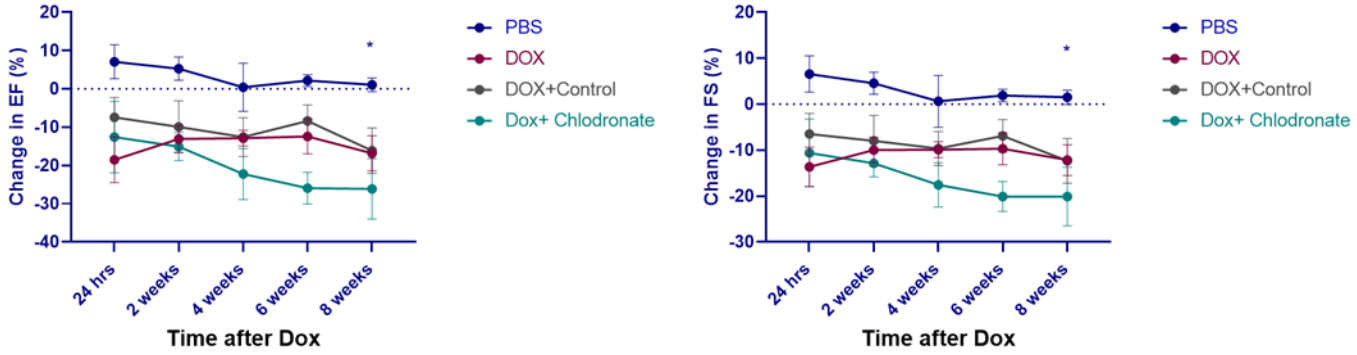

Figure 13: Monocytes and Macrophages in Dox-induced cardiotoxicity. Ejection fraction (EF) and fractional shortening (FS) were quantified by echocardiography up to 8 weeks after therapy. Data are presented as mean ± SEM, n = 5 each, $p < 0.05$, one-way ANOVA analysis with Tukey comparison was used to compare the groups.

ROS

We investigated ROS levels during and after Dox treatment by measuring the intensity of Dichlorofluorescein dye using flow cytometry. While no increase was seen 6 hrs post Dox, we observed a significant increase in circulating ROS levels 24 hrs after 1 dose of Dox. This increase did not persist at the 1-week timepoint. The increase in ROS was also seen 24 hrs after dose 4 of Dox indicating that this is a consistent acute response to Dox therapy (Figure 13).

Since ROS is generated by neutrophils and can augment the generation of neutrophil extracellular traps and pro-inflammatory cytokines [156], further investigation into ROS levels in our neutrophil depleted mice would help understand the relationship between ROS and neutrophils in Dox-induced cardiotoxicity.

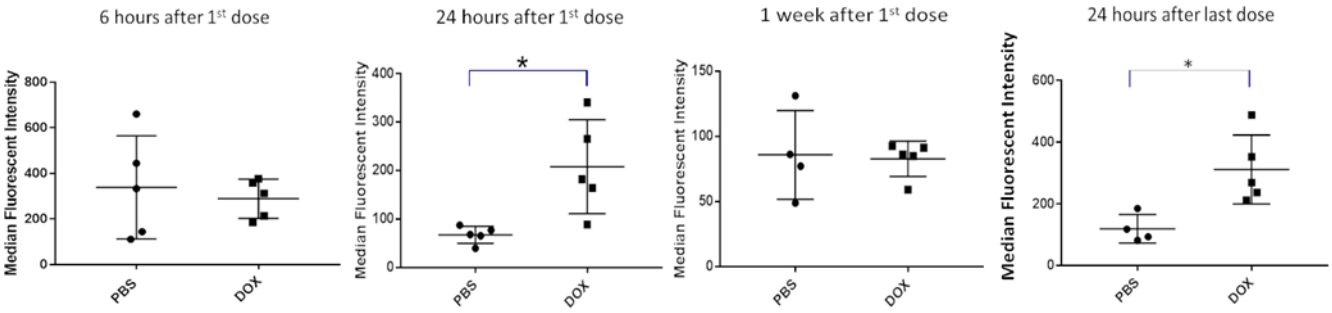

Figure 14: ROS analysis in blood post Dox treatment. ROS analysis was done measuring Dichlorofluorescein dye by flow cytometry at different time points after treatment. Data are presented as mean ± SEM, n=5 each, *p<0.05, a Mann-Whitney U-test was used to compare two groups.

Cardiac Fibroblasts

In the context of Dox-induced cardiotoxicity we did a preliminary investigation on the effects of Dox on cardiac fibroblasts. We first isolated primary fibroblasts from mouse hearts and then treated these fibroblasts with Dox for 24 hrs. We then collected the cells and isolated RNA and then analyzed cytokines and chemokines associated with immune cell recruitment and inflammation by qPCR. We found a significant increase in CXCL-1 and Granulocyte macrophage colony-stimulating factor (GM-CSF) (Figure 14). CXCL-1 is a strong neutrophil chemoattractant. Reports have also indicated that CXCL1 may aggravate cardiac fibrosis by a pro-inflammatory effect [157]. Additionally in CXCR2 (receptor for CXCL1) knockout mice where TGFβ1 and p-Smad 2/3 were suppressed, it was found that α-SMA levels were decreased suggesting that CXCL1 may be mediating cardiac fibrosis though TGF-Smad2/3 signaling pathway [158]. GM-CSF promotes myeloid cell development and maturation [159]. In studies of Kawasaki disease, a pediatric heart disease that is characterized by cardiac inflammation and infiltration of neutrophils and monocytes, it was found that cardiac fibroblasts were a major source of GM-CSF. Additionally, GM-CSF was found to drive cardiac inflammation as it functioned as a pro-inflammatory cytokine. It was found to be rapidly and selectively expressed in the heart during initial disease progression and switched on inflammatory gene profile of resident macrophages[160].

It is quite clear that cardiac fibroblasts play a major role in heart diseases. Hence, a deeper investigation into the role of these cells in Dox-induced cardiotoxicity is warranted based on the preliminary data and previous studies in heart diseases.

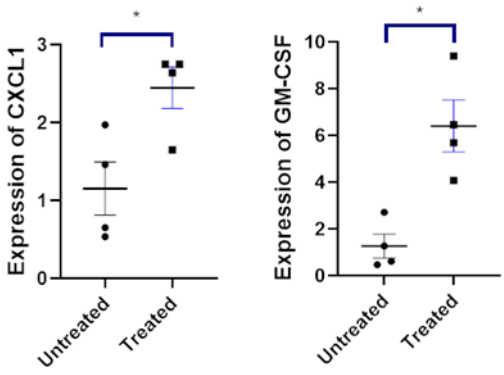

Figure 15: Evaluation of cytokines in cardiac fibroblasts. Expression levels of CXCL-1 and GM-CSF were evaluated using qPCR analysis. Data are presented as mean ± SEM, n=4 each, *p<0.05, a Mann-Whitney U-test was used to compare two groups.

Chapter Six: Methodology

This chapter is based upon “Bhagat A, Shrestha P, Jeyabal P, Peng Z, Watowich SS, Kleinerman ES. Doxorubicin-induced cardiotoxicity is mediated by neutrophils through release of neutrophil elastase. Front Oncol. 2022 Aug 10; 12:947604” as a part of CC-BY Creative Commons attribution license.

Materials and Methods:

Mice: Our juvenile cardiotoxicity mouse model was used to determine the role of neutrophils in Dox-induced cardiotoxicity [33,34]. As Dox-induced cardiotoxicity has been shown to be increased in females, female 4-6 weeks old C57BL/6 mice were acquired from Experimental Radiation Oncology at MD Anderson Cancer Center (Houston, TX, USA). Female 4-6 weeks old Balb/c mice were acquired from Charles River Laboratory, Frederick. NE-deficient mice ($NE^{-/-}$) of C57BL/6 background were kindly provided by Dr Stephanie Watowich. All mice were maintained in a pathogen-free animal facility and used in accordance with IACUC approved protocols. All experiments were performed in mice on Balb/c background, except for experiments involving neutrophil elastase, for which C57BL/6 mice were used.

Echocardiography: Anaesthetized mice were assessed for cardiac function using transthoracic echocardiography (Vevo 3100 echocardiography with a 40MHz linear signal transducer and 550D probe; VisualSonics, Toronto, CA). M-mode short axis images were recorded at the level of the papillary muscles. The left ventricular (LV) muscle was bisected to obtain the optimal M-Mode(multimodal) selection. For each mouse, at least five B-mode and five M-mode images were recorded. All images

were saved for analysis**.** Conventional echocardiographic measurements of the left ventricular function included ejection fraction (EF), fractional shortening (FS), end-diastolic dimension (LVID(d)), end-systolic dimension (LVID(s)), and anterior and posterior wall thickness. For long axis B-mode measurements, the endocardium was traced beginning from the mitral valve and excluding the papillary muscle. EF and FS were calculated by Vevo Lab software and is expressed as change from baseline measurement that is taken before Dox treatment.

Neutrophil depletion: InVivoPlus anti-mouse Ly6G(IA8) (Bioxcell BP) antibody was used to deplete neutrophils. On days 2 and 9 of the experiment, anti-mouse Ly6G (500 µg) was administered intra-peritoneally. To confirm successful depletion blood samples were collected via retro-orbital bleeding. The red blood cells were lysed using ACK lysis buffer and subsequently washed with phosphate-buffered saline (PBS). The subsequent single cell suspension was incubated in PBS containing anti-mouse Ghost Violet Dye 510 (Tonbo Biosciences 13-0870) for 15-30 min at 4°C to identify dead cells. This was followed by incubation in PBS with 2% FBS (FACS buffer) containing FcR block for 10 minutes at 4°C. Subsequently, samples were stained with fluorescently conjugated antibodies against murine cell surface markers for 90 min at 4°C using the following reagents: anti-mouse CD45 PECy7 (Tonbo Biosciences), anti-mouse Ly6G FITC (BioLegend) and anti-mouse CD11b APC-Cy7(BioLegend). Stained single-cell suspensions were analyzed on a BD LSR Fortessa (BD Biosciences). Data analysis was performed using FlowJo v10 software (FlowJo, Ashland, OR, USA)

Administration of Doxorubicin in vivo: Doxorubicin from TEVA/Actavis(2mg/mL) was resuspended in PBS to make up to a total volume of 100 µL at a dosage of 2.5mg/kg. Resuspended Doxorubicin was administered to the mice intravenously via the tail vein twice a week for 2 weeks on days 4, 6, 11 and 13 as previously described[33,34].

Collection of Heart Sections: On day 14, 24h after the last dose of doxorubicin mice were euthanized and hearts were removed and split into two sections. One section was stored at -80°C (the optimal cutting temperature medium) to generate slides for immunofluorescence staining. The other section was chopped into small pieces (~2 mm) with a razor. Heart pieces were incubated in 2 mL Hanks' Balanced Salt solution (HBSS). Type 2 collagenase (Worthington) was added at a dilution of 1:10 to the heart pieces in HBSS and incubated for 30 min in a shaking incubator at 37°C and 125 RPM. Digested cell suspensions were passed through 70 µm mesh filters; cells were subsequently washed with PBS. Following the wash, red blood cells were digested using ACK lysis buffer and then washed again with PBS in preparation for antibody staining.

Immune Profiling by antibody staining and flow cytometry: Single-cell suspensions were incubated in PBS containing anti-mouse Ghost Violet Dye 510 (Tonbo Biosciences) for 15-30 min at 4°C to identify dead cells. This is followed by incubation in PBS buffer with 2% FBS (FACS buffer) containing FcR block for 10 min at 4°C. The samples were then stained for 90 min at 4°C with the following antibodies against murine cell surface markers: anti-mouse CD45 PECy7 (Tonbo Biosciences), anti-mouse Ly6G FITC (BioLegend), anti-mouse F4/80 APC

(eBioscience), anti-mouse CD11b APC-Cy7(BioLegend), anti-mouse Ly6C PerCP/Cy5.5(BioLegend). The stained single-cell suspensions were then analyzed using a BD LSR Fortessa (BD Biosciences). Data analysis was performed using FlowJo v10 software (FlowJo, Ashland, OR, USA).

Real-Time PCR: Quantitative real-time reverse transcription polymerase chain reaction (RT–PCR) was conducted to verify the changes in mRNA expressions. Extraction of the total RNA from cardiac tissues was performed utilizing the TRIzol (Invitrogen, MO, USA) reagent. The list of the primer sequences used in the study: mouse CXCL-1:(Forward:5'- ACCCGCTCGCTTCTCTGT-3), (Reverse: 5'-AAGGGAGCTTCAGGGTCAAG-3).

Immunofluorescence staining: Frozen heart sections were fixed with acetone and then incubated with anti-mouse Ly6G antibody (Abcam), anti-mouse CD31 (BD Pharmingen) and anti-mouse NG2 (Santa Cruz Biotech. Fluorescence microscopy (Leica Microsystems) was used to analyze the slides. At least five different microscopy fields from different heart samples were examined using SimplePCI 6.0 software (Hamamatsu), and the average expression was quantified to determine relative expression.

Masson Trichrome stain: Heart sections from mice were embedded in paraffin and then fixed in Bouin's solution. Following fixation, sections were stained using the Sigma-Aldrich Trichrome Stain kit (Procedure No: - HT15), and images of stained slides taken using the Hamamatsu Nanozoomer. At least five different fields from different heart samples were analyzed using Leica Microsystems software (LAS X), and the average expression was quantified to determine relative expression.

Bone Marrow derived neutrophils: Femur and tibia from 4 to 6-week-old C57BL/6 control mice and NE-/- mice were collected and flushed with RPMI 1640 with 10% FBS and 1% penicillin/streptomycin. Histopaque 1119 and 1077 were used to create a density gradient to separate neutrophils from other immune cells. Purity of neutrophils was verified using flow cytometry.

Neutrophil Trans-well Migration Assay: The neutrophils were stained with CFSE (0.5 µM) dye and added to a 3 µm trans-well filter placed over the wells for 2 h. Transmigration was observed using an Incucyte system. Fluorescent intensity was quantified using Incucyte System S3 software. A chemoattractant for neutrophils: WKYMVm (conc: 100 nM) was used.

TUNEL assay: Apoptotic cells were assessed using TUNEL staining with a DeadEnd Fluorometric TUNEL system (Promega) according to manufacturer's instructions. Slides were fixed in 4% formaldehyde and then sections were incubated in TdT reaction mix for 1 h at 37^{o} C in the dark. Slides were then rinsed thrice with PBS and observed under a fluorescence microscope. The number of apoptotic cells was determined by counting the cells that were positive for both green fluorescence and DAPI using ImageJ analysis software.

Western blotting: Isolated hearts were homogenized, and the protein lysates obtained were run on a 7.5% polyacrylamide gel. The primary antibodies used for blotting included: cleaved caspase-3, caspase-3 and GAPDH (Cell Signaling Technology). Chemiluminescence was detected using ChemiDoc System (BioRad).

NE Inhibitor Treatment: Mice were injected with AZD9668 intraperitoneally twice a day(100mg/kg) for the duration of Dox treatment[161] .

Statistics: Prism 8 software (GraphPad Software, San Diego, CA, USA) was used to perform statistical analyses. Data are shown as mean ± the standard error of the mean. An unpaired, two-tailed *t* test or Mann-Whitney test was used to compare two groups. A one-way ANOVA with Tukey comparison was performed to compare more than two groups. Differences were considered significant when $p<0.05$.

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VITA

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