The Influence of Conventional Phthalate Plasticizers Versus Emerging and Novel Non-Phthalate Plasticizers on Recovery From Cardiac Injury Using a Mouse Model of Surgically Induced Myocardial Infarction

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August 2020

A Thesis

Submitted to McGill University

In Partial Fulfillment

Of the Requirements of the Degree of

Master of Science

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I. Abstract

Phthalates, plasticizers that make plastics soft and malleable, escape from medical devices. Whether they impede patient recovery is unknown. According to the 2019 Canadian Health Measures Survey, phthalates were determined to present a significant risk to human health, causing harm to many bodily systems. Replacement of current phthalates, such as di-ethylhexyl phthalate (DEHP) and butyl-benzyl phthalate (BBzP), with stable, biocompatible plasticizers remains a promising option. Tris(2-ethylhexyl) trimellitate (TOTM), a commercially available mellitate plasticizer, is the most common replacement. Several novel, “green” plasticizers, including di-heptyl succinate (DHS) and di-octyl succinate (DOS), demonstrate limited elution from polyvinyl chloride *in vitro* and low toxicity *in vivo*. However, the safety of TOTM, DHS, and DOS is not well established in the context of patient recovery. To study the direct sex-specific effect of clinically based chemical exposures during a model of cardiac recovery, we exposed male and female mouse models of cardiac surgery to DEHP, BBzP, TOTM, DOS, and DHS, and assessed survival, cardiac structure and function, immune cell infiltration, and markers of inflammation. To examine the direct effects cells involved in wound healing, we treated human THP-1 macrophages with the plasticizers *in vitro*. *In vivo*, male mice treated with DEHP and TOTM had greater cardiac dilation, reduced cardiac function, and increased infiltration of neutrophils, monocytes, and macrophages suggesting impaired recovery. In contrast, the impact of DEHP and TOTM was reduced in female mice and male mice treated with non-phthalate plasticizers, DOS and DHS. Moreover, treatment of human THP-1 macrophages with phthalate-based plasticizers induced a dysfunctional proinflammatory macrophage phenotype. The data indicates that males are affected by phthalates and mellitates to a greater extent than females. These results suggest that replacing current plasticizers with non-phthalate-based plasticizers may improve patient recovery, especially in the male population. In our assessment of commercially available and novel alternatives, DHS provides the most promising possibility for a non-toxic biocompatible plasticizer.

Il a été démontré que les phtalates, plastifiants qui rendent les plastiques souples et malléables, s'échappent des dispositifs médicaux et nuisent par le fait même au rétablissement des patients. L'Enquête canadienne sur les mesures de la santé 2019 a déterminé que les phtalates présentent un risque important pour la santé humaine, causant des dommages à de multiples systèmes corporels. Le recours à des plastifiants stables et biocompatibles pour remplacer les phtalates actuels tels que le phtalate de di-éthylhexyle (DEHP) et le phtalate de butyl-benzyle (BBzP) est une option prometteuse pour optimiser la convalescence des patients. Le tri-octyl-trimellitate (TOTM), un plastifiant mellitate disponible sur le marché, est le substitut le plus courant. Plusieurs nouveaux plastifiants «verts», y compris le succinate de di-heptyle (DHS) et le succinate de di-octyle (DOS), démontrent une élution limitée du chlorure de polyvinyle *in vitro* et une faible toxicité *in vivo*. Cependant, la sécurité du TOTM, du DHS et du DOS n'est pas bien établie en ce qui concerne le rétablissement des patients. Afin d'étudier l'effet direct et spécifique au sexe d'une exposition chimique clinique, nous avons exposé des souris témoins mâle et femelle, modèles de chirurgie cardiaque, au DEHP, BBzP, TOTM, DOS et DHS, et évalué la survie, la structure et la fonction cardiaques, l'infiltration de cellules immunitaires et les marqueurs de l'inflammation. Afin d'examiner l'impact direct des plastifiants sur la réponse inflammatoire, nous avons traité des macrophages THP-1 humains avec les plastifiants *in vitro*. *In vivo*, les souris mâles traitées avec le DEHP et le TOTM avaient une plus grande dilatation cardiaque, une fonction cardiaque réduite et une infiltration accrue de neutrophiles, de monocytes et de macrophages suggérant une récupération altérée. En revanche, les souris traitées avec des plastifiants sans phtalate, soit le DOS et le DHS, ont démontré une récupération améliorée. De plus, le traitement des macrophages THP-1 humains avec des plastifiants à base de phtalates a induit un phénotype de macrophage pro-inflammatoire dysfonctionnel. Les données indiquent que les mâles sont plus affectés par les phtalates et les mellitates que les femelles. Ces résultats suggèrent que le remplacement des plastifiants

actuels par des plastifiants sans phtalate pourrait améliorer la récupération des patients, en particulier chez les hommes. Selon notre évaluation des alternatives disponibles sur le marché, le DHS est le plastifiant biocompatible le plus prometteur.

II. Acknowsledgements

I would like to extend my sincere and heartfelt gratitude towards all of the individuals that have supported with the writing and completion of this research project. Firstly, I would like to express my gratitude to my supervisor, Dr. Lorraine Chalifour, for her unwavering patience, support, and guidance throughout this research project. Secondly, for my dad, Ivan, and sister, Rebecca, who not only encourage me but are a major reason that for the opportunities I am able to have today. I am thankful for my partner, Camille, for her endless positivity and continuous source of inspiration. I extend my gratitude towards my friends, for not only tolerating me, but also joining me on endless runs and cycling adventures. Finally, I would like to thank all of the members of the LDI AQ. You have all been incredibly kind, patient, and supportive.

III. Contribution to original knowledge

Our study directly compares the sex-specific impact of phthalate versus non-phthalate plasticizer exposure on the ability to heal from a clinically relevant cardiovascular insult, myocardial infarction (MI). Overall, our data are consistent with a model where phthalate (DEHP) and a common phthalate alternative (TOTM) are toxic to the injured heart and where the adverse impact on cardiac healing post-MI is centered on increasing inflammation and activation of the NLRP3 inflammasome in the infarct. Furthermore, we are the first to identify that post-surgery males are disproportionately affected by DEHP and TOTM exposure, compared to that of their female counterparts. In our evaluation of the safety and toxicity of non-phthalate plasticizer alternatives, the succinate-based plasticizer, DHS, most closely resembled vehicle treated mice and displayed reduced inflammation and improved healing compared to that of phthalate and mellitate plasticizers. We are the first to evaluate the efficacy of succinate-based plasticizers in this setting and determine that DHS is a safer alternative to current conventional plasticizers for both males and females.

IV. Contribution of Authors

M.Sc. candidate Adam Schwendt coordinated the research, carried out the experiments and data analysis, and drafted the manuscript. Dr. Lorraine Chalifour conceived of the study, participated in research coordination, assisted with experimental data collection and analysis, and edited the thesis manuscript. Mr. JiJun Shang assisted with experimental data collection. Dr. Richard Leask participated in research coordination and provided novel succinate-based plasticizers.

Abbreviations

BBzP, butyl benzyl phthalate; CVD, cardiovascular disease; DAMP, damage associated molecular pattern; DEHP, di(2-ethylhexyl phthalate); DHxS, di-hexyl succinate; DHS, di-heptyl succinate; DOS, di-octyl succinate; MI, myocardial infarction; NLRP3, Nod-, LRR- and pyrin domain-containing protein 3; PRR, pattern recognition receptor; PVC, polyvinyl chloride; TOTM, trioctyle trimellitate.

V. Introduction

A. Phthalates: everyday plasticizers

Polymers are a large class of materials that consist of many small molecules, monomers, that are linked together to form long chains (3). The global use of polymers continues to increase in nearly every area of modern living (4, 5). Additives commonly added to plasticizers include plasticizers, coloring agents, flow aids, heat stabilizers, and solvents. As a result of the widespread and growing use of additives, there is an increasing interest and concern of the impact that they have on humans, animals, and the environment.

Phthalates and phthalate esters are synthetic diesters of phthalic acid, first introduced as additives in the production of plastics in the 1920s. Phthalates are a large group of compounds used as liquid plasticizers that can be found in a wide variety of products and applications, including medical tubing and devices, gelling agents, adhesives, lubricants, cosmetics, dispersants, and emulsifying agents. They are also used in many household and consumer goods including PVC (polyvinyl chloride) interior surface coverings, food wrappings, shower curtains, nail polish, plastic goods, and kitchen plastic ware (6). Plasticized PVC, however, is commonly found in medical equipment, such as hospital tubing and IV bags, food wrapping, wire and cable insulation, and automobile parts (7). Approximately 90% of phthalates are used as plasticizers and the remaining 10% are found in solvents, inks, waxes, adhesives, cosmetics, insecticides, and pharmaceuticals (8). Plasticizers increase the spacing

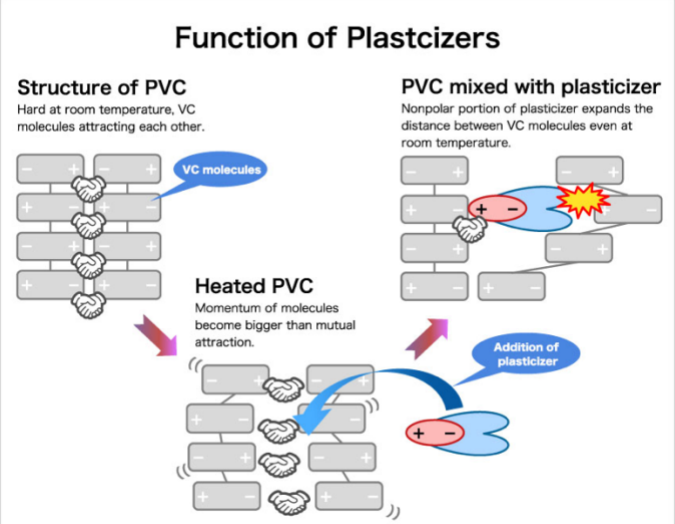

Figure 1. Function of Plasticizers.
VC (vinyl chloride) is rigid at room temperature and maintains its shape. The introduction of plasticizers prevents VC molecules from coming close together, softening the material. Adapted from (1).

between chains of crystalline polymers to make them more flexible, and, thereby, durable (9) (Figure 1). Because phthalates are not chemically bound to the plastic polymer chains, phthalates can migrate within the material and, even, leach into the environment. Furthermore, since these plasticizers are polar and relatively small molecules compared to large polymer chains, they are easily dissolvable in aqueous environments, therefore, contributing to the risk of exposure. Human exposure to phthalates occurs through the ingestion of contaminated food and water, inhalation, and dermal contact (10, 11). Indeed, phthalates are detectable in human urine, serum, and milk samples (12, 13), and the estimated daily exposure to one major phthalate, di(e-ethylhexyl)phthalate (DEHP), ranges from 3-30 *μg/kg/d* (14). Biomonitoring studies have been conducted in order to establish trends in phthalate exposure. The most recent study of 11,071 participants from the United States indicated that the metabolic byproducts of BBzP and DEHP were detected in nearly all participants (15). The US Environmental Protection Agency has expressed concern towards the pervasive human and environmental exposure to phthalates due to due to their toxicity (16).

B. Phthalate types

Phthalates are the most commonly used plasticizers, and within phthalates, DEHP is regarded as the most consequential (17). Phthalate plasticizers, such as DEHP, are synthesized from phthalic acid, in which the two carbonyl groups are in an ortho-position in respect to each other (Figure 2). Phthalic acid is esterified with various alcohols, but most commonly with 2-ethyl hexanol, to form liquid DEHP (18). DEHP is the most common plasticizer and accounts for approximately 50% of all plasticizers used in PVC, the most prevalent brittle plastic polymer (17).

Various interactions between the small plasticizer (I.e. DEHP) and the polymer (I.e. PVC) result in the desired plasticization (19). In the presence of plasticizers, long PVC chains interact less, and the material is rendered more flexible and malleable (Figure 1). To achieve

this, a large quantity of DEHP is generally required. For example, plasticized PVC can contain up to 40% of plasticizer by weight (20). DEHP has been labeled a ubiquitous environmental contaminant for several decades as a result of its leaching from materials (21). DEHP is particularly well investigated due to the extent of its toxic effects (18). Not only does DEHP pose significant health risks, in fact, DEHP produces stable metabolites that have been shown to be more toxic than DEHP itself (22-25).

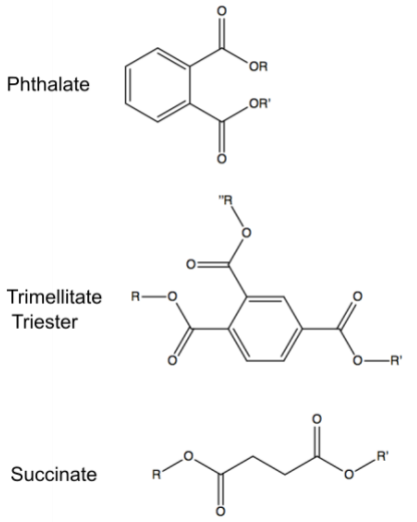

Figure 2. Chemical structure of plasticizers. Chemical structure for common plasticizers including phthalates (BBzP and DEHP), TOTM, and succinates (DOS and DHS)

Butyl benzyl phthalate (BBzP), also a member of phthalic acid esters, is classified by the US Environmental Protection Agency as a priority environmental pollutant and toxicant. BBzP is an aromatic ester manufactured by the reaction of the monobutyl ester of phallic acid with benzyl chloride (Figure 2). BBzP is mainly used as a plasticizer for PVC (26). Most people are exposed to BBzP primarily through food, but it can also occur through air and water (27).

C. Health concerns linked to phthalate exposure

The environmental and health consequences caused by phthalate exposure have attracted extensive global attention. The US Environmental Protection Agency has raised concern regarding substantial human and environmental phthalate exposures and has designated phthalates as priority environmental pollutants (16). In fact, these concerns and the growing evidence of health impacts has led to the ban of phthalate use in children's toys in the European Union (28), the USA (29), and Canada (30). Phthalates have been classified as potent toxicants and endocrine-disrupting chemicals based on their ability to interfere with normal reproductive function and hormone signaling (31). Similar to hormones, phthalates

exhibit complex dose-response curves, and they can act at extremely low concentrations to perturb normal physiological processes (32). Information on the toxicity of phthalates stems from a large database of studies in laboratory cells and animal models. Furthermore, many epidemiology studies have examined associations between phthalate exposure and various adverse health effects. Together, these findings indicate that phthalates can perturb normal cardiovascular, renal, digestive, hepatic, reproductive, metabolic, and developmental processes. Furthermore, the International Agency for Research on Cancer (IARC) classifies DEHP as a possible cause of cancer. The National Toxicology Program (NTP) says that DEHP "is reasonably anticipated to be a human carcinogen" (33). Similarly, a report by the WHO indicates that BBzP possess carcinogenic properties (26).

i. Developmental Effects

Disturbances to regular hormonal levels during development can perturb ontogenic processes. Phthalate exposure can emulate or antagonize hormonal systems and change developmental trajectories (34). The negative impacts of phthalate exposure present early in development and persist throughout adulthood (35). The disruptive nature of phthalates on normal development, notably reproductive and neurological, has been consistently documented. Human epidemiological studies and animal models indicate potential associations between maternal phthalate exposure and preterm birth (36), male genital anomalies (37-39), altered timing of puberty (40), and delayed mental, psychomotor, and neuroendocrine development (41). The repercussions of developmental phthalate exposure extend beyond reproduction and neurodevelopment. In fact, a plethora of evidence has established a strong connection between exposure and the disruption of glucose and lipid homeostasis, and metabolic disorders, including obesity and diabetes mellitus (42). The diabetogenic and obesogenic action of phthalates also presents a significant risk factor for cardiovascular disease (CVD).

ii. Immune Effects

In addition to interference with normal developmental trajectories, phthalate exposure has been shown to be associated with immune risks. The Comparative Toxicogenomic Database, phthalates were found to interact with 483 genes and proteins. Five of the top ten toxicity networks were found to be involved in inflammation (43). Robust epidemiological and experimental data indicates that phthalate exposure may result in an increased risk of enhanced immune responses (6) and respiratory problems (44, 45), namely wheezing and asthma (46, 47). In these studies, elevated immune responses include increase in immune cells in bronchoalveolar lavage fluid and lymph nodes, immunoglobulins, cell infiltration, airway remodeling in lungs, and airway responsiveness. Furthermore, phthalates influence cytokine secretion from both monocytes/macrophages and T cells and induce a proinflammatory phenotype. Together, phthalates appear to impact human innate and adaptive immunity and cell differentiation, regeneration, and inflammatory processes (48).

iii. *Cardiovascular Effects*

Phthalate exposure has emerged as a potential contributor to cardiovascular dysfunction (27). In fact, the cardiovascular system is particularly susceptible to phthalates and cardiotoxicity is one of the top phthalate toxicity categories (49). Robust epidemiological and experimental studies continue to indicate a complex and widespread influence of phthalates on the cardiovascular system.

Cardiomyocytes, the cells that make cardiac muscle, are connected to neighboring myocytes via intercalated disks, a highly organized framework consisting of mechanical and electrical connections (50). Intercalated disks are responsible for the integration of electrical and mechanical activity across the myocardial tissue, and, hence, proper function of the heart. Disruption of these intercellular junctions can increase the incidence of cardiac arrhythmias (51). Chemicals that disrupt cell-cell communication can have adverse consequences on intercellular

signaling and stability. Disrupted gap junction intercellular coupling can result in slowed electrical conduction in hearts, which was reported in neonatal cardiomyocytes exposed to phthalates (52). DEHP exposure slows electrical conduction in cardiomyocytes in a dose-dependent manner, with increased exposure exacerbating an arrhythmogenic phenotype (52). In rodents, DEHP-treatment has been shown to induce hepatocellular carcinomas, which may be a result of DEHP's inhibition of intercellular communication (53). Similarly, DEHP treatment inhibits gap junction intercellular communication in other cell types, including lung fibroblasts (54) and Sertoli cells (55).

The influence of phthalates on the cardiovascular system is not limited to cardiac arrhythmias. In fact, DEHP exposure has also been associated with cardiac malformations. Maternal exposure of DEHP led to congenital heart defects and altered gene expression of important cardiac transcription factors in mice such as MEF2C, GATA4, and CHF (56). These transcription factors are critical to cardiac development and the expression of sarcomere genes (57). Moreover, parental occupational exposure to phthalates was shown to be associated with an increased risk of congenital heart defects, including patent ductus arteriosus, and atrial and ventricular septal defects (58). Furthermore, parental phthalate exposure has been shown to be associated with ventricular septal defects in children (59).

Phthalate exposure directly disrupted cardiac electrophysiology and contractile performance in multiple experimental models. A clinically relevant dose of DEHP exposure results in a rapid cardiodepressive effect, whereby treatment completely inhibits the spontaneous beating of chick cardiomyocytes after 30 min and results in 97-98% cell death within 24h of treatment (60). Rat hearts treated with doses of DEHP equivalent to that escaping from stored blood bags experienced a similar cardiodepressive effect, including a dramatic decrease in heart rate, coronary flow, and systolic tension (61). Moreover, atrioventricular conduction and repolarization time were significantly slowed in hearts exposed to DEHP (61). A comparable reaction can be observed in human cardiomyocyte automaticity, in which DEHP

exposure causes a decline of spontaneous beating rate (62). A phthalate dose comparable to patient plasma levels after exchange transfusion, increased atrioventricular conduction time, and slowed epicardial conduction velocity (63). The slowed conduction velocity may be a factor of impaired gap junction intercellular coupling, which has been reported in DEHP exposed cardiomyocytes (52, 64).

The underlying mechanisms of phthalate exposure on the cardiovascular system remain largely elusive. DEHP-treatment alters the mRNA expression of ion channels and calcium-handling genes in neonatal cardiomyocytes (65). Changes in gene expression may contribute to the phenotypic observations in cardiomyocytes and heart tissue, considering that both automaticity and contractility are sensitive to changes in calcium ion homeostasis (64). In support of this notion, DEHP-treated cardiomyocytes exhibited reduced spontaneous beating rate, decreased calcium transient amplitude, and altered calcium transient dynamics (62). Furthermore, phthalate exposure has been shown to inhibit potassium channels that are critical for cardiac repolarization and the resting membrane potential (63, 66).

Phthalates have been implicated in interfering with nuclear receptors and modulation of gene expression (67). DEHP exposure upregulates PPARα to increase the use of fatty acid substrates in cardiomyocytes (65). As a consequence of this upregulation, exposure to DEHP results in metabolic remodeling of cardiomyocytes, namely cardiac cells increase their dependence on fatty acids for energy production (65). Chronic dependence on fatty acids is associated with an accumulation of lipid intermediates, lactate, protons, and reactive oxygen species – a dependence that can sensitize the heart to ischemic injury and ventricular dysfunction. A disruption in cardiomyocyte metabolism caused by DEHP exposure results in an increase in inflammatory and oxidative stress markers (68).

Phthalate exposure during early life can lead to vascular adaptations that increase the risk of cardiovascular disease later in life (69). For example, an increased urinary phthalate concentration has been shown to be associated with elevated systolic blood pressure (70), a

potential byproduct of increased cortisol (71) or increased oxidative stress (72). Additional studies have identified an association between urinary phthalate levels and biomarkers of oxidative stress (73-75). In elderly populations, the risk of coronary heart disease, atherosclerosis, and downstream complications, including MI, increases with the concentration of circulating phthalates (76, 77).

In conclusion, environmentally and clinically relevant doses of phthalate exposure have emerged as a potential contributor to cardiovascular dysfunction. The current understanding of phthalate cardiovascular toxicity stems from a collection of epidemiological and experimental studies that indicate a widespread influence, including increased risk of arrhythmias, coronary heart disease, atherosclerosis, malformations, increased blood pressure, and reduced cardiac function. The impact of phthalates on the cardiovascular system is particularly concerning in clinical settings and carries important safety implications, especially considering that changes in blood pressure and heart rate variability are risk factors for cardiac complications.

D. Impact on cardiac performance and expression

In summary, because phthalates structurally resemble natural hormones, they can exert endocrine-disrupting qualities and interfere with a range of important physiological processes. As a result, phthalates have a global impact on human health that is multifactorial. Epidemiological studies and data from cellular and animal models have reported associations between phthalate exposure and a broad collection of health conditions, including developmental impairments, metabolic disturbances, reproductive disorders, inflammatory conditions, carcinogenic properties, and cardiovascular disease (78, 79). Of the myriad of complications, the latter is particularly alarming. The patients who are most susceptible to hospital-based phthalate exposure are often at increased risk for cardiovascular complications, including those undergoing transfusions or support procedures. Local and systemic interactions

with phthalates may add an extra obstacle to patients that are prone to secondary complications.

Approximately 38,000 Canadians a year undergo cardiac surgery. 25% of coronary bypass graft (CABG) require acute care or hospital readmission within 65-days of discharge to treat serious adverse events (80-82). Despite the rapid progression and development of cardiovascular therapies, the number of patients with cardiovascular disease and its comorbid conditions continues to grow. Despite the use of high-intensity statin treatment and other standard-of-care measures still face an unacceptable burden of recurrent cardiovascular events remains (83). Whether phthalates and other common plasticizers released from medical devices required to perform surgery and support patients during recovery adversely influence wound healing or contribute to poor outcomes remains largely elusive.

E. Medical equipment

Cardiovascular surgeries are common major surgeries that necessitate the substantial use of medical devices and, thus, expose at risk populations to a large quantity of phthalates. In adults, isolated CABG surgery is the most frequently performed cardiac surgery followed by aortic valve replacement (AVR) and CAB+AVR surgery. These surgical interventions are assisted by the use of a cardiopulmonary bypass (CPB machine) to allow the surgeon to operate on an arrested heart. While DEHP has been banned for use in children's toys (28-30), it remains heavily used in hospital equipment due to its low cost (84). Infusion pumps, indwelling catheters, draining tubing, enteral feeding tubes, endotracheal tubes, and intravenous bags used for blood product and drug delivery may remain in place for 24 hours or longer (85, 86). Additionally, the plastic circuitry of the CPB machine, including its blood reservoir, filters, oxygenator, and extensive tubing (~20-30 feet) contains phthalate plasticizers. Some of these devices remain in place after surgery in the ICU and continue to leech plasticizers into the patient. Medical devices can consist of up to 40% DEHP in intravenous bags and 80% DEHP in

medical tubing. As a consequence of their hydrophobicity, phthalate esters are highly susceptible to leaching when in contact with blood, plasma, and other lipophilic solutions (87, 88). Of the incorporated DEHP, up to 21% can leach into patients (89). In fact, in a Belgian study of 35-adult patients in the intensive care unit, metabolites of DEHP were increased 100- to 1000-fold (90). Similarly, our recent study quantified the elution of ten different phthalates in a clinical setting; within 12-hours of cardiac surgery, and found DEHP was increased ~1600-fold and BBzP increased beyond their Tolerable Daily Limits (91). The greatest increase in phthalate concentration following cardiac surgery was observed with metabolites of DEHP, which increased 1000 to 1300-fold and those of BBzP, which increased 2000- to 3000-fold (92). Meanwhile, medical procedures in critically ill neonates were found to increase a patient's cumulative phthalate exposure to levels that were 4000- to 16000-times higher than deemed safe (93). These elevated phthalate concentrations can persist in the blood for hours to weeks, depending on the course of treatment (94).

Concentrations of DEHP in leachates from medical equipment are significantly higher than that of food and water (18). Indeed, the most intense exposure of individuals to DEHP occurs in hospital patients. Incidental plastic chemical exposure during medical procedures is essentially unavoidable, yet the direct implications on patient health remain unclear.

F. Phthalates and tissue healing

Monocytes, macrophages, and neutrophils are the protagonist immune cells of mammalian tissue repair. Soft tissue injury repair mechanisms are preserved in evolution and essentially equivalent in mice and humans (95-99). In all tissues, regardless of whether the trauma is a result of surgery, reperfusion injury, or cell damage, injury repair proceeds in the same path in the absence of infection, also referred to as "sterile inflammation." Mammalian wound healing initiates with blood stasis, followed by three overlapping phases: inflammation, proliferation, and remodeling. Within seconds to minutes after blood vessel injury, platelets

arrive and promote clot formation. Additionally, factors are released that attract immune cells and thereby begin the inflammatory phase. Chemokines and damage-associated molecular pattern molecules (DAMPs) released from dead and dying cells after surgical trauma, reperfusion injury, and other damage stimulate circulating monocytes and neutrophils to migrate to the wound (100). Neutrophils produce proteases and pro-inflammatory cytokines. At the wound site, monocytes and macrophages (MΦ) phagocytose dead cells and debris, and secrete proteolytic enzymes that release the growth factors necessary for future angiogenesis, tissue repair, and recovery (98). This marks the beginning of the proliferative phase. During the remodeling stage, the extracellular matrix matures and increases in tensile strength.

A dysfunctional, excessive infiltration or impaired macrophage response negatively impacts tissue repair, however. The coordinated infiltration of neutrophils, monocytes, and macrophages (MΦ) is critical for wound healing in all mammals regardless of the injury (95-97, 100-102). Infiltrating cells respond to spatiotemporal cues and transform within the wound niche to address the multidimensional demands of complete healing. Monocytes and macrophages respond to chemokines such as CCL2 (97, 98, 100), which acts on the macrophage receptor CCR2 (103, 104). The monocyte / macrophage phenotype is nuanced, and at its extremes labelled as pro-inflammatory (M1-type) or anti-inflammatory (M2-type). Pro-inflammatory monocytes / macrophages, or M1-type, are the most abundant cell type in the wound within 1-2 days (105). Pro-inflammatory macrophages stimulate the release of reactive oxygen species and nitric oxide, inflammatory cytokines (including TNFα and IL-1β), display phagocytic activity, and remove dead cells by efferocytosis (104, 106). Their secretion of matrix metalloproteinases (MMP) allows the release of growth factors such as TGFβ, which then stimulates collagen production and induces angiogenesis (107). Together, these activities expedite the elimination of damaged cells and debris. Afterwards, M1-type macrophages transform into the anti-inflammatory M2-type that promote the deposition of supporting extracellular matrix proteins and angiogenesis. The transition between pro-inflammatory and anti-inflammatory repair is the

decisive step that dictates whether wound healing stalls in the acute injury phase or progresses towards resolution.

In consideration of the complex inflammatory cascade that dictates wound healing, an important question becomes: how does exposure to disruptive chemicals, such as phthalate plasticizers, impact the pro-inflammatory and anti-inflammatory processes that underlie effective tissue repair? Laboratory and population-based studies have linked increasing phthalate exposure to increased prevalence of inflammation-related disease during cardiovascular disease (72, 108), indicating that phthalate exposure is pro-inflammatory. In fact, in a mouse model of cardiac surgery, phthalate exposure is linked to reduced survival, greater cardiac dilation, reduced cardiac function, and increased infiltration of neutrophils, monocytes, and macrophages, suggesting impaired recovery (91). Similarly, treatment of human macrophages with DEHP or DEHP metabolites increases pro-inflammatory cytokine production, reduced phagocytic activity, and increased MMP secretion (91, 109, 110). These data suggest that phthalate exposure promotes a macrophage population that is pro-inflammatory and dysfunctional. Thus, the accumulated evidence suggests that acute or chronic exposure to phthalates may reduce appropriate monocyte infiltration, promote a proinflammatory response, and impair cardiac function, thereby impeding patient recovery.

G. Danger associated molecular pattern molecules (DAMPs) in wounds activate NLR Family Pyrin Domain Containing 3 (NLRP3)

Migrating monocytes encounter Damage Associated Molecular Patterns (DAMPs) at the wound site (111). DAMPs include mitochondrial DNA or RNA, released as a result of cell destruction, and heat shock proteins or degradation products of fibronectin, secreted by damaged cells. DAMPs are recognized by highly conserved germline-encoded immune signaling receptors, pattern recognition receptors (PRRs). Notable subtypes of PRRs include: C-type lectin receptors (CLRs), Toll-like receptors (TLRs), Retinoic acid-inducible gene I (RIG-I-

)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and AIM-like receptors (ALRs) (112). PRRs are classified based on location and function. TLRs, such as TLR4, are located on the plasma membrane. Intracellular PRRs are exemplified by NLR Family Pyrin Domain Contain 3 (NLRP3). TLR-type receptors respond to extracellular signals and bacterial molecules. Meanwhile, intracellular PRRs function as a “back-up” and provide a synergistic response to persistent insults. provide a synergistic response to persistent insults.

NLRP3, the best studied intracellular PRR, is predominantly expressed by monocytes and macrophages (113, 114). In fact, NLRP3 is the main sensor of sterile inflammation signals and is the primary PRR activated by tissue damage (115). Increased NLRP3 inflammasome activity is associated with a variety of chronic diseases linked to inflammation and ageing (112). In multiple models, including post-MI injury, NLRP3-specific inhibition reduces inflammasome activity (116-120), and reduces infarct size and preserves cardiac function (121-125). Overall, increased NLRP3 inflammasome activity is detected in diseases linked with pro-inflammatory monocytes and macrophages.

The NLRP3 inflammasome is a multi-protein complex, which consists of NLRP3 (the receptor), NEK7 (an assembly aid specific to NLRP3), ASC (adaptor protein), and Caspase-1 (effector protein). Inflammasome activation proceeds in two steps: The first or “priming” step (Signal 1) involves the increase of NLRP3 inflammasome component mRNAs as a result of TLR4:IL-1R → MyD88:IRAK4 → NF-κB:AP-1 signaling. NF

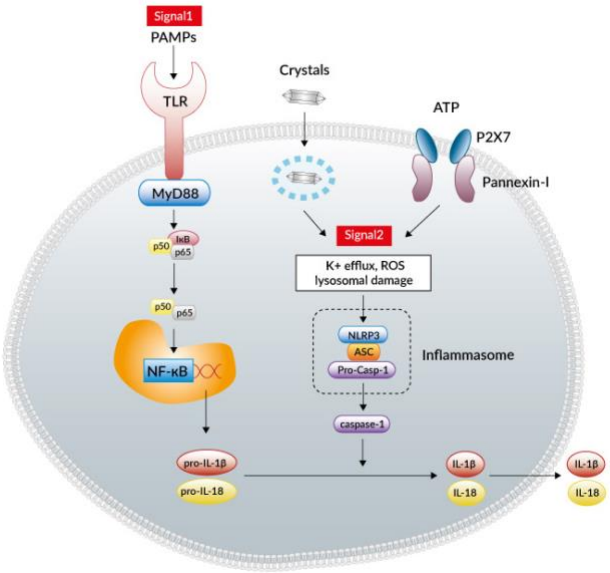

Figure 3. NLRP3 Inflammasome Activation.
The activation of NLRP3 initiates the formation of a multi-protein complex known as the inflammasome. Activation results in the recruitment and activation of pro-caspase-1 and subsequent processing of IL-1β into its mature form. Adapted from (2).

κB is a transcription factor that regulates genes responsible for innate and adaptive immune responses. The second step involves the assembly of NLRP3, NEK7, ASC, and pro-caspase-1 in the cytosol into the active inflammasome (117) (Figure 3). The assembly of the NLRP3 inflammasome is induced by a variety of agents that increase K^+ efflux or provoke lysosomal damage (115). After the complex is formed, p45kD pro-Caspase-1 is cleaved to p20kD capsase-1. Caspase-1 activity cleaves pro-interleukins IL-1β and IL-18, releasing their mature, pro-inflammatory forms. Secreted IL-1β binds IL-1R, which promotes the expression of other inflammatory genes and amplifies the response.

The connection between phthalates and inflammasome induction is supported by *in vitro* evidence. Treatment of human THP-1 monocytes with high dose DEHP promotes NF-κB nuclear translocation, indicating increasing priming, and increased IL-1β secretion, a marker of inflammasome activation (110). Together, the association between phthalate exposure and inflammasome activation, and the well-documented connection between inflammasome activity and disease suggests that phthalates induce a proinflammatory phenotype through the NLRP3 pathway.

H. Summary: Risks associated with phthalate exposure

While phthalate-based plasticizers remain widespread in common materials and commercial products, accumulating evidence continues to indicate negative health and environmental impacts associated with phthalate exposure. To summarize, the health repercussions are multifarious and complex. To date, phthalates have been labelled as endocrine disrupting chemicals that perturb normal development and reproduction, exacerbate disease, and carry carcinogenic potential. Furthermore, DEHP has been shown to be rather resistant to biodegradation (126-129). Once DEHP is in the environment, it strongly bonds to organic matter deep in soils and aqueous environments (130). Despite the presence of

microorganisms capable of biodegrading DEHP, strong bonding to suspended soils makes mineralization of DEHP a slow process.

I. Green plasticizer alternatives: rationale and necessity

In consideration of the health and environmental repercussions associated with phthalate use, it is critical to replace phthalates with less problematic alternatives. Several candidate “green” plasticizers were designed based on DEHP’s biodegradation pathways to avoid producing breakdown biproducts known to be toxic or persistent. These alternatives include diesters based on succinic acid, maleic acid, and fumaric acid, which resemble phthalates structurally, and are esterified with linear alcohols to avoid the buildup of DEHP’s biproducts following biodegradations. These compounds were found to be effective plasticizers and rapidly biodegradable. Succinates biodegrade most rapidly from the candidate alternatives (131).

Thorough testing in diverse contexts is needed to validate these replacement compounds to ensure their utility as plasticizers, and to provide sufficient evidence that they are, in fact, a safer alternative to current phthalate-based plasticizers. A greener, safer plasticizer should have the following characteristics: good plasticizing capacity, rapid biodegradability, and low to no toxicity (132). Together, these factors will carry the potential to either drive the industry to adopt them and/or regulatory agencies to ban the use of phthalates in favor of the alternatives.

Trimellitate triesters, namely tri(2-ethylhexyl) trimellitate (TOTM) (essentially DEHP with an added ester function) is a phthalate resembling alternative (Figure 2). TOTM is the most common replacement plasticizer for DEHP in clinical settings, albeit its safety is not well established(133, 134). A lower rate of elution from PVC would support that TOTM is a more stable and safer alternative to DEHP. However, testing from isolated PVC samples has shown greatly contrasting estimates for TOTM escape(134, 135). A major weakness of elution studies

is that they assess plasticizer escape *in vitro*, with the use of single device or pieces of devices. Although instructive, these data do not accurately reflect human exposure to multiple devices simultaneously in clinical settings. *In vivo* studies with animal models offer the potential to more accurately indicate the safety of phthalate alternatives.

Recent technological advances in biobased production have generated effective, benign, biobased plasticizers using naturally derived end products. While phthalates are petroleum-based, di-ester succinates are produced from the fermentation of biomass. Since phthalate synthesis requires the products of petroleum refinement, a shift towards succinic acid-based plasticizers reduces carbon offsets and the corresponding footprint (136). Moreover, diesters of succinate acid are involved in the mammalian citric acid cycle and, as natural metabolites, are candidates for less toxic alternatives to current phthalate-based plasticizers (Figure 2). Succinates with varying lengths of alkoxyl chains have been shown to be compatible with PVC (137), including di-hexyl succinate (DHS) and di-octyl succinate (DOS). In fact, 6 to 8 carbon alkoxyl chains were shown to be the most efficient succinate-based plasticizers (137). The percent plasticizer leached decreases with increasing total chain length of succinates. However, no difference was found between a 7-carbon chain and an 8-carbon chain. The reduction in leaching is due to the increase in hydrophobicity of the compounds as the length of the alkyl chain increases. As the plasticizer's structure becomes increasingly non-polar, the plasticizer is less likely to leach out into a polar solvent. Several of these "green" succinate-based plasticizers, including di-heptyl succinate (DHS) and (DOS), have been developed at McGill University by Dr. Richard Leask's research group (126). (138). In an elution study for common plasticizers, di-hexyl succinate and di-octyl succinate leached 0% in the first week. By three weeks, DHxS (6-carbon) leached 0.33%, while DOS (8-carbon) leached 0%. As a group, succinates leached less than commercially available phthalates, dibenzoates, fumarates, and maleates (131), making them worthy of consideration as replacements for DEHP. To date, DHS demonstrates little elution from PVC *in vitro* and no toxicity *in vivo* in reproductive tissue,

marking it a promising candidate as a safe alternative to currently used plasticizers. Similarly, in a two-generational study, DOS was shown to exhibit no acute toxicity or reproductive toxicity (136). Testing the impact of these plasticizers *in vivo* is an objective of this study.

J. Sex-specific response to phthalates and wound healing

The consequences of phthalate exposure are complex and varied. The responses to endocrine disrupting chemicals and tissue damage can be sex-specific. Indeed, the repercussions associated with phthalate exposure appear more pronounced in males (139, 140). In addition to maladaptive outcomes on the male reproductive system, DEHP exposure interferes with proper neurodevelopment in male rats (139). Meanwhile, in female rats DEHP induces microRNA production that preserves development (141). Phthalate exposure has been shown to be associated with gestational outcomes, thyroid function, and body fat distribution in a sex-dependent manner (140, 142, 143). Perinatal phthalate exposure was shown to increase body weight and adipocyte size in male rodents (144).

Correspondingly, inflammasome signatures are often sex- and disease-specific. In general, females generate a more robust and potentially protective humoral and cell-mediated response after antigenic challenges than their male counterparts. In cardiovascular patients, male peripheral blood mononuclear cells (PBMCs) were found to express significantly higher mRNA levels of *AIM2, NLRP3, ASC (PYCARD), Caspase-1,* and *IL-1β* than females (145). These findings indicate that male PMBCs (lymphocytes and monocytes) display a systemic proinflammatory state with primed inflammasomes that may contribute to disease pathogenesis. Emerging evidence has indicated that estrogen can act as an inflammatory protective factor to suppress NLRP3-mediated neuroinflammation in the hippocampus (146). On the contrary, testosterone is believed to promote innate immune cell activation and production of proinflammatory cytokines (147). Whether phthalates exacerbate a proinflammatory phenotype in a sex-dependent manner remains unknown.

Immune-mediated tissue recovery has also been shown to be sex-specific. Recovery from surgery in rodents can be improved through NLRP3 ablation in males, whereas NLRP3 ablation yields no improvements in females (148). This data indicates that post-surgical inflammatory processes appear to progress in an NLRP3-dependent fashion in males, but not in females. Moreover, females seem to possess an estrogen-mediated downregulation of proinflammatory inflammasome responses. The differences between male and female wound healing extend beyond the inflammasome response. Males display delayed and less regenerative basement membrane type VII collagen, worse patterns of elastin regeneration, and decreased elastase activity than females (149). Moreover, wound proteolytic activity is greater in females than in males. In fact, in wound healing studies, being male was identified as a primary risk factor (150). In the context of cardiovascular disease, inflammatory pathways contribute to the progression of adverse remodeling and are activated upon cardiac injury. The Y chromosome contains several genes involved in macrophage activation (151). Higher numbers of neutrophils and macrophages in males lead to an increased extent of inflammation, and together with a reportedly higher activity of matrix metalloproteinases (MMPs). This can cause premature breakdown of collagen and therefore cardiac rupture and slowed healing after injury (152). Moreover, TLR, JAK-STAT, Wnt, TNF, TGF-β, and BMP cardiac inflammatory pathways are activated to a lesser extent in females than in men, and male patients show increased expression of various inflammatory proteins upon LV remodeling (153). Furthermore, female hearts post-MI show lower rates of apoptosis and necrosis than males, which could be due to the presence of apoptosis-related genes on the Y chromosome (151). In summary, sex dimorphism is an important consideration in wound healing and in designing effective therapeutic interventions for patient recovery in medical settings.

To date, most clinical studies on cardiovascular disease have primarily included men, and the knowledge based on CVD and common treatments in women has been largely based on extrapolation. Although cardiovascular disease is more prevalent in men than in women for

all age groups, and women tend to acquire those diseases approximately 10 years later in life (153), women have higher mortality trends and experience more complications (154). Female sex is a predictor for early morbidity and mortality in the perioperative period for CABG surgery, with increased risk for death and postoperative complications than males. Conversely female sex may provide a survival benefit after 30 days postoperatively (155).

K. Conclusion

The ever-increasing use of polymers and polymer additives in everyday life and the fact that they are often not listed on product labels, makes plasticizer exposure nearly unavoidable. Consumers, infants and children, and hospital patients are all at risk for phthalate exposure. The population of individuals exposed to plasticizers in the greatest quantities, however, is comprised of medical patients, including post-surgery adults and premature neonates – highly susceptible individuals to the toxic consequences of plasticizer exposure. Thus, phthalate exposure represents a major public health concern and a significant threat to vulnerable populations.

The impact of increased exposure to phthalates that escape from medical devices and poor wound healing and/or adverse patient outcomes has not been considered. The sex-dependent impact of phthalates on tissue repair and inflammatory processes also remains elusive. Moreover, novel replacement plasticizers, such as TOTM or diesters of succinic acid, remain untested for their toxicity in this setting. Furthermore, the consequences of plasticizer escape on recovery in post-surgery individuals remains largely unknown.

Although some differences are evident, the pharmokinetics of phthalates are sufficiently similar in mice and humans that mice remain the standard model to assess the impact of human exposure to common plasticizers (156). This project employs a mouse model of cardiac surgery paired with the THP-1 human monocytic cell line to test novel non-phthalate-based plasticizers as promising candidates for improved patient outcomes in clinical settings. The objectives of this

master's project are to characterize the sex-specific impact of DEHP, TOTM, DOS, and DHS on survival and weight loss, heart function and dilation, markers of polarization, inflammasome activation, and the immune cell population *in vivo*. Moreover, I assess the impact of DEHP, BBzP, TOTM, DOS, and DHS on the expression of inflammasome genes and release of pro-inflammatory proteins in macrophages *in vitro.*

In conclusion, the aforementioned findings led me to hypothesize that phthalates synergize with Damage-associated molecular patterns (DAMPS) released from injured tissues to enhance inflammasome activation. The rank of inflammasome activation from most to least will be DEHP > TOTM >>> DHS & DOS. I hypothesize that the novel succinate-based plasticizers (DHS and DOS) will resemble VEH-treated mice and induce the least cardiac dilation, and active p17kD IL-1β compared with DEHP or TOTM. The high amounts of plasticizers that occur post-surgery will cause an increase in neutrophil, monocyte, and macrophage infiltration and in the pro-inflammatory monocyte and macrophage (MΦ) population that contributes to poor patient recovery. The consequences of phthalate exposure will be more pronounced in males than in females. Moreover, treatment of human THP-1 macrophages with phthalate-based plasticizers will induce a dysfunctional proinflammatory macrophage phenotype and DHS and DOS will resemble the VEH treatment.

VI. Materials and Methods

A. Materials

Parent phthalates, di-(2-ethyl hexyl) phthalate (DEHP, >99.5% pure, CAS 117-81-7), benzyl butyl phthalate (BBzP, 98% pure, CAS 26761-40-0) and tri-octyl trimellitate (TOTM, 99% pure, CAS 3319-31-1), were purchased from Sigma-Aldrich. Peanut oil (CAS 8002-03-7), was purchased from Sigma-Aldrich. Diheptyl succinate (DHS) and dioctyl succinate (DOS) were prepared by Dr. Richard Leask (Chemical Engineering Department, McGill University).

B. THP-1 male human monocyte cell line

The male-human monocyte cell line, THP-1, was cultured in RPMI media containing 10% FBS and antibiotics. For 6-well plate experiments, 1.5×10^6 cells/well were cultured for 48h in media containing PMA (50ng / mL media), to induce macrophage (MΦ) differentiation. The media was then replaced to remove PMA and 8 hours later, vehicle or chemicals were added at the indicated concentrations. DEHP, DHS, DOS, BBzP, or TOTM were dissolved in absolute ethanol and serially diluted in ethanol before use. Ethanol addition was similar regardless of concentration and did not exceed 0.4% of the media volume. After culture for 24h, the media was collected, and RNA or protein were isolated from washed adherent cells.

C. Animal manipulation

The Lady Davis Institute Animal Care Committee reviewed and approved the animal use protocol with animal experiments performed according to the guidelines of the Canadian Council on Animal Care. C57bl/6N mice (Charles River, St. Constant, Québec) were used. Mice were fed a Harlan Teklad Global 2018 diet and housed in polycarbonate cages with ¼’ corncob bedding and were exposed to a 12-h dark/light schedule.

D. Myocardial infarction surgery and treatments post-MI

The surgery core of the Lady Davis Institute performed the cardiac surgery (157, 158). Male (>21g) and female (>19g) mice between 3 and 5 months of age were anesthetized with isoflurane and intubated. Injection of slow release buprenorphine provided analgesia for the next 3 days. The left anterior descending coronary artery, approximately 1 mm distal to the left atrial appendage, was ligated permanently using a 7-0 silk suture. This surgery places approximately 40% of the left ventricle at risk for infarction (159). Animals were allowed to recover in a heated chamber and then returned to general housing.

The formula, human equivalent dose = [mouse equivalent dose x (km for mice/km for humans)], where the km for mice is 3 and the km for humans is 37, was used to calculate the mouse equivalent of the human exposure (160). The amounts of chemicals were chosen to reflect the median of patient exposure 12-h post-surgery (91). Mice were randomly selected to the different treatment options before surgery. Treatments began immediately after surgery and continued for 3 days.

DEHP, DHS, DOS, BBzP, TOTM, and vehicle (peanut oil) were dissolved in ethanol and diluted into peanut oil. An equal volume, 25 µL, of vehicle (peanut oil) or chemical diluted in peanut oil was delivered daily by micro-pipettor directly into the oral cavity.

On day 3 post-MI, differences in weight change were recorded. After echocardiography, mice were euthanized, the hearts excised and immediately or portions stored at -80°C until RNA or protein expression analyses.

E. Echocardiography

On day 3 post-MI, mice were anesthetized with isoflurane and echocardiography was performed using a VEVO 770 sonograph (VisualSonic, Toronto, Ontario). EKV-gated acquisitions of the long axis view were collected, and proprietary software was used to outline the LV wall in diastole and systole as previously described (91, 157, 158). These single plane-derived dimensions permitted calculations to estimate LV area, LV volume in systole and diastole,

stroke volume, ejection fraction and cardiac output (161). Similarly, EKV-gated acquisitions of the short axis view of the short axis view at the level of the papillary muscles allowed calculation of the fractional area change (FAC) .

F. Flow cytometry

To equate exposure with inflammatory cell infiltration on a cell-by-cell basis, single cell suspensions of whole heart tissue were prepared, and flow cytometry performed as previously described (91, 157). Briefly, mice were euthanized on day 3 post-MI, the whole heart dissected into small pieces and then placed in an enzyme mixture containing 675 U/ml collagenase I, 18.75 U/ml collagenase XI, and 9 U/ml hyaluronidase (Sigma-Aldrich) at 37°C for 90 min. The washed cell pellet was suspended in 40% Percoll, layered onto 80% Percoll and centrifuged at 2000xg for 20 min. Cells were collected from the interface, pelleted and incubated with Am-Cyan Live/Dead Fixable Dead Cell Stain (Molecular Probes, Carlsbad, California) for 30 min at 4°C, with 2.4G2 hybridoma (FC Receptor Block, ATCC: HB-197) for 30 min at 4°C, and then stained with fluorescently labeled antibodies for 30 min at 4C. The following antibodies were used. Brilliant Violet 785-conjugated anti-CD45 (30-f11, BioLegend), Brilliant Violet 650-conjugated anti-MHCII (M5/114.15.2, BD Biosciences), phycoethythrin/Dazzle 594-conjugated CD64 (x54-5/7.1, BioLegend), efluor450-conjugated anti-CD11b (MI/70, eBioscience), APC-efluor780-conjugated anti-CD11c (N418, eBioscience), Alexa 488-conjugated anti-Ly6G (RB6-8c5, eBioscience), APC-conjugated anti-Ly6C (AL-21, BD Biosciences), and phycoethythrin-conjugated anti-MerTK (2b10c42, BioLegend). To further characterize macrophage infiltration, CD64 MerTK macrophages were sub-fractionated to discriminate M1-type macrophages (CD38 positive) and M2-type macrophages (CD206 positive). Paraformaldehyde fixed samples were re-suspended in 300 µl FACS buffer and 10 µl of 123count e-beads (cat No. 01-1234, Affymetrix) added. Samples were analyzed by flow cytometry using an LSR Fortessa Cell

Analyzer (BD Biosciences, San Jose, California). Data were analyzed using FlowJo software v10.1 (Tree Star, Inc, Ashland, Oregon).

G. Expression analyses:

To quantify RNA expression changes in cells or tissues, n=6 per group, RNA purification was performed with a GENEzol TriRNA Pure Kit according to manufacturer's instructions (Genaid, New Taipei City, Taiwan). Briefly, samples were homogenized in GENEzol and DNA-free RNA was collected following DNase I digestion, RNA wash, and RNA elution.

First-strand cDNA was prepared using a 5X All-In-One RT MasterMix kit from ABM (Richmond, BC). Quantitative PCR was performed using technical duplicates or triplicates, a Green-2-Go qPCR Mastermix-Low ROX mix (cat. No. QPCR002-L, BioBasic Canada, Markham, Ontario), gene-specific primers and Applied Biosystems 7500 Fast sRT-PCR system (Life Technologies). Data were normalized to the housekeeping gene *hGapdh* for THP-1 cells and *mGAPDH* for mouse tissues. Fold change in gene expression was determined by the $2^{-\Delta\Delta Ct}$ method in comparison with expression in VEH-treated samples.

H. Protein isolation from heart tissue and cells

To examine protein expression, the ischemic area of the LV, or THP-1 cell pellets, were homogenized in RIPA buffer (1% NP-40, 50 nM Tris (pH 7.4), 0.5% deoxycholate, 159 nM NaCl, 0.1% SDS, 10mM sodium metabisulfite, proteinase inhibitor cocktail (Roche, Indianapolis, Indiana, and 1 mM PMSF) with a tissue homogenizer. Protein was measured using the Bradford Protein Determination Assay (BioRad, Hercules, California) against a standard curve prepared using bovine serum albumin as per the manufacturer's instructions.

I. Immunoblots

Protein expression was measured using a standard immunoblotting (IB) method. Briefly, proteins (10 µg) were separated using SDS-page and electrophoretically transferred to Immobilon P membrane (Millipore, Bedford, Massachusetts). Membranes were blocked, incubated overnight at 4°C with mouse anti-Caspase-1 (Adipogen) or rabbit anti-IL-1β antibody (Adipogen, 1:5000). Then with anti-mouse or anti-rabbit secondary antibody complexed to horseradish peroxidase and the interaction revealed using chemiluminescent detection kits (Pierce Chemical Co, Rockford, Illinois). Several exposures were collected onto x-ray film. After immunoblotting, membranes were washed, permanently stained with Coomassie Brilliant Blue, destained, and scanned using NIH Image J analysis software (NIH, Rockville, Maryland). Expression of Caspase-1 and IL-1β was corrected for any differences in protein loading by normalizing with the amount of protein detected on the stained membrane.

J. Statistical analyses

Differences between exposure groups as was evaluated by Student's *t* test for pairwise comparisons and one-way ANOVA or two-way ANOVA as appropriate for groups of >2 and the Tukey-Kramer post hoc test. A two-tailed *p* value of <.05 was considered significant. A Bonferroni correction was used for multiple comparisons.

A. Genes

Markers of polarization
NOS-2 (M1)
CCR7 (M1)
Egr-1 (M1)
CXCL10 (M1)
IL-6 (M1)
Fizzl-1 (M2)
IL-10 (M2)
CL3L (M2)
CD206 (M2)
CD163 (M2)
CCL22 (M2)
Chemokine receptor and ligand
CCR2
CCL2
Macrophage markers
MerTK (M1)
MMP9
Mfge8
Inflammasome receptors
NLRP3
NLRP6
AIM2
P2xR7
TLR4
NLRC4
IL-1R
CD36
Inflammasome pathway
Myd88
IRAK4
IL-1β
Caspase-1

K. Primers

Primer		Sequence
HUMAN GAPDH	Forward Primer	GAAGGTGAAGGTCGGAGTC
	Reverse Primer	GAAGATGGTGATGGGATTTC
HUMAN CXCL10	Forward Primer	GAATCGAAGGCCATCAAGAA
	Reverse Primer	CCTCTGTGTGGTCCATCCTT
HUMAN IL-6	Forward Primer	CACACAGACAGCCACTCACC
	Reverse Primer	TTTTCTGCCAGTGCCTCTTT
HUMAN CD206	Forward Primer	CTACAAGGGATCGGGTTTATGGA
	Reverse Primer	TTGGCATTGCCTAGTAGCGTA
HUMAN CD163	Forward Primer	GAGCTGAGGCTAGTGGATGG
	Reverse Primer	GAGCTGAGGCTAGTGGATGG
HUMAN CCL22	Forward Primer	GCCGTGATTACGTCCGTTAC
	Reverse Primer	GCTCTTCATTGGCTCAGCTT
HUMAN NLRP3	Forward Primer	CCACAAGATCGTGAGAAAACCC
	Reverse Primer	CGGTCCTATGTGCTCGTCA
HUMAN NLRC4	Forward Primer	TGCATCATTGAAGGGGAATCTG
	Reverse Primer	GATTGTGCCAGGTATATCCAGG
HUMAN IL-1R	Forward Primer	AAGGTGGAGGATTCAGGACA
	Reverse Primer	TCTCCTGCAACGGGTAGTTT
HUMAN TLR4	Forward Primer	CCTGACTCCTCAAGTCCAGAA
	Reverse Primer	ACAGAAATGGGTCGTTCATCAAA

HUMAN AIM2	Forward Primer	AGCAAGATATTATCGGCACAGTG
	Reverse Primer	GTTCAGCGGGACATTAACCTT
HUMAN P2xR7	Forward Primer	TATGAGACGAACAAAGTCACTCG
	Reverse Primer	GCAAAGCAAACGTAGGAAAAGAT
HUMAN IL-1β	Forward Primer	CCACAGACCTTCCAGGAGAA
	Reverse Primer	GTGATCGTACAGGTGCATCG
HUMAN Caspase-1	Forward Primer	GGCTCAGAAGGGAATGTCAA
	Reverse Primer	TCACCCCACTCTATCCTTG
HUMAN Mfge8	Forward Primer	ACCTGTTTGAGACCCCTGTG
	Reverse Primer	ATCTGCTTGTCAGGGATGCT
HUMAN MMP9	Forward Primer	TTC AGG GAG ACG CCC ATT TC
	Reverse Primer	AAC CGA GTT GGA ACC ACG AC
HUMAN C36	Forward Primer	TGCCTCTCCAGTTGAAAACC
	Reverse Primer	CTCCCTTCTTTGCATTTGCT

Primer		Sequence
MOUSE GAPDH	Forward Primer	CATGGCCTTCCGTGTTCCTA
	Reverse Primer	GCGGCACGTCAGATCCA
MOUSE NOS-2	Forward Primer	TTCACCCAGTTGTGCATCGACCTA
	Reverse Primer	TCCATGGTCACCTCCAACACAAGA
MOUSE CCR7	Forward Primer	AACCAAAAGCACAGCCTTCC
	Reverse Primer	ACGTTTTTCCTGGGTTTCCC

MOUSE Egr-1	Forward Primer	CAGTCCCATCTACTCGGCTG
	Reverse Primer	CAGTCCCATCTACTCGGCTG
MOUSE Fizzl-1	Forward Primer	TCCAGCTAACTATCCCTCCACTGT
	Reverse Primer	GGCCCATCTGTTCATAGTCTTGA
MOUSE IL-10	Forward Primer	GCTCTTACTGACTGGCATGAG
	Reverse Primer	CGCAGCTCTAGGAGCATGTG
MOUSE CL3L	Forward Primer	GGGCATACCTTTATCCTGAG
	Reverse Primer	CCACTGAAGTCATCCATGTC
MOUSE CCR2	Forward Primer	TGGCTGTGTTTGCCTCTCTA
	Reverse Primer	CCTACAGCGAAACAGGGTGT
MOUSE CCL2	Forward Primer	TTAAAAACCTGGATCGGAACCAA
	Reverse Primer	GCATTAGCTTCAGATTTACGGGT
MOUSE MerTK	Forward Primer	CAGGGCCTTTACCAGGGAGA
	Reverse Primer	TGTGTGCTGGATGTGATCTTC
MOUSE MMP9	Forward Primer	TCACACGACATCTTCCAGTACC
	Reverse Primer	ACCTCATGGTCCACCTTGTTC
MOUSE Mfge8	Forward Primer	TTGGGAAGGCTGGATAATCAGG
	Reverse Primer	GTGATGATTCCTGTCACTTGCC
MOUSE NLRP3	Forward Primer	TGC TCT TCA CTG CTA TCA AGC CCT
	Reverse Primer	CA AGC CTT TGC TCC AGA CCC TAT
MOUSE NLRP6	Forward Primer	CTCGCTTGCTAGTGACTACAC
	Reverse Primer	AGTGCAAACAGCGTCTCGTT

MOUSE AIM2	Forward Primer	ACCCGCAGTGACAATGACTT
	Reverse Primer	TGTTCTGCCACCATCTGTTT
MOUSE P2xR7	Forward Primer	TGTGAAGTCTCTGCCTGGTG
	Reverse Primer	TGTCCCCTAGTCGGAAGATG
MOUSE Myd88	Forward Primer	AGGACAAACGCCGGAACTTTT
	Reverse Primer	GCCGATAGTCTGTCTGTTCTAGT
MOUSE IRAK4	Forward Primer	CATACGCAACCTTAATGTGGGG
	Reverse Primer	GGAACTGATTGTATCTGTCGTCG
MOUSE IL-1β	Reverse Primer	GAAATGCCACCTTTTGACAGTG
	Forward Primer	TGGATGCTCTCATCAGGACAG
MOUSE Caspase-1	Forward Primer	GCCCAAGCTTGAAAGACAAG
	Reverse Primer	GGCCTTCTTAATGCCATCAT

VII. Results

A. Survival and weight loss

i. Impact of exposure on survival and weight loss post-MI in males

The impact of phthalate versus non-phthalate plasticizer exposure on recovery from MI is unknown. To investigate the potential of mellitate- and succinate-based plasticizers as an alternative to phthalate plasticizers and to assess recovery from a major wound *in* vivo, we exposed mice to the mouse equivalent of the 12h human chemical exposure as they recovered from MI (Figure 4A) (91). Mice were euthanized on day 3 post-MI to interrogate the immediate recovery period.

To evaluate the impact of plasticizer exposure on recovery post-MI in a clinical setting we began by recording body weight at the time of MI and at the time of euthanasia 3 days later. Survival to day 3 was also monitored. The results show that DEHP and TOTM treated males lost more weight compared to that of the other chemical cohorts (Figure 4C), although these differences did not reach significance. No major differences in survival post-MI were observed among the groups, (Figure 4D).

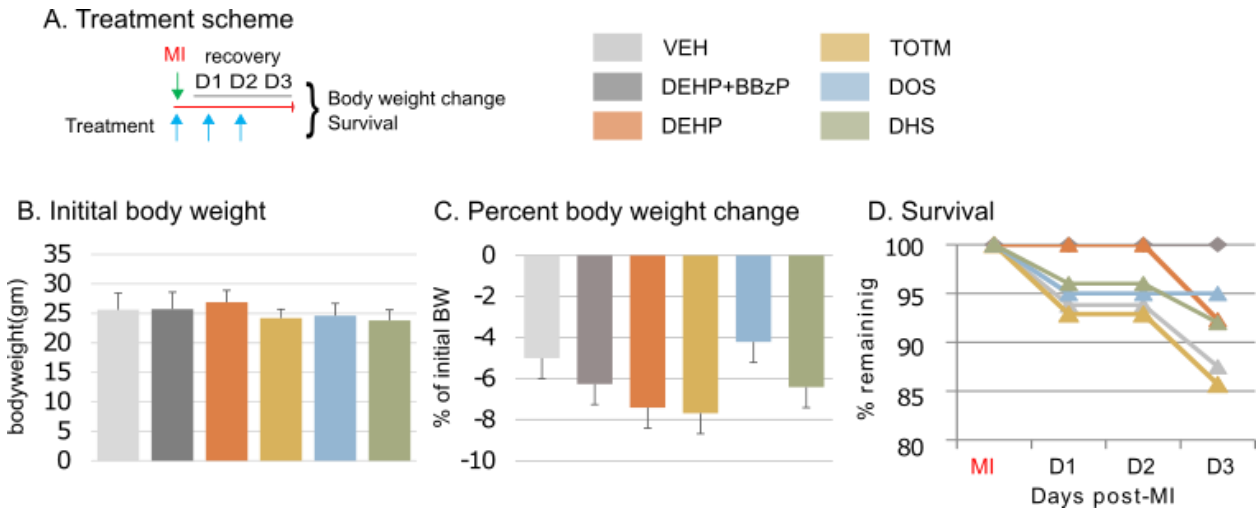

Figure 4. Impact of exposure on survival and weight loss post-MI in males.
Influence of phthalate and non-phthalate plasticizer exposure on survival and weight loss after MI. A, treatment scheme. Surgery was performed on male C57bl/6N mice to create a permanent blockage in the coronary artery. Cohorts were treated with VEH (peanut oil), DEHP+BBzP, DEHP, TOTM, DOS, or DHS (25 mg/kg/day). On day 3 post-MI, mice were euthanized. Survival post-MI was observed over time and body weight change was recorded on day 3 post-MI. B, Initial body weight. C, Percent body weight change. D, Survival of mice post-MI over time. Data are the mean ± SEM. A *p* value of <0.05 was considered significant.

ii. Impact of exposure on survival and weight loss post-MI in females

The trajectory of recovery in health after an acute MI is different between males and females (162). The factors responsible for the underlying sex-specific differences in post-MI outcomes remain largely elusive, however (163). Moreover, the sex-specific consequences of plasticizer exposure on recovery are unknown. To characterize the sex-specific impact of plasticizer exposure on post-surgery recovery, we treated female mice to the mouse equivalent of the 12h human chemical exposure as they recovered from MI (Figure 5A).

We observed no differences in body weight change (Figure 5C) or survival (Figure 5D) between females exposed to phthalate, mellitate, or succinate plasticizers. Female mice tended to fare better than their male counterparts after cardiac surgery, however. Females lost less weight post-MI (Figure 4C and Figure 5C) and experienced greater rates of survival for each chemical treatment group (Figure 4D and Figure 5D). Notably, the percentage of weight loss in female DEHP and TOTM treated mice versus VEH-treated female mice was significantly less weight than the percentage of body weight lost by male DEHP and TOTM treated mice versus VEH treated male mice. Specifically, DEHP and TOTM exposed males lost over 3 times more body weight than DEHP and TOTM treated females. These data indicate that DEHP and TOTM exposure has a greater impact on recovery in males than females. In contrast, DOS and DHS exposed animals resembled the VEH for both males and females.

In summary, we observed that all male exposure groups, including VEH, had increased mortality and weight loss compared to that of female mice post-MI. Hence, our data indicates that, overall, males experience lower rates of recovery post-MI. DEHP and TOTM exposure had

the largest impact on body weight loss in males. Meanwhile, female mice exposed to DEHP and TOTM did not experience the same body weight loss as their male counterparts, indicating that DEHP and TOTM exposure carries a sex-specific risk. In contrast to TOTM and DEHP, succinate-based plasticizers (DOS and DHS) resembled the VEH treatment group for both sexes.

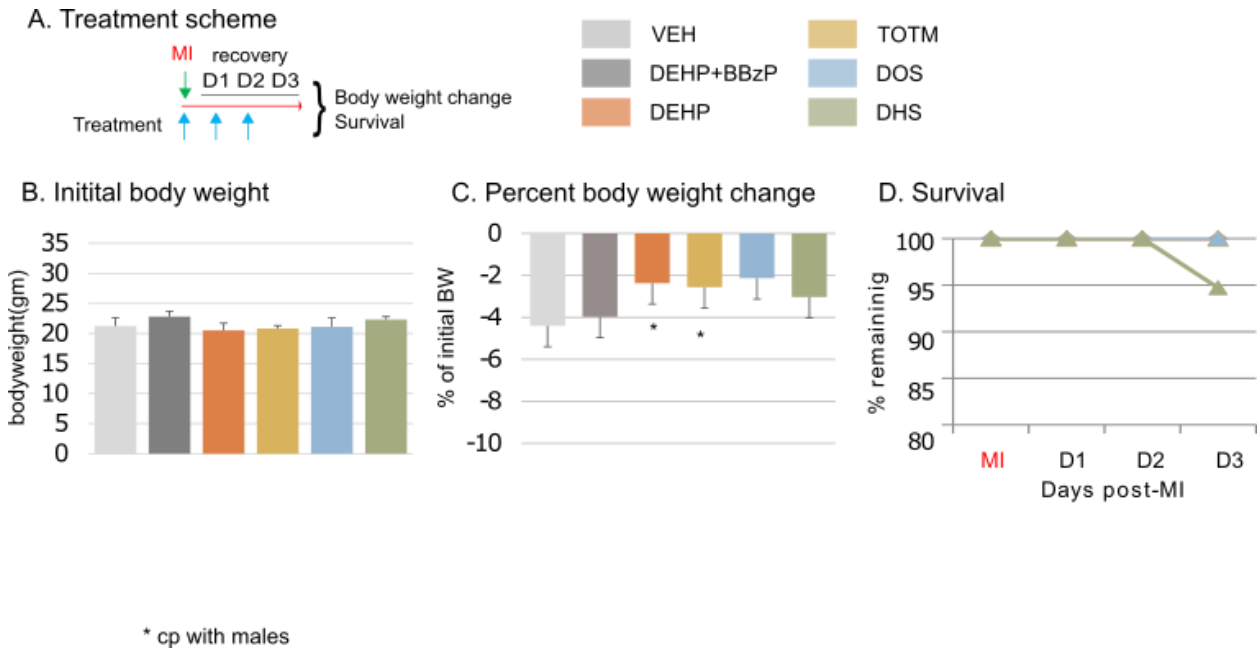

Figure 5. Impact of exposure on survival and weight loss post-MI in females.
Influence of phthalate and non-phthalate plasticizer exposure on survival and weight loss after MI. A, treatment scheme. Surgery was performed on female C57bl/6N mice to create a permanent blockage in the coronary artery. Cohorts were treated with VEH (peanut oil), DEHP+BBzP, DEHP, TOTM, DOS, or DHS (25 mg/kg/day). On day 3 post-MI, mice were euthanized. Survival post-MI was observed over time and body weight change was recorded on day 3 post-MI. B, Initial body weight. C, Percent body weight change. D, Survival of mice post-MI over time. Only two colours visible due to line overlay. Data are the mean ± SEM. A *p* value of <0.05 was considered significant and indicated by an * in comparison with male mice of the same cohort.

B. Heart dilation and function

i. Impact of exposure on heart dilation and function in males detected by echocardiogram

The influence of phthalate versus non-phthalate plasticizers on cardiac structure and function post-MI has never been considered in this context. To evaluate whether the weight loss in TOTM and DEHP exposed males is associated with damaged cardiac structure and function,

mice were anesthetized on day 3 after MI with isoflurane and echocardiography was performed using a VEVO 770 sonograph. The data reveal that the left ventricle (LV) area and volume (Figure 6B), was slightly elevated in systole and diastole in DEHP+BBzP, DEHP, and TOTM exposed mice compared to that of VEH, DOS, and DHS mice, indicating greater cardiac dilation. While these results did not reach significance, DEHP and TOTM treated animals experienced the greatest increase in LV area and LV volume. On the contrary, LV area and volume for DOS and DHS exposed animals did not exceed that of the VEH (Figure 6B).

To evaluate heart function, stroke volume (SV, volume of blood pumped out by the LV during each systolic contraction), cardiac output (CO, volume of blood pumped by the heart, equal to the heart rate X stroke volume), functional area change (FAC, global left ventricle systolic function), and ejection fraction (EF, percentage of blood leaving LV each contraction were measured (Figure 6C). The data show that DEHP+BBzP, DEHP, and TOTM treated mice had reduced SV, CO, FAC, and EF (Figure 6C). In summary, male mice exposed to phthalate and mellitate plasticizer experienced greater cardiac dilation and reduced cardiac function. However, no statistically significant changes in cardiac dilation or cardiac function for DOS and DHS exposed animals compared with that of the VEH treatment group were observed.

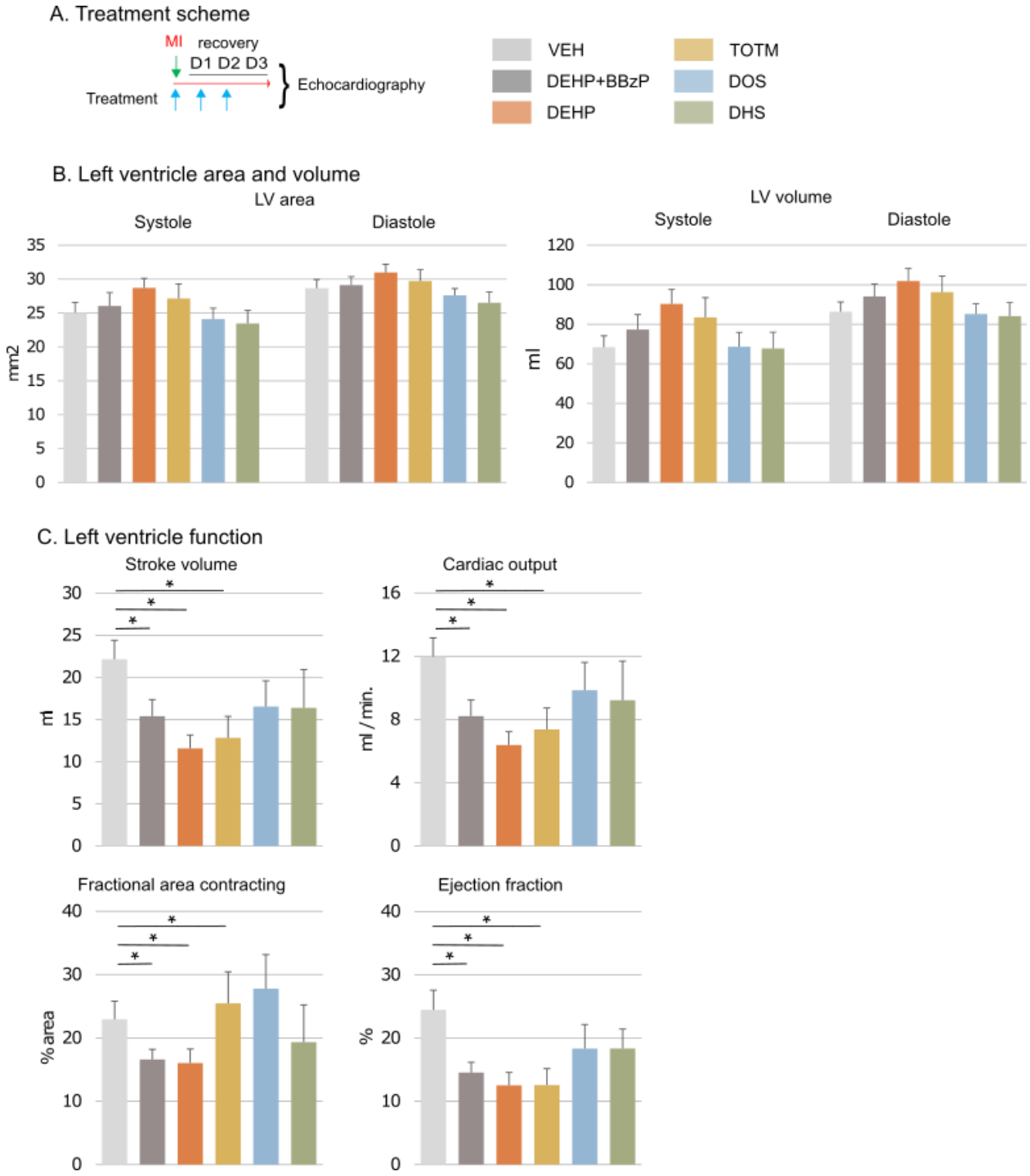

Figure 6. Impact of exposure on heart dilation and function post-MI in males.

Influence of phthalate exposure on cardiac structure/function remodeling after an MI. A, Treatment scheme. Echocardiography acquisitions were obtained on day 3 using a VEVO 770 sonograph. The left ventricle (LV) internal area and volume in systole and diastole were calculated using the long axis view and EKV-gated acquisitions. Single plane-derived dimensions allowed calculations to estimate LV area, LV volume in systole and diastole, stroke volume, ejection fraction and cardiac output. Similarly, EKV-gated acquisitions of the short axis view at the level of the papillary muscles allowed calculation of the fractional area change (FAC). B, Left ventricle area and volume in systole and diastole. C, Left ventricle function. Data are the mean ± SEM. A *p* value of <.05 was considered significant and is indicated by an * in comparison with VEH.

ii. Impact of exposure on heart dilation and function in females detected by echocardiogram

To assess the sex-specific impact of plasticizers on cardiac structure and function, male and female echocardiography results were compared. Similar to the results of the survival and body weight data, plasticizer exposure in female mice yielded no significant differences in cardiac structure between the different treatment groups (Figure 7B). Similarly, there were no observable differences in cardiac function among females exposed to phthalate versus non-phthalate plasticizers (Figure 7C).

In comparison with males, LV area was significantly lower in female mice exposed to phthalates (DEHP+BBzP and DEHP) compared to that of their male counterparts (Figure 6B and Figure 7B). Similarly, LV volume was significantly lower in female DEHP+BBzP, DEHP, and TOTM exposed mice compared with males of the same treatment groups (Figure 6B and Figure 7B). Thus, while DEHP+BBzP, DEHP, and TOTM male mice experienced greater cardiac dilation, phthalate and mellitate exposure did not alter cardiac structure in females. Furthermore, FAC was significantly greater in DEHP+BBzP females and EF was significantly greater in TOTM females compared with males (Figure 6C and Figure 7C).

To summarize, males treated with phthalate and mellitate plasticizers experienced greater cardiac dilation and reduced cardiac function compared with VEH treatment mice. This finding was not replicated when females experienced the same treatment. Meanwhile, cardiac structure and function for DOS and DHS exposed mice resembled the VEH treatment group for both sexes. Here, it is evident that phthalate and mellitate plasticizers possess a greater impact on cardiac structure and function in males.

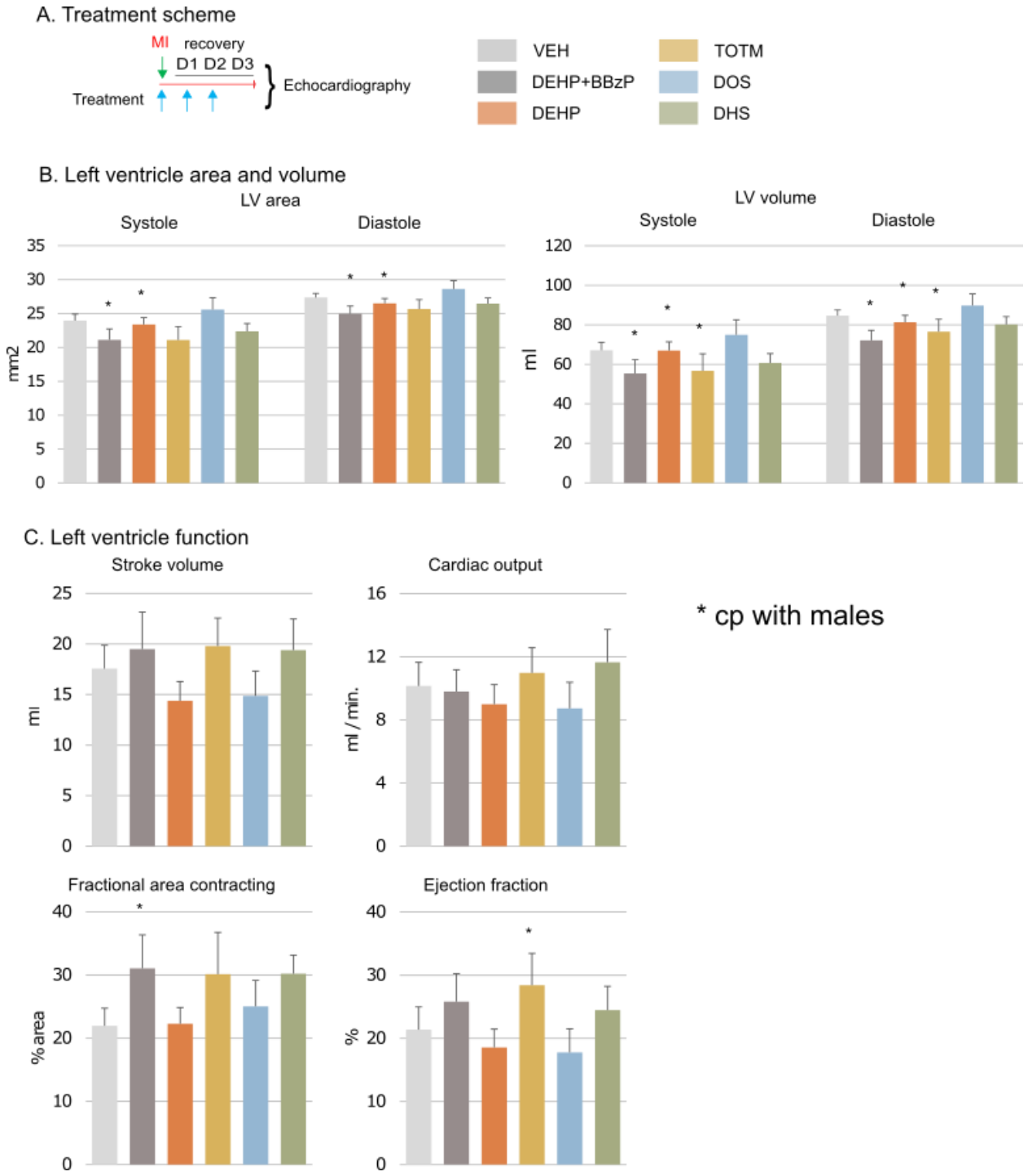

Figure 7. Impact of exposure on heart dilation and function post-MI in females.
Influence of phthalate exposure on cardiac structure/function remodeling after an MI. A, Treatment scheme. Echocardiography acquisitions were obtained on day 3 using a VEVO 770 sonograph. The left ventricle (LV) internal area and volume in systole and diastole were calculated using the long axis view and EKV-gated acquisitions. Single plane-derived dimensions allowed calculations to estimate LV area, LV volume in systole and diastole, stroke volume, ejection fraction and cardiac output. Similarly, EKV-gated acquisitions of the short axis view at the level of the papillary muscles allowed calculation of the fractional area change (FAC). B, Left ventricle area and volume in systole and diastole. C, Left ventricle function. Data are the mean ± SEM. A *p* value of <.05 was considered significant and is indicated by an * in comparison with male mice.

C. Immune cell population

i. Impact of exposure on innate immune cells post-MI in males

Following an MI, resident innate immune cells rapidly coordinate their function to contain inflammation by removing debris and promoting cardiomyocyte regeneration (164). To evaluate whether the changes in cardiac structure and function in phthalate and mellitate exposed males is a byproduct of a dysfunctional immune response the characteristics of the immune cells infiltrating the infarcts post-MI on cell-by-cell basis was established. Flow cytometry of single cells isolated from whole hearts used established cell markers and a gating strategy previously described (157) and enumerated the numbers of infiltrating neutrophils, monocytes, and macrophages. DEHP and TOTM treated mice had a significantly higher number of CD45+ cells than that of VEH, DOS, and DHS exposed animals, suggesting increased infiltration of immune cells (Figure 8B). To identify which immune cells infiltrate the wound, CD45+ cells were further characterized by their typical cell staining. Specifically, neutrophils (CD11b+Ly6G+) and monocytes (Cd11b+Ly6C+) were identified. Monocytes were subclassified into CD11b+Ly6c-high, and CD11b+Ly6C-low phenotypes. Macrophages were identified as CD64+MerTK+. Male mice exposed to DEHP had greater numbers of macrophages and neutrophils and TOTM exposed males had greater numbers of macrophages, monocytes, neutrophils, and Ly6c-high monocytes infiltrate the infarct. Thus, the data indicate that exposure to DEHP and TOTM enhances the infiltration of proinflammatory innate immune cells after an MI (Figure 8B). In contrast, immune cell infiltration in DOS and DHS treated males resembled that present in VEH treated males.

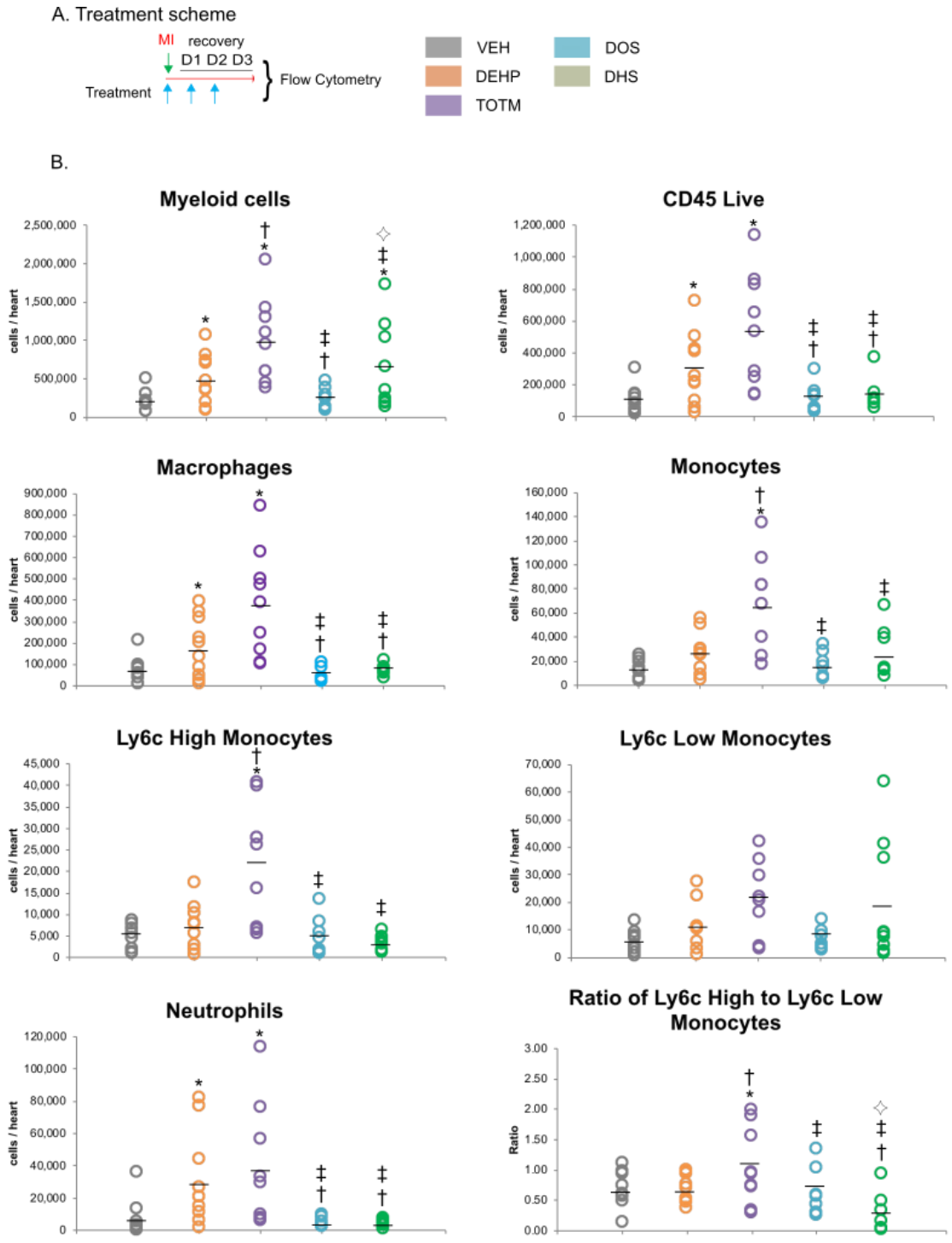

Figure 8. Impact of exposure on innate immune cells post-MI in males.

Influence of phthalate versus non-phthalate plasticizer exposure on innate immune cells post-MI in males detected by flow cytometry. A, Treatment scheme. Flow cytometry of single cell preparation isolated form whole hearts post-MI. Single cell preparations were prepared form the hearts of mice on day 3 post-MI. The bar represents the average number/heart. B, The number of indicated innate immune cell types in the heart of each mouse is indicated. Each circle represents an individual mouse. Neutrophils (CD11b+Ly6G+) and monocytes (CD11b+Ly6C+) were identified. Monocytes were then subclassified into CD11b+Ly6c-high, and CD11b+Ly6c-low. Macrophages were identified as CD64+MerTK+. A *p* value of <.05 was considered significant. * denotes comparison with VEH; † denotes comparison with DEHP; ‡ denotes comparison with TOTM; and ◇ denotes comparison with DOS.

ii. Impact of exposure on innate immune cells post-MI in females

To evaluate the sex-specific impact of phthalate versus non-phthalate plasticizer exposure on immune cell infiltration into the infarct post-MI, flow cytometry of single cells isolated from whole hearts in females was performed (Figure 9). In contrast to males, who experienced an increase in CD45+ cells in response to DEHP and TOTM exposure, there were no differences in the number of CD45+ cells between the chemical cohorts in females (Figure 9B). Moreover, neither phthalate, mellitate, nor succinate plasticizer exposure yielded an increase in macrophages, monocytes, or neutrophils in comparison with the VEH in females (Figure 9B). In females, DEHP exposure was associated with reduced macrophage numbers.

In comparison to females, DEHP and TOTM exposure in males caused a 347% and 292% increase in CD45+ cells per heart respectively. Furthermore, DEHP treated males experienced a 512% increase in macrophages, 260% increase in monocytes, and 35-fold increase in neutrophils. Similarly, males exposed to TOTM had a 358% increase in macrophages, 223% increase in monocytes, and 327% increase in neutrophils.

In summary, innate immune cell infiltration for DOS and DHS treated mice resembled the VEH for both male and female mice. In contrast, however, males exposed to DEHP and TOTM had a greater infiltration of macrophages, monocytes, and neutrophils. In consideration of our data, phthalate and mellitate plasticizers appear to enhance a proinflammatory innate immune cell infiltration in a sex-dependent manner. Moreover, regardless of sex, exposure to succinate-base plasticizers does not increase proinflammatory innate immune cell infiltration in comparison with VEH treated mice.

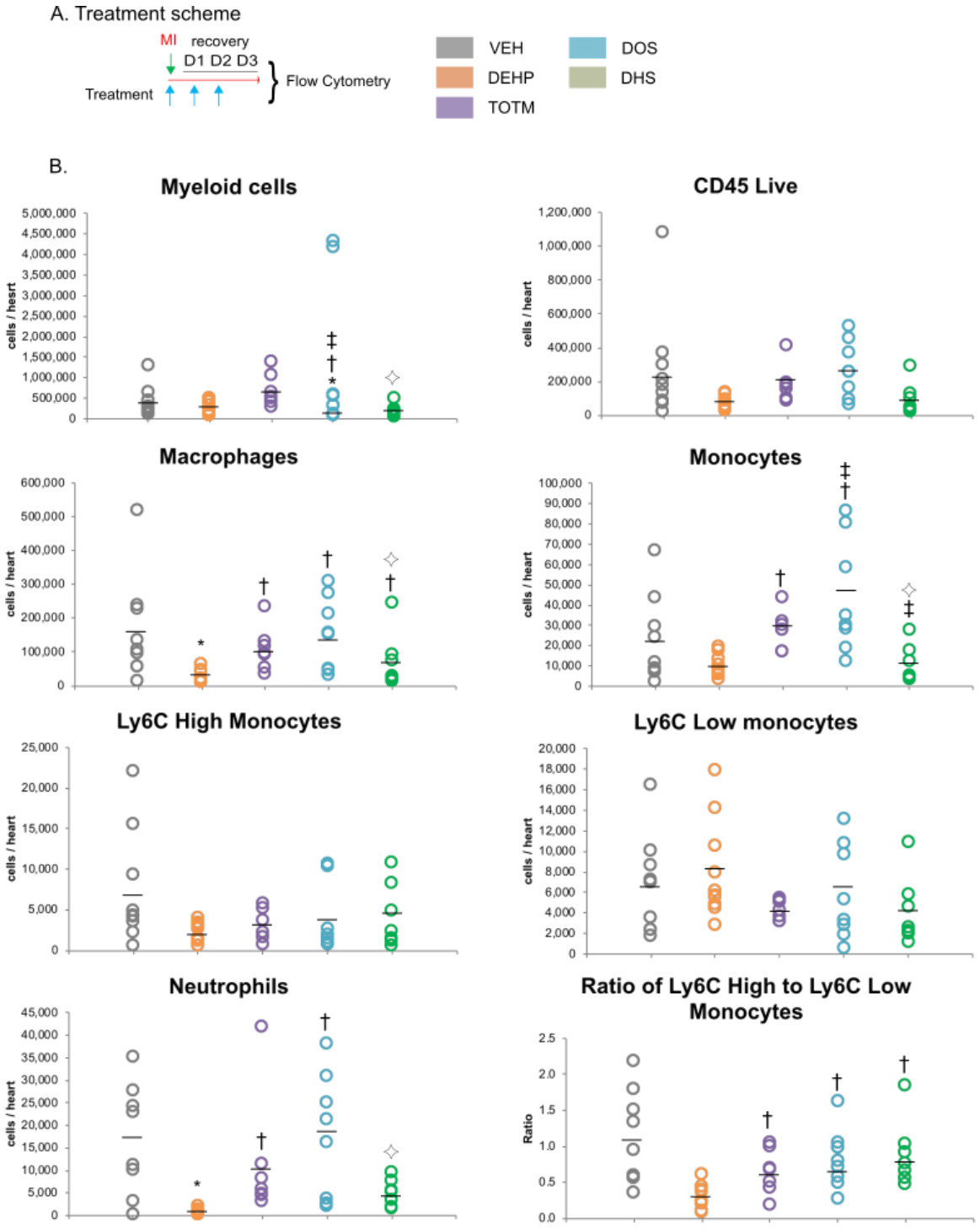

Figure 9. Impact of exposure on innate immune cells post-MI in females.

Influence of phthalate versus non-phthalate plasticizer exposure on innate immune cells post-MI in females detected by flow cytometry. A, Treatment scheme. Flow cytometry of single cell preparation isolated form whole hearts post-MI. Single cell preparations were prepared form the hearts of mice on day 3 post-MI. The bar represents the average number/heart. B, The number of indicated innate immune cell types in the heart of each mouse is indicated. Each circle represents an individual mouse. Neutrophils (CD11b+Ly6G+) and monocytes (CD11b+Ly6C+) were identified. Monocytes were then subclassified into CD11b+Ly6c-high, and CD11b+Ly6c-low. Macrophages were identified as CD64+MerTK+. A *p* value of <.05 was considered significant. * denotes comparison with VEH; † denotes comparison with DEHP; ‡ denotes comparison with TOTM; and ◇ denotes comparison with DOS.

iii. Impact of exposure on polarization markers expressed by macrophages post-MI in males

To further sub-classify the population of macrophages infiltrating the infarct post-MI, flow cytometry using CD38 and CD206 cell markers (Figure 10) was performed. Classically activated and proinflammatory macrophages, sometimes called M1 MΦ, are characterized in human monocytes by increased expression of cluster of differentiation 38 (CD38). Alternatively, activated and anti-inflammatory MΦ, sometimes called M2 MΦ, are characterized by increased expression of cluster of differentiation factor 206 (CD206). Male mice exposed to TOTM had a significantly higher percentage of CD38+ and CD206+ macrophages compared to that of VEH, DEHP, DOS, and DHS mice (Figure 10B). Moreover, DOS treated mice experienced a significantly higher percentage of CD38+ expressing macrophages compared to VEH and DHS (Figure 10B). Thus, the data indicates that TOTM exposure induces a dysfunctional proinflammatory macrophage population classified by increased expression of M1 and M2 markers in the macrophages. Meanwhile, the population of macrophages that infiltrate the wound in DOS exposed males tend to be of the M1 proinflammatory type. Macrophages isolated from mice exposed to DHS had significantly less M1 macrophages than both TOTM and DOS, further indicating that DHS does not exacerbate the infiltration of a proinflammatory immune cell population. Finally, while DEHP exposed males tend to experience a greater infiltration of macrophages, they appear to be neither the M1 nor M2 type markers.

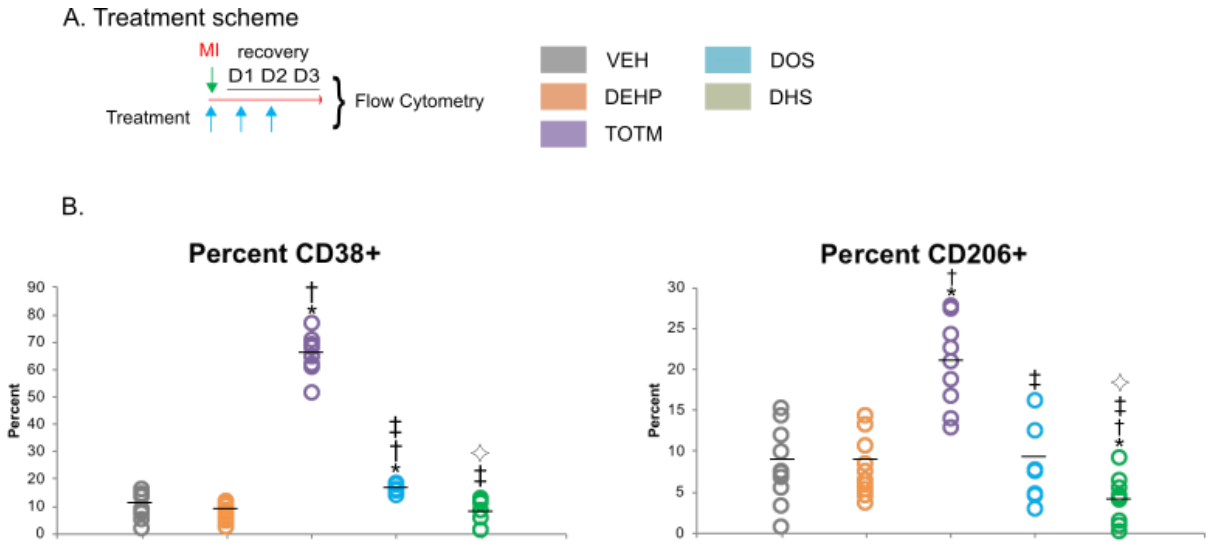

Figure 10. Impact of exposure on polarization markers expressed by macrophages post-MI in males.
Influence of phthalate versus non-phthalate plasticizer exposure on polarization markers expressed by macrophages post-MI in males detected by flow cytometry. A, Treatment scheme. Flow cytometry of single cell preparation isolated form whole hearts post-MI. Single cell preparations were prepared form the hearts of mice on day 3 post-MI. The bar represents the average number/heart. B, The number of indicated macrophage types in the heart of each mouse is indicated. Each circle represents an individual mouse. CD38+ M1 macrophages and CD206+ M2 macrophages were identified. A *p* value of <.05 was considered significant. * denotes comparison with VEH; † denotes comparison with DEHP; ‡ denotes comparison with TOTM; and ◇ denotes comparison with DOS.

iv. Impact of exposure on polarization markers expressed by macrophages post-MI in females

Flow cytometry was similarly performed for M1 and M2 macrophage markers in females (Figure 11). Neither phthalate, mellitate, nor succinate plasticizers had a higher percentage of M1 macrophages compared to the VEH. DOS and DHS treated females had a significantly higher percentage of anti-inflammatory M2 macrophages compared to VEH, DEHP, and TOTM treated females (Figure 11B). Furthermore, DHS had a significantly higher M2 population that that of DOS exposed females.

In summary, male DEHP and TOTM treated mice had the greatest infiltration of proinflammatory innate immune cells, namely monocytes, macrophages, and neutrophils. Additionally, a larger percentage of macrophages from TOTM and DOS exposed males expressed a marker associated with the proinflammatory M1 type. Meanwhile, neither phthalate, mellitate, nor succinate exposure increased innate immune cell infiltration in female mice,

relative to the VEH. In female mice, however, DOS and, to a greater extent, DHS exposed mice had a higher percentage of alternatively activated and anti-inflammatory M2 macrophages. In consideration of our data, DEHP and TOTM continues to display a sex-specific impact whereby exposure in males results in an increased proinflammatory immune cell population and a greater percentage of M1 macrophages in mellitate exposed animals. Meanwhile, DHS exposed animals, regardless of sex, had immune cell numbers comparable to the VEH and DHS exposed females had the greatest percentage of anti-inflammatory macrophages, indicating that DHS exposure does not evoke an excessive proinflammatory response.

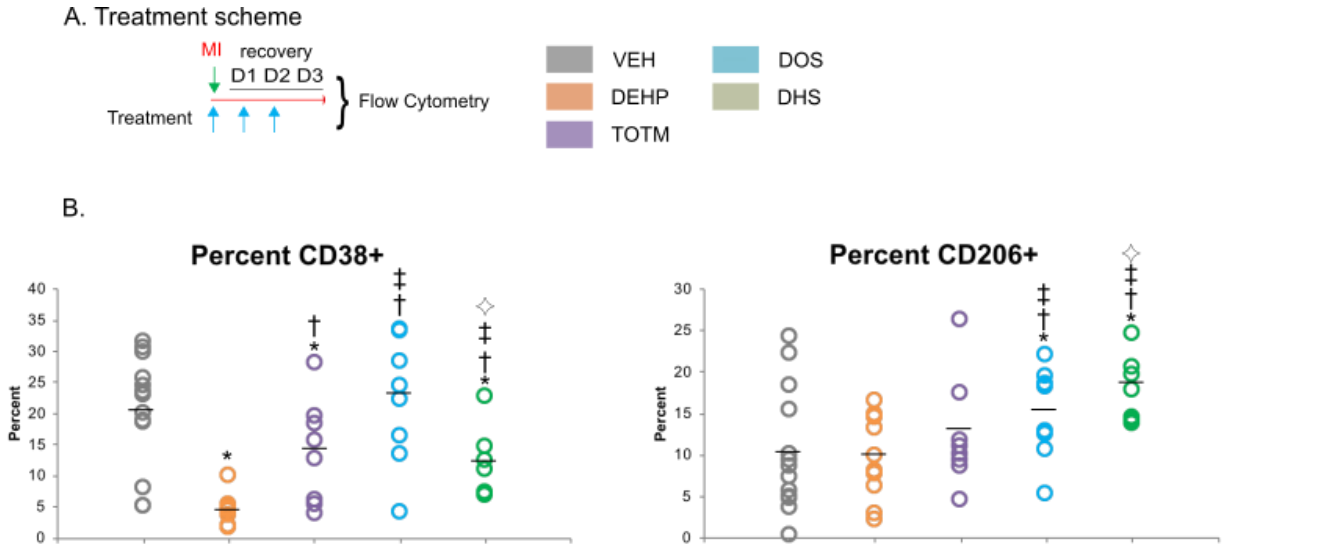

Figure 11. Impact of exposure on polarization markers expressed by macrophages post-MI in females.
Influence of phthalate versus non-phthalate plasticizer exposure on polarization markers expressed by macrophages post-MI in females detected by flow cytometry. A, Treatment scheme. Flow cytometry of single cell preparation isolated form whole hearts post-MI. Single cell preparations were prepared form the hearts of mice on day 3 post-MI. The bar represents the average number/heart. B, The number of indicated macrophage types in the heart of each mouse is indicated. Each circle represents an individual mouse. CD38+ M1 macrophages and CD206+ M2 macrophages were identified. A *p* value of <.05 was considered significant. * denotes comparison with VEH; † denotes comparison with DEHP; ‡ denotes comparison with TOTM; and ◇ denotes comparison with DOS.

D. THP-1 human monocyte cell line toxicity assays

i. Impact of exposure on polarization marker expression *in vitro*

The data suggest that macrophages entering the wounded heart may be induced to a proinflammatory cell type in vivo and that this may be more enhanced in males. To further evaluate the impact of phthalate versus non-phthalate plasticizer exposure on healing, the human male monocyte cell line, THP-1, differentiated into MΦ by culture in PMA was treated with plasticizers. We exposed the human macrophages to phthalate and non-phthalate plasticizer alternatives at a chemical concentration that reflects a normal range of human patient exposure (165).

Healing from any injury requires monocytes and macrophages (MΦ) to remove debris and dead cells, and to promote healing and repair (100, 166). Classically activated and proinflammatory MΦ, sometimes called M1 MΦ, are characterized in human monocytes by increased expression of chemokines (such as C-X-C motif chemokine 10, CXCL10) and interleukins (such as Interleakin-1, IL-6). Alternatively activated and anti-inflammatory MΦ, sometimes called M2 MΦ, are characterized by increased expression of chemokines (C-C chemokine ligand 22, CCL22), and cluster of differentiation factors CD206 and CD163.

To evaluate polarization toward the proinflammatory M1-type phenotype, we quantified CXCL10 and IL-6 expression (Figure 12B). DEHP, TOTM, BBzP, and DOS exposure led to increased CXCL10 expression when compared with DHS. CXCL10 expression was highest in DEHP, followed by TOTM, BBzP, and DOS treatment groups. Meanwhile, IL-6 expression was highest in DEHP treated cells and lowest in the 100-fold dilution of DHS. To assess polarization toward the repair, M2-type phenotype, we quantified CD206, CD163, and CCL2 expression (Figure 12C). Interestingly, DEHP exposure led to the highest expression of CD206, comparable to LPS treated cells. DOS displayed the greatest increase in CD163 expression. TOTM and BBzP exposure resulted in the greatest increase in CCL2 expression.

In summary, DEHP exposure in human macrophages appears to induce a dysfunctional proinflammatory phenotype whereby both M1 and M2 markers are overexpressed. Similarly, BBzP, TOTM, and DOS exposure results in an increased expression of M1 and M2 markers, albeit to a lesser extent. Meanwhile, the succinate-based plasticizer, DHS, most closely resembles VEH treated macrophages.

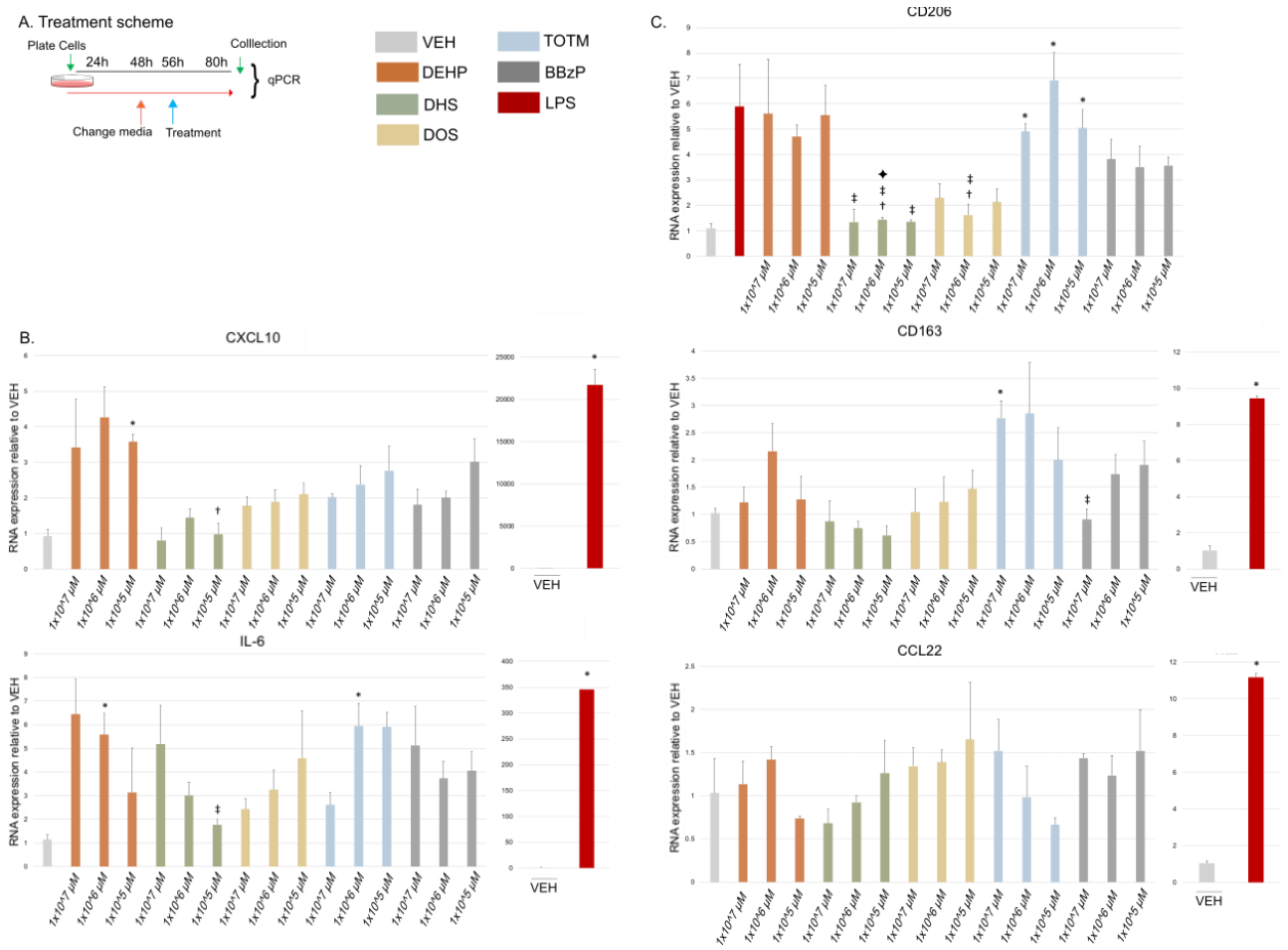

Figure 12. Impact of exposure on expression of polarization genes *in vitro*

Influence of phthalate and non-phthalate alternative exposure *in vitro* on expression of polarization genes on THP-1 macrophages. A, Treatment scheme. THP-1 monocytes were differentiated into macrophages by culture for 48h in PMA-containing media and then treated for 24h with the indicated chemicals. RNA was isolated and qPCR was performed using the indicated gene-specific primers. B, M1-specific markers. C, M2-specific markers. The bar graphs show the combined results. Expression of VEH treated cells was equal to 1.0. Human THP-1 macrophages were cultured and treated in triplicates as shown above. Data are the mean ± SEM. A *p* value of <0.05 was considered significant. * denotes comparison with VEH; † denotes comparison with DEHP; ‡ denotes comparison with TOTM; ✦ denotes comparison with BBzP; and ◇ denotes comparison with DOS. Results shown are representative of two independent experiments.

ii. Impact of exposure on expression of inflammasome genes in macrophages *in vitro*

Inflammasomes are cytosolic multiprotein sensors and receptors of the innate immune system responsible for the activation of Caspase-1 and induction of inflammatory responses to a variety of stimuli, including irritants (167). Monocyte-derived macrophages have been shown to be responsible for inflammasome activation and, hence, propagation of inflammatory responses (168). Thus, to further classify the impact of plasticizer exposure on macrophages and to better characterize the underlying inflammatory response, we quantified the expression of inflammasome receptors (Figure 13), inflammasome pathway components (Figure 14), and macrophage markers (Figure 15) *in vitro.*

Inflammasome names denote the protein forming the scaffold, namely of the NLR family (NLRP3 and NLRC4) and non-NLR family (AIM2). The inflammatory response is not limited to inflammasome pathways, however, and is instead characterized by the coordinated activation of disparate signaling pathways that respond to diverse stimuli, including the NF-κB pathway (TLR4), interleukin family of cytokines (IL-1R), and extracellular ATP-sensing purinoceptors (P2xR7). Expression of NLRP3, NLRC4, IL-1R, TLR4, and P2xR7 was consistently lowest in DHS treated macrophages and highest in DEHP exposed macrophages (Figure 13). Similarly, expression of phagocytosis genes Mfge8, MMP9, and CD36 was the lowest for DHS (Figure 14B). Furthermore, TOTM treated macrophages exhibited increased expression of NLRP3, IL-1R, and AIM2 in comparison to VEH treated cells. Meanwhile, DOS exposure increased macrophage expression of NLRP3, TLR4, and AIM2. Finally, BBzP treatment consistently displayed elevated inflammasome receptor expression compared to that of VEH treated macrophages. These data indicate that DHS exposure results in the lowest level of inflammasome and macrophage gene expression compared to the other plasticizers, indicating that it is a strong candidate for a safe non-toxic plasticizer alternative.

Inflammasomes form a scaffold to recruit the inactive zymogen pro Caspase-1. Oligomerization of pro Caspase-1 protein induces its auto-proteolytic cleavage into active Caspase-1 (169). Active Caspase-1 is a cysteine-dependent protease that cleaves precursor cytokine pro IL-1β generating biologically active cytokine IL-1β.(170) Quantification of IL-1β and Caspase-1 expression revealed that macrophages exposed to the succinate-based plasticizer, DHS, displayed the lowest levels of IL-1β and Caspase-1 expression compared with that of the other chemical treatments (Figure 14B). DOS and BBzP treatment resulted in the greatest expression of IL-1β. The differences were less pronounced for Caspase-1 expression, although the trends were similar. DEHP, DOS, and BBzP exposure induced the greatest expression of the inflammasome pathway marker Caspase-1.

In summary, our THP-1 cell line data indicates that DHS is the least toxic of the plasticizer chemicals. DHS treated macrophages displayed the lowest expression of pro-inflammatory, inflammasome, and macrophage markers compared to the rest of the chemical exposure groups. Meanwhile, DEHP exposed macrophages showed the greatest increase in expression of pro-inflammatory, inflammasome, and macrophage markers. Similarly, TOTM, BBzP, and DOS exposed macrophages displayed elevated expression of inflammatory genes, although to a lesser extent to that o DEHP. Together, the data further indicates the potential of DHS as a less hazardous alternative to current phthalate-based plasticizers.

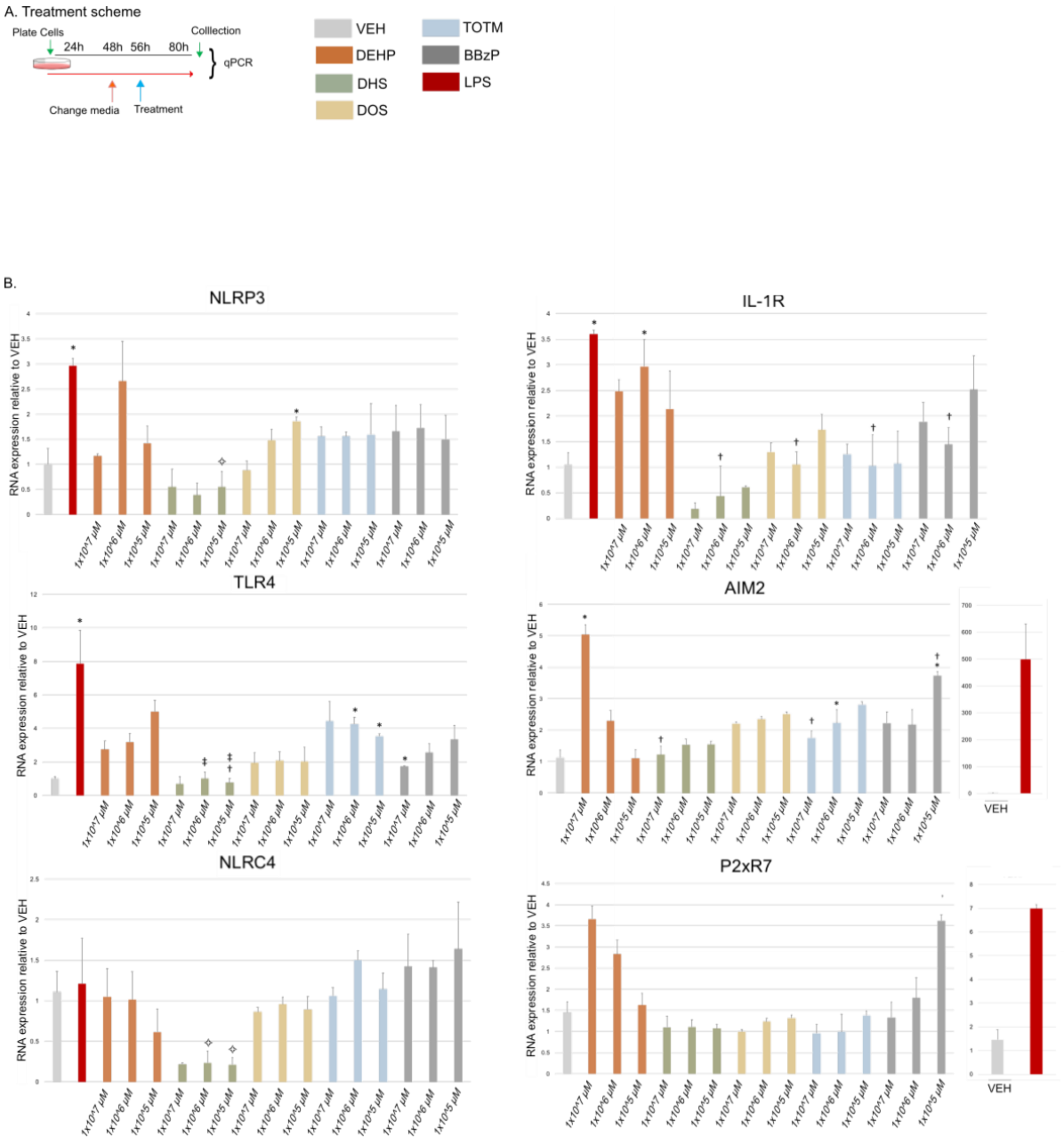

Figure 13. Impact of exposure on expression of inflammasome receptor genes *in vitro*

Influence of phthalate and non-phthalate alternative exposure *in vitro* on expression of inflammasome receptor genes on THP-1 macrophages. A, Treatment scheme. THP-1 monocytes were differentiated into macrophages by culture for 48h in PMA-containing media and then treated for 24h with the indicated chemicals. RNA was isolated and qPCR was performed using the indicated gene-specific primers. B, Inflammasome receptor markers. The bar graphs show the combined results. Expression of VEH treated cells was equal to 1.0. Human THP-1 macrophages were cultured and treated in triplicates as shown above. Data are the mean ± SEM. A *p* value of <0.05 was considered significant. * denotes comparison with VEH; † denotes comparison with DEHP; ‡ denotes comparison with TOTM; ✦ denotes comparison with BBzP; and ◇ denotes comparison with DOS. Results shown are representative of two independent experiments.

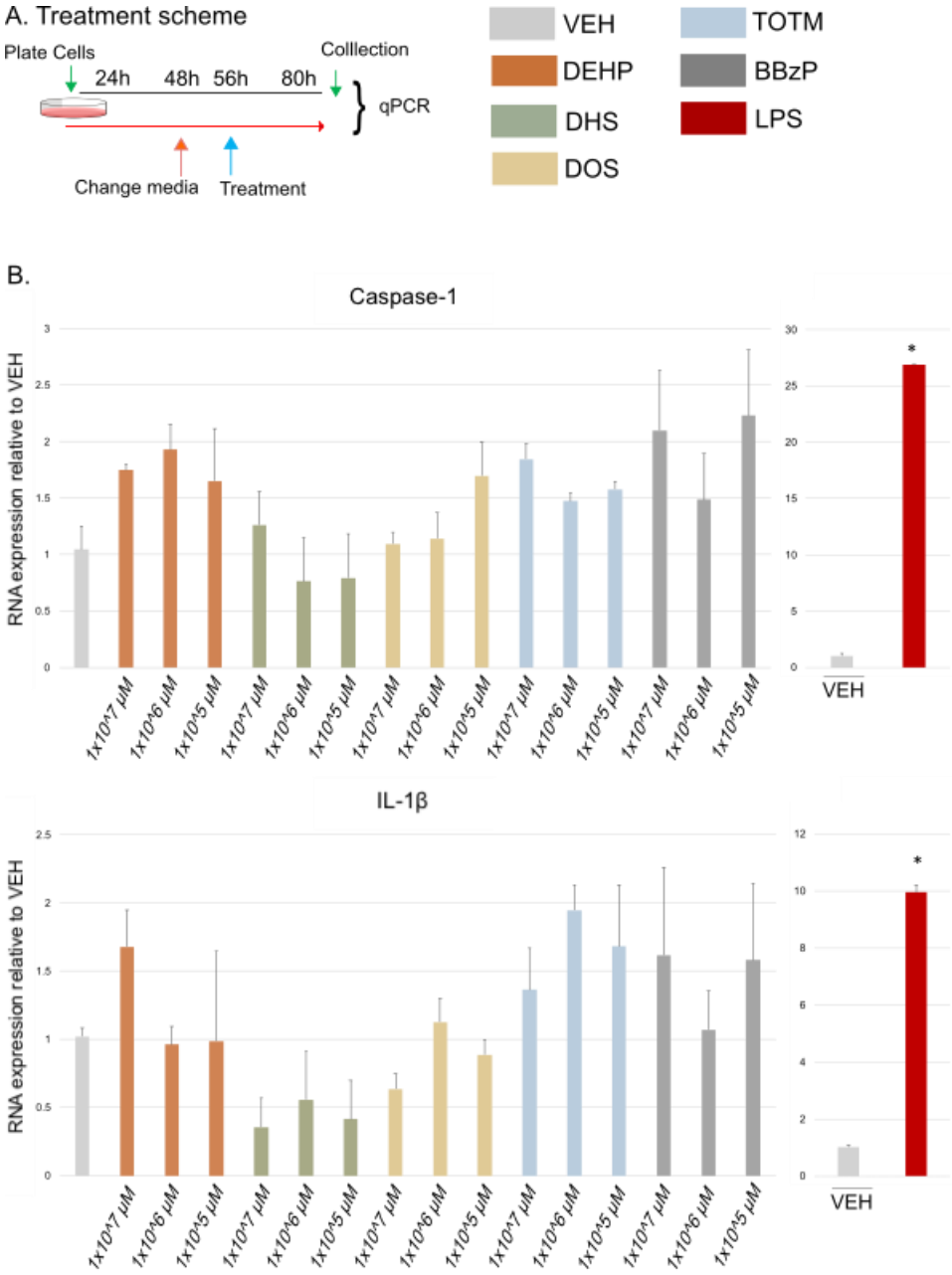

Figure 14. Impact of exposure on expression of inflammasome pathway genes *in vitro*

Influence of phthalate and non-phthalate alternative exposure *in vitro* on expression of inflammasome pathway genes on THP-1 macrophages. A, Treatment scheme. THP-1 monocytes were differentiated into macrophages by culture for 48h in PMA-containing media and then treated for 24h with the indicated chemicals. RNA was isolated and qPCR was performed using the indicated gene-specific primers. B, Inflammasome pathway markers. The bar graphs show the combined results. Expression of VEH treated cells was equal to 1.0. Human THP-1 macrophages were cultured and treated in triplicates as shown above. Data are the mean ± SEM. A *p* value of <0.05 was considered significant. * denotes comparison with VEH; † denotes comparison with DEHP; ‡ denotes comparison with TOTM; ✦ denotes comparison with BBzP; and ◇ denotes comparison with DOS. Results shown are representative of two independent experiments.

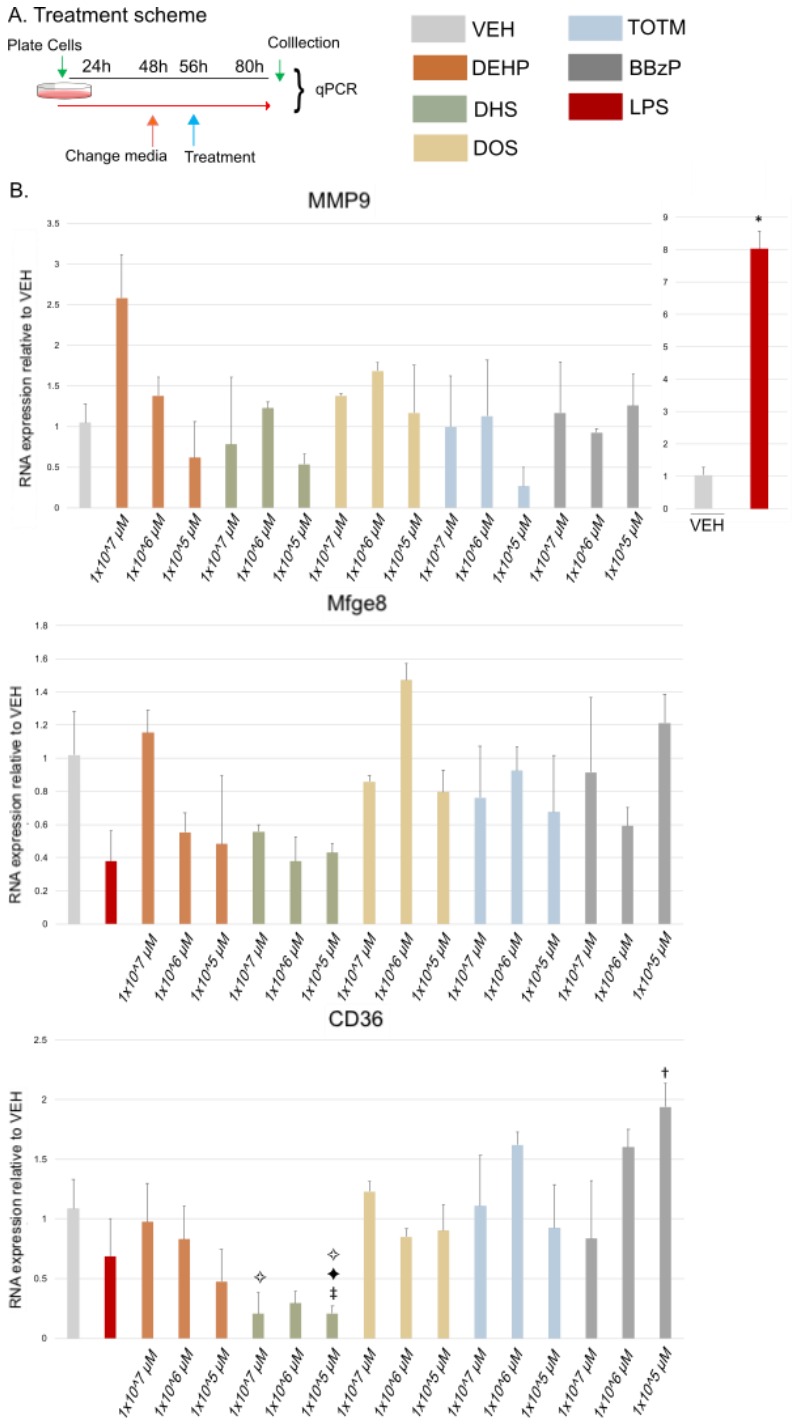

Figure 15. Impact of exposure on expression of macrophage genes *in vitro*

Influence of phthalate and non-phthalate alternative exposure *in vitro* on expression of inflammasome receptor genes on THP-1 macrophages. A, Treatment scheme. THP-1 monocytes were differentiated into macrophages by culture for 48h in PMA-containing media and then treated for 24h with the indicated chemicals. RNA was isolated and qPCR was performed using the indicated gene-specific primers. B, Macrophage markers. The bar graphs show the combined results. Expression of VEH treated cells was equal to 1.0. Human THP-1 macrophages were cultured and treated in triplicates as shown above. Data are the mean ± SEM. A p value of <0.05 was considered significant. * denotes comparison with VEH; † denotes comparison with DEHP; ‡ denotes comparison with TOTM; ✦ denotes comparison with BBzP; and ◇ denotes comparison with DOS. Results shown are representative of two independent experiments.

iii. Release of IL-1β and Caspase-1 into media

IL-1β is an important response factor of activated macrophages and a key mediator of inflammation. Overproduction of IL-1β and Caspase-1 is linked to an exaggerated proinflammatory phenotype. Activated macrophages rapidly release large amounts of IL-1β and inflammasome components including Caspase-1 (171). To further evaluate the potential of DHS as a replacement alternative for DEHP, we compared the release of IL-1β and Caspase-1 protein into media (Figure 16). Pro IL-1β and Active IL-1β were both considerably higher in the media of DEHP treated macrophages compared to that of DHS treated macrophages. Similarly, protein levels of both Pro Caspase-1 and Active Caspase-1 were higher in media from DEHP treated macrophages. In concert with the observed increase in RNA expression of IL-1β and Caspase-1 in DEHP treated macrophages and the increased release of IL-1β and Caspase-1 protein into media, the data indicates that DEHP induces an exaggerated proinflammatory response in comparison to the candidate green plasticizer, DHS.

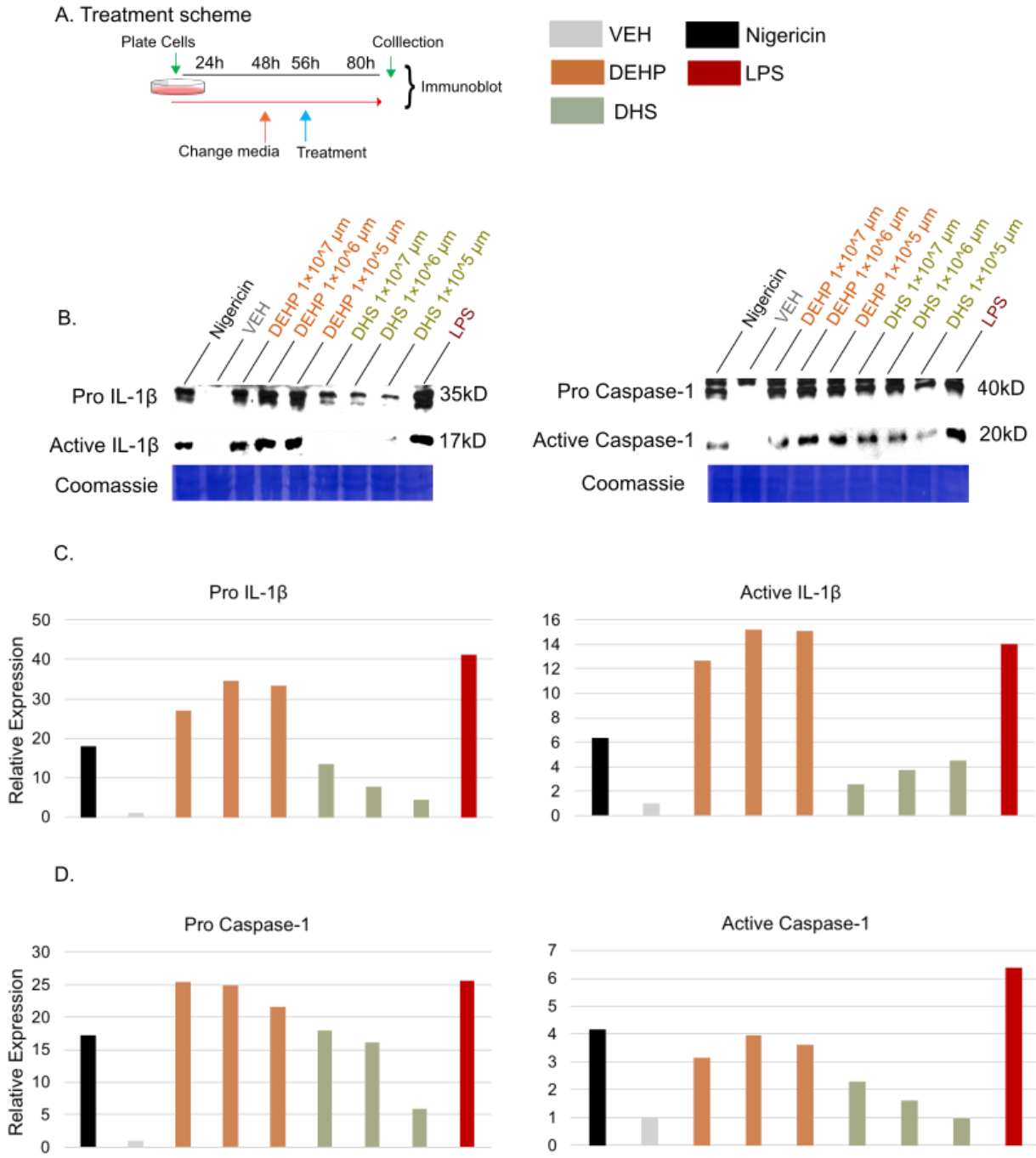

Figure 16. Impact of exposure on IL-1β and Caspase-1 release into media

Impact of DEHP versus DHS exposure on release of IL-1β and Caspase-1 release into media. A, Treatment scheme. THP-1 monocytes were differentiated into macrophages by culture for 48h in PMA-containing media and then treated for 24h with the indicated chemicals. Protein was isolated from the media and immunoblots were performed. B, Representative blots for expression of IL-1β and Caspase-1. Expression was calculated relative to the stained and scanned Coomassie Blue stained membrane, which was used as a loading control. The bar graphs show the combined results. Expression of VEH treated cells was equal to 1.0. C, Release of Pro IL-1β and Active IL-1β into media. D, Release of Pro Caspase-1 and Active Caspase-1 into media. Results shown are representative of two independent experiments.

E. *In vivo* RNA expression analysis

i. Impact of exposure on the expression of inflammasome genes in heart tissue in males

To characterize further the cell types in the infarct milieu, RNA was isolated from the infarct and probed for expression of macrophage polarization and inflammasome markers *in vivo* (Figure 17 and 18). Expression of M1-marker NOS-2 was significantly higher in DEHP treated mice compared to DOS and DHS treated mice, which resembled VEH exposed mice. Similarly, TOTM treated mice displayed elevated levels of NOS-2 (Figure 17B) compared to that of VEH, DOS, and DHS treated mice. TOTM treated mice exhibited the greatest expression of the M1 marker CCR7, which was significantly higher than that of VEH exposed mice. Expression of CCR7 was second highest for DEHP mice, while DOS and DHS treated mice resembled VEH mice. Expression of M1 marker Egr-1 was highest in DEHP males, which was significantly higher than that of DHS males. Expression of Egr-1 was also elevated in TOTM treated mice, while DOS resembled VEH exposed mice. Similar to that of the *in vitro* macrophage toxicity assay, M2 markers Fizzl-1 and IL-10 were also elevated in DEHP and TOTM treated mice, which were significantly higher than that of DOS and DHS exposed mice (Figure 13B). Thus, the expression of both M1 and M2 polarization markers were significantly elevated in DEHP and TOTM treated mice compared to that of VEH, DOS, and DHS exposed mice.

Following injury, circulating C-C chemokine receptor type 2 (CCR2) monocytes are actively recruited to an injury in response to chemokines such as to the monocyte chemoattractant chemokine CC-C motif ligand 2 (CCL2) ligand. (172). To test the idea that DEHP and TOTM exposure increases immune cell accumulation, CCR2 and CCL2 expression levels were evaluated in heart infarcts. Similar to the flow cytometry analysis of innate immune cell infiltration, DEHP treated mice displayed significantly higher expression of CCR2 compared to VEH, DOS, and DHS exposed mice (Figure 17C). Expression of CCR2 was also elevated in

TOTM treated mice. Moreover, expression of CCL2 in DEHP exposed mice was significantly higher than that of DOS and DHS mice.

Matrix metalloproteinases (MMP) secreted by myeloid cell and other cardiac cells can increase cardiac dilation post-MI due to excessive degradation of the supporting extracellular matrix (52, 173). To determine if greater MMP9 activity was a factor in the cardiac dysfunction in DEHP and TOTM treated mice (Figure 6), MMP expression was quantified. Indeed, MMP9 expression was significantly higher in DEHP mice compared to that of DOS and DHS treated mice (Figure 13D). Expression of MMP9 was also elevated in TOTM exposed mice. Macrophage activities in the infarct include phagocytosis, scavenging of apoptotic and dead cells, and removal of extracellular debris. To assess the expression of phagocytosis-related proteins, MerTK and Mfge8 was measured. Expression of MerTK (also an M1 marker) was significantly higher in DEHP mice compared to that of VEH, TOTM, DOS, and DHS mice. Expression of Mfge8 was highest in TOTM treated mice (Figure 17D).

The expression of inflammasome markers NLRP3, NLRP6, AIM2, and P2xR7 was highest for DEHP treated mice followed by TOTM exposed mice (Figure 18B). Expression of NLRP3 and P2xR7 was significantly higher in DEHP mice compared to that of VEH, DOS, and DHS treated mice. Furthermore, expression of inflammasome pathway genes Myd88, IRAK4, IL-1β, and Caspase-1 was highest for DEHP treated mice and second highest for TOTM treated mice. The expression of IL-1β and Caspase-1 was significantly higher in DEHP treated mice compared to both DOS and DHS exposed males. In consideration of our *in vivo* RNA expression analysis, it is evident that DEHP exposure induces the greatest expression of proinflammatory markers. Furthermore, TOTM exposure also results in an over-expression of proinflammatory genes, albeit to a slightly lower extent than that of DEHP. Nevertheless, succinate-based plasticizers, DOS and DHS, most closely resemble VEH treated animals.

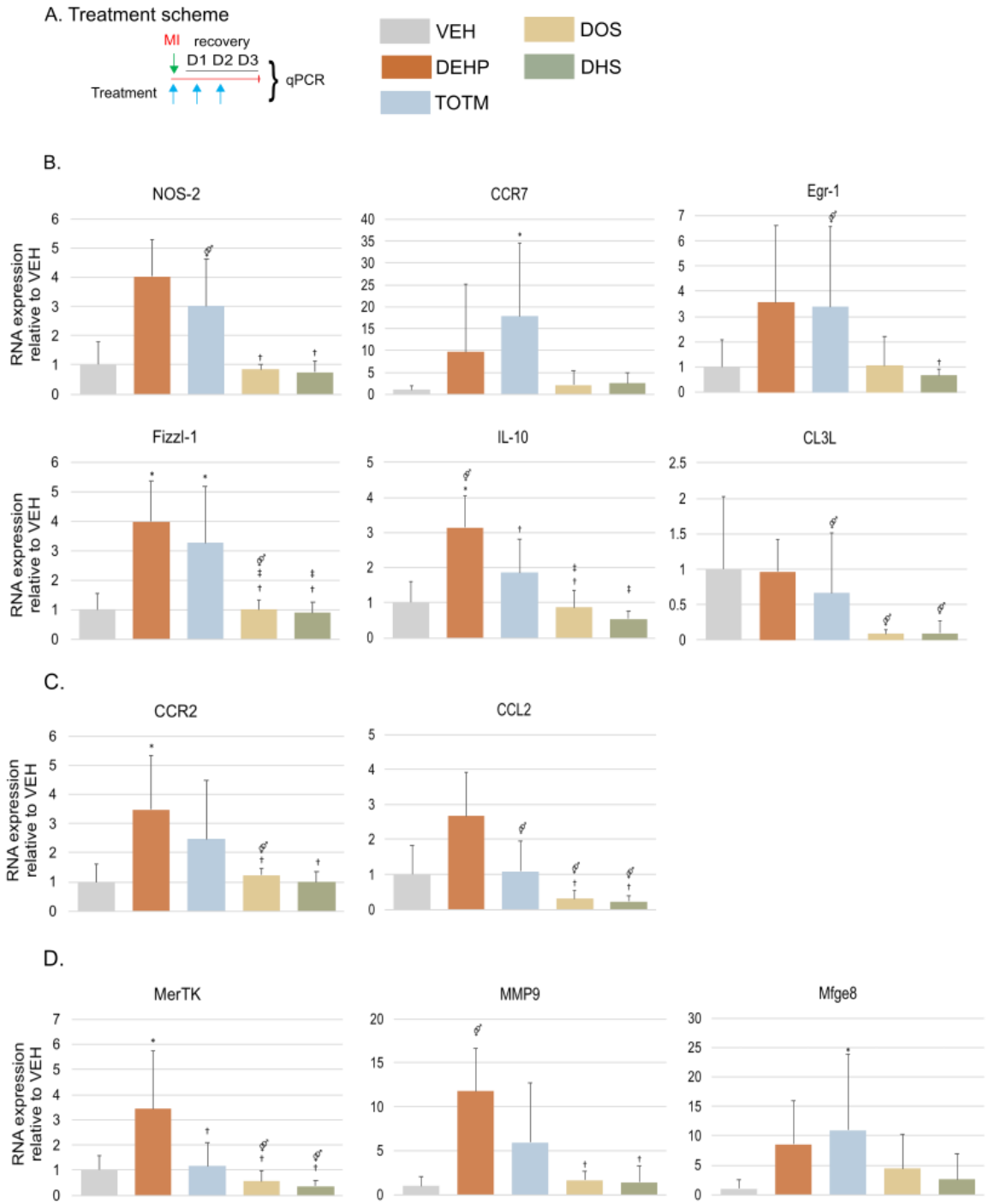

Figure 17. Impact of exposure on expression of polarization, chemokine, and macrophage genes in male heart tissue

Impact of phthalate versus non-phthalate plasticizer exposure on expression of inflammasome genes in heart tissue detected by qPCR. A, Treatment scheme. Surgery was performed on male C57bl/6N mice to create a permanent blockage in the coronary artery. Cohorts were treated with VEH (peanut oil), DEHP, TOTM, DOS, or DHS (25 mg/kg/day). On day 3 post-MI, mice were euthanized, and heart tissue was excised. RNA was isolated and qPCR was performed using the indicated gene-specific primers. B, Polarization markers. C, Chemokine markers. D, Macrophage markers. Expression of VEH treated mice was equal to 1.0. Data are the mean ± SEM. A *p* value of <0.05 was considered significant. * denotes comparison with VEH; † denotes comparison with DEHP; ‡ denotes comparison with TOTM; and ◇ denotes comparison with DOS. VEH (n=5); DEHP (n=5); DHS (n=5); TOTM (n=5); and DOS (n=4).

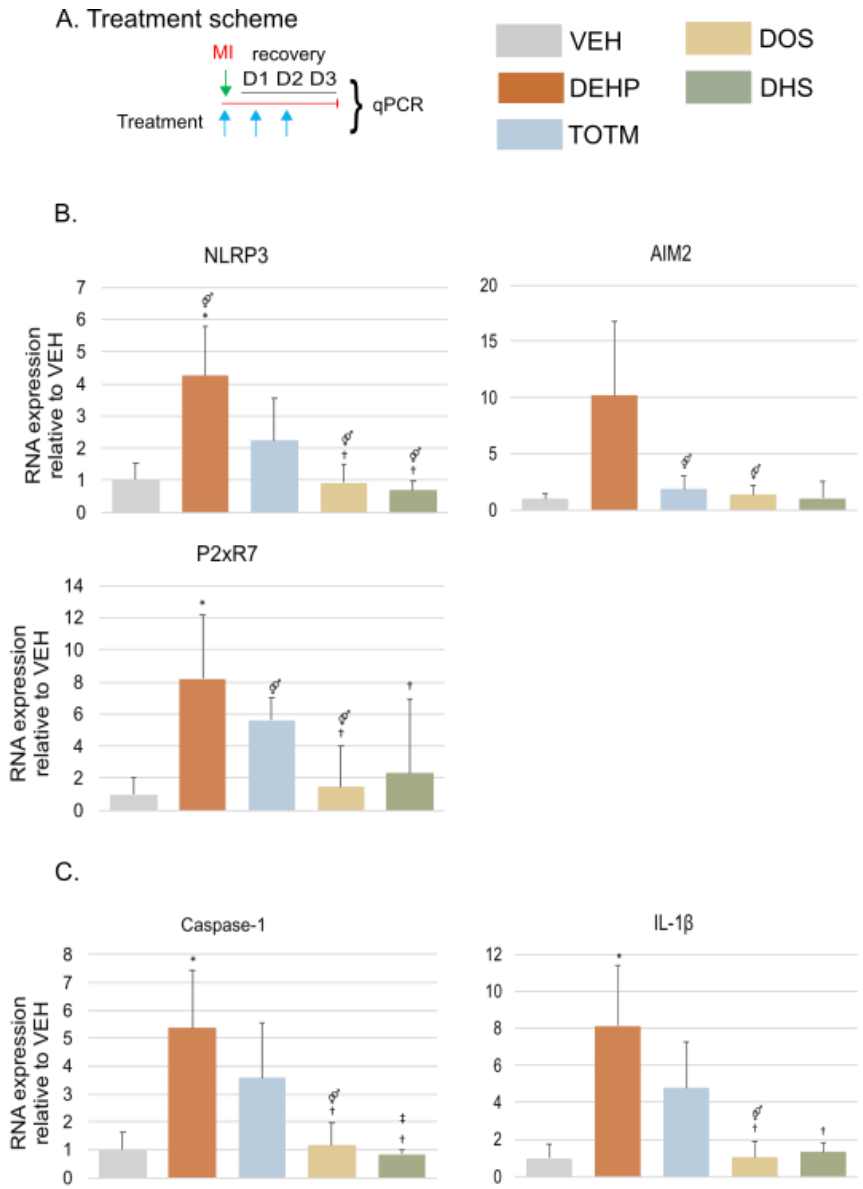

Figure 18. Impact of exposure on expression of inflammasome genes in male heart tissue

Impact of phthalate versus non-phthalate plasticizer exposure on expression of inflammasome genes in heart tissue detected by qPCR. A, Treatment scheme. Surgery was performed on male C57bl/6N mice to create a permanent blockage in the coronary artery. Cohorts were treated with VEH (peanut oil), DEHP, TOTM, DOS, or DHS (25 mg/kg/day). On day 3 post-MI, mice were euthanized, and heart tissue was excised. RNA was isolated and qPCR was performed using the indicated gene-specific primers. B, Inflammasome receptor markers. C, Inflammasome pathway markers. Expression of VEH treated mice was equal to 1.0. Data are the mean ± SEM. A *p* value of <0.05 was considered significant. * denotes comparison with VEH; † denotes comparison with DEHP; ‡ denotes comparison with TOTM; and ◇ denotes comparison with DOS. VEH (n=5); DEHP (n=5); DHS (n=5); TOTM (n=5); and DOS (n=4).

ii. Impact of exposure on the expression of inflammasome genes in heart tissue in females

Next, I evaluated the impact of plasticizer exposure on the expression of polarization, chemokine, macrophage, and inflammasome markers in female mice. Similar to that of males, expression of M1 polarization markers NOS-2, CCR7, and Egr-1 was highest for DEHP treated females (Figure 19B). Expression of M1 markers was lower in TOTM treated females compared to that of their male counterparts. In TOTM males, expression of NOS-2 was 267% higher, expression of CCR7 was 623% higher, and Egr-1 was 345% higher. Expression of M2 marker Fizzl-1 was elevated in DEHP treated females (Figure 19B). In agreement with the flow cytometry data, I detected an increased expression of the M2 markers IL-10 and CL3L in TOTM, DOS, and DHS treated females, indicating a tendency towards alternatively activated macrophages in these non-phthalate-based plasticizer cohorts; this data did not reach significance, however.

Additionally, the expression of chemokine receptor CCR2 and ligand CCL2 was lowest in DHS treated females (Figure 19C). MerTK, MMP9, and Mfge8 expression was highest in DEHP treated females and lowest in DHS females, although the differences did not reach significance (Figure 19D). In comparison with males, MMP9 expression was 302% higher in DEHP males and 304% higher in TOTM males compared to their female counterparts, suggesting a potential factor for the differences in cardiac dilation between DEHP and TOTM treated males and females (Figure 7). Similarly, the macrophage marker Mfge8 was expressed 280% more in DEHP males and 370% more in TOTM males.

Similar to that of male mice, DEHP exposed females expressed significantly higher NLRP3 compared to that of VEH and DHS treated mice. AIM2 expression was significantly higher in DEHP, TOTM, and DOS mice (Figure 16B). Moreover, P2xR7 expression in TOTM and DOS exposed mice was significantly higher than VEH, DEHP, and DHS mice. Inflammasome marker expression was consistently low in DHS treated females (Figure 20C).

Caspase-1 expression in DEHP, TOTM, and DOS treated females was significantly higher than that of DHS females.

In summary, the data further indicates that the differences of phthalate versus non-phthalate plasticizer exposure are less pronounced in females compared to that of their male counterparts. Regardless of sex, however, DHS treated animals consistently scored the lowest on gene markers of inflammation. Thus, in consideration of sex-specific responses, the data appears to indicate that the ranking for plasticizer safety in the context of surgical recovery from MI is: DHS > DOS >>> TOTM > DEHP.

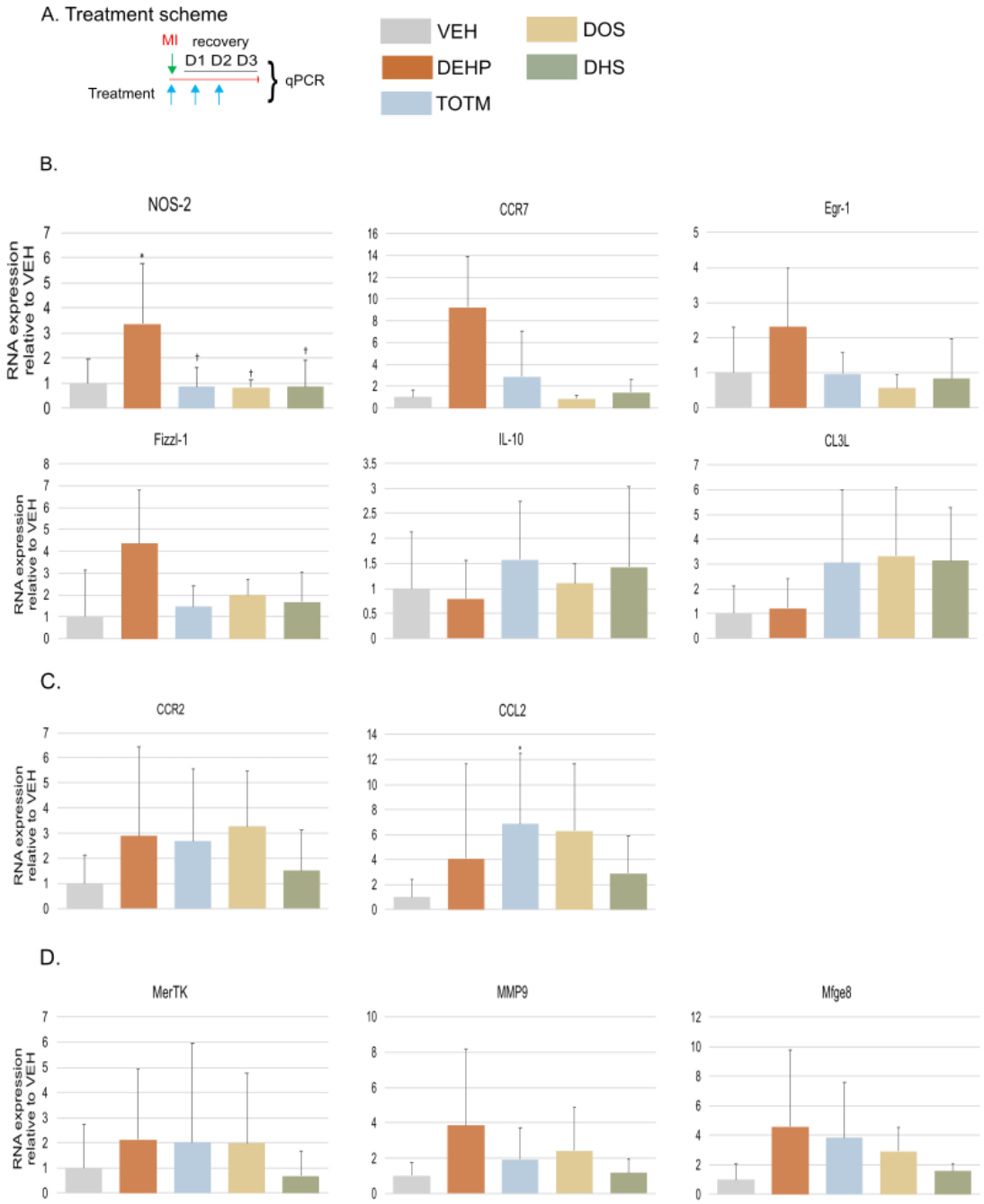

Figure 19. Impact of exposure on expression of polarization, chemokine, and macrophage genes in female heart tissue

Impact of phthalate versus non-phthalate plasticizer exposure on expression of inflammasome genes in heart tissue detected by qPCR. A, Treatment scheme. Surgery was performed on female C57bl/6N mice to create a permanent blockage in the coronary artery. Cohorts were treated with VEH (peanut oil), DEHP, TOTM, DOS, or DHS (25 mg/kg/day). On day 3 post-MI, mice were euthanized, and heart tissue was excised. RNA was isolated and qPCR was performed using the indicated gene-specific primers. B, Polarization markers. C, Chemokine markers. D, Macrophage markers. Expression of VEH treated mice was equal to 1.0. Data are the mean ± SEM. A *p* value of <0.05 was considered significant. * denotes comparison with VEH; † denotes comparison with DEHP; ‡ denotes comparison with TOTM; and ◇ denotes comparison with DOS. DHS (n=6); VEH (n=5); DEHP (n=5); TOTM (n=4); and DOS (n=3).

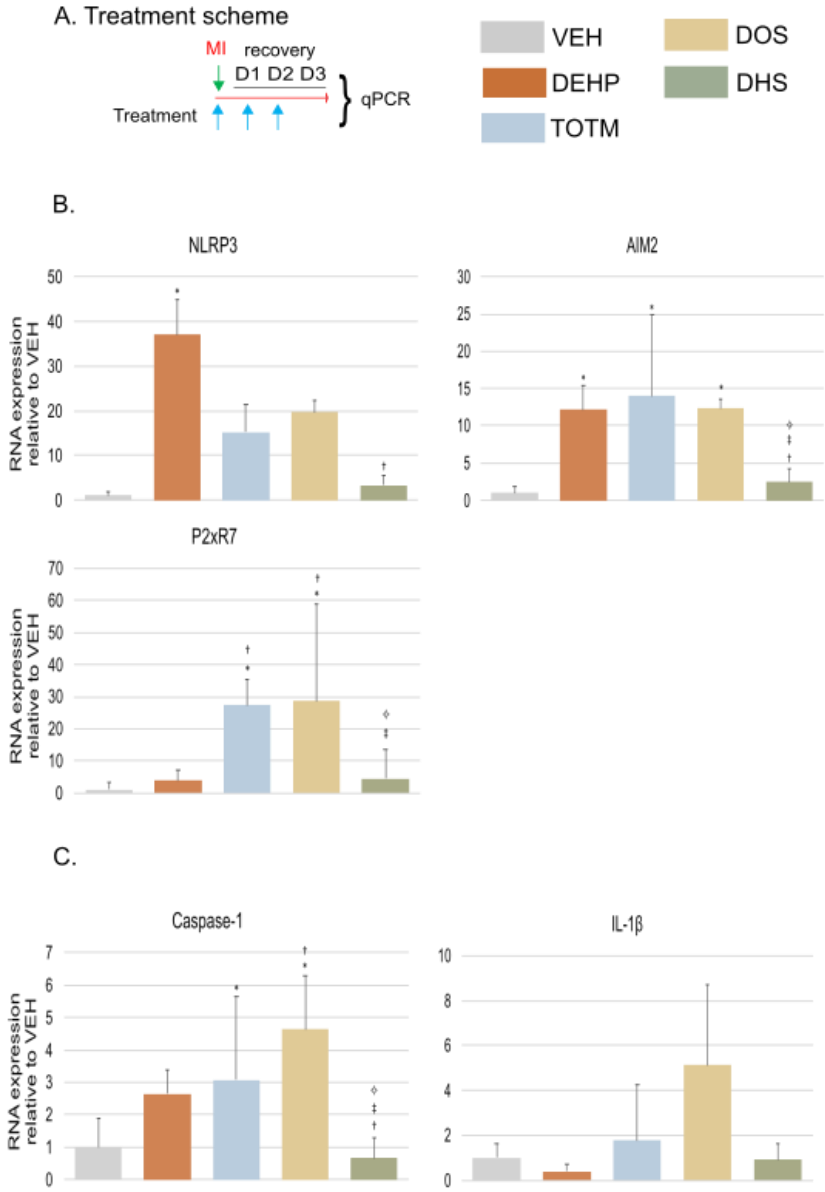

Figure 20. Impact of exposure on expression of inflammasome genes in female heart tissue

Impact of phthalate versus non-phthalate plasticizer exposure on expression of inflammasome genes in heart tissue detected by qPCR. A, Treatment scheme. Surgery was performed on female C57bl/6N mice to create a permanent blockage in the coronary artery. Cohorts were treated with VEH (peanut oil), DEHP, TOTM, DOS, or DHS (25 mg/kg/day). On day 3 post-MI, mice were euthanized, and heart tissue was excised. RNA was isolated and qPCR was performed using the indicated gene-specific primers. B, Inflammasome receptor markers. C, Inflammasome pathway markers. Expression of VEH treated mice was equal to 1.0. Data are the mean ± SEM. A *p* value of <0.05 was considered significant. * denotes comparison with VEH; † denotes comparison with DEHP; ‡ denotes comparison with TOTM; and ◇ denotes comparison with DOS. DHS (n=6); VEH (n=5); DEHP (n=5); TOTM (n=4); and DOS (n=3).

F. *In vivo* protein expression analysis

i. Impact of exposure on markers of inflammasome activation in heart tissue in males

After observing an increase in RNA and protein expression of IL-1β and Capase-1 *in* vitro for DEHP and TOTM exposure, and to further evaluate the efficacy of DHS as a replacement plasticizer for DEHP,Casapse-1 and IL-1β protein within the infarct were quantified (Figure 21 and 22). Both pro Caspase-1 and active Caspase-1 were elevated in comparison with VEH treated mice (Figure 17C). In contrast, DHS treated males had significantly lower pro Caspase-1 and active Caspase-1 compared with that of VEH-treated mice (Figure 17C). Pro IL-1β and active IL-1β were significantly higher in DEHP exposed males. Again, DHS treated males had significantly lower pro IL-1β and active IL-1β compared to the VEH group (Figure 22).

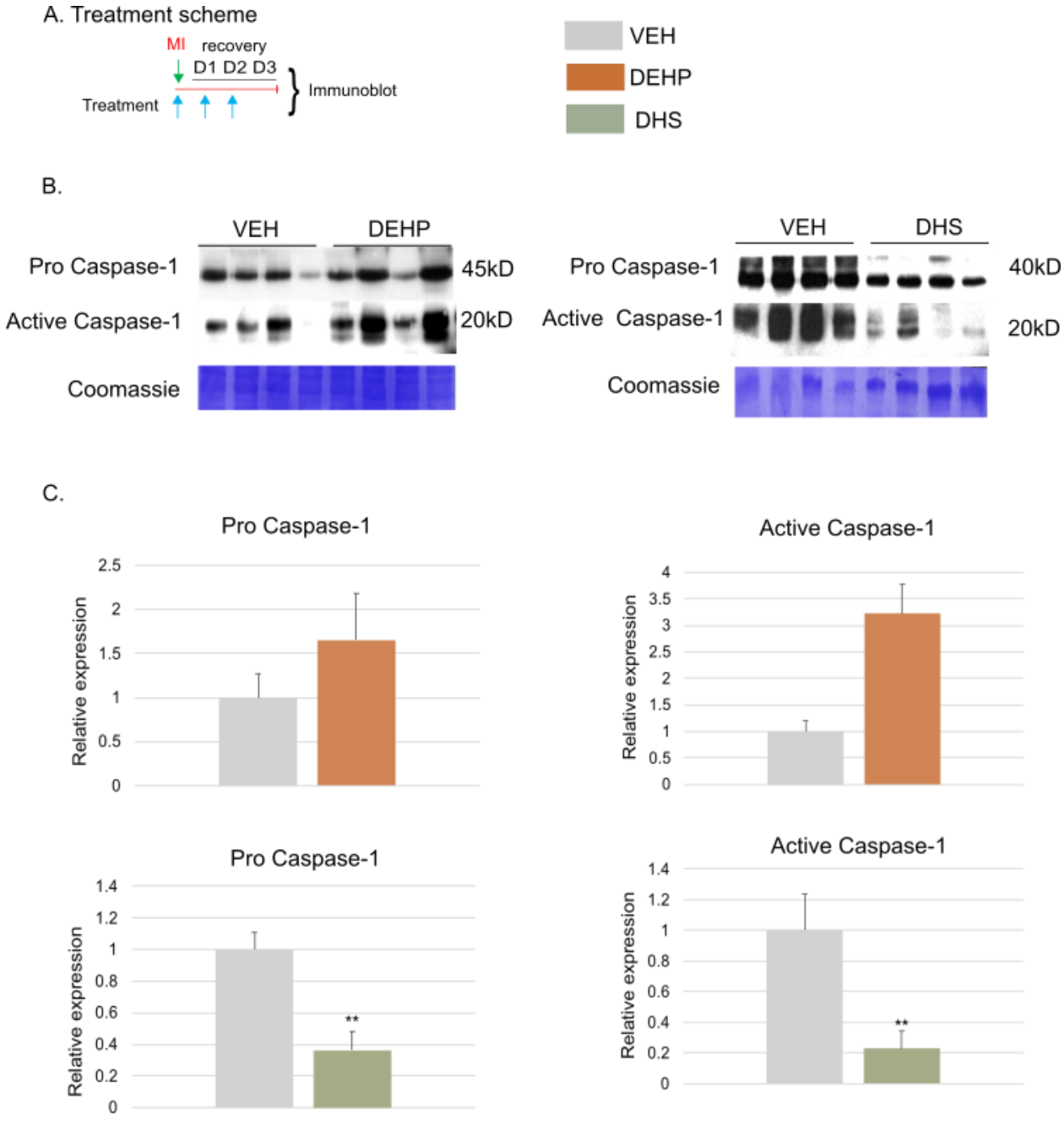

Figure 21. Impact of exposure on Caspase-1, a marker of inflammasome activation in male heart tissue

Impact of phthalate versus non-phthalate plasticizer exposure on Caspase-1 expression in male heart tissue detected by immunoblot. A, Treatment scheme. Surgery was performed on male C57bl/6N mice to create a permanent blockage in the coronary artery. Cohorts were treated with VEH (peanut oil), DEHP, TOTM, DOS, or DHS (25 mg/kg/day). On day 3 post-MI, mice were euthanized, and heart tissue was excised. Protein was isolated and immunoblots were performed. B, Representative blots for expression of Caspase-1. Expression was calculated relative to the stained and scanned Coomassie Blue stained membrane, which was used as a loading control. Expression of VEH treated mice was equal to 1.0. C, Expression of Pro Caspase-1 and Active Caspase-1. Data are the mean ± SEM. A *p* value of <0.05 was considered significant and is indicated by an * in comparison with VEH mice. VEH (n=4); DEHP (n=4); and DHS (n=4).

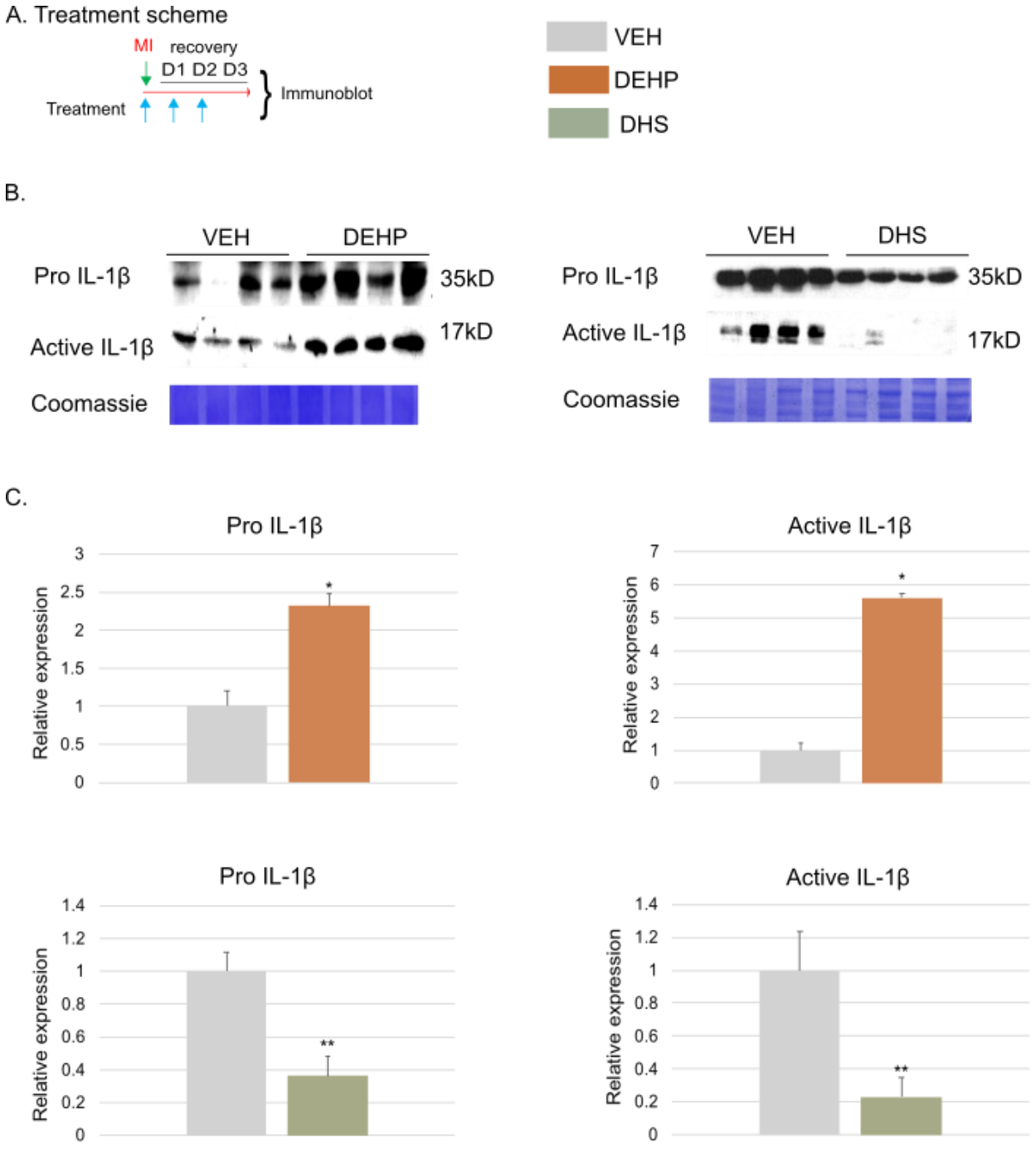

Figure 22. Impact of exposure on IL-1β, a marker of inflammasome activation in male heart tissue

Impact of phthalate versus non-phthalate plasticizer exposure on IL-1β expression in male heart tissue detected by immunoblot. A, Treatment scheme. Surgery was performed on male C57bl/6N mice to create a permanent blockage in the coronary artery. Cohorts were treated with VEH (peanut oil), DEHP, TOTM, DOS, or DHS (25 mg/kg/day). On day 3 post-MI, mice were euthanized, and heart tissue was excised. Protein was isolated and immunoblots were performed. B, Representative blots for expression of IL-1β. Expression was calculated relative to the stained and scanned Coomassie Blue stained membrane, which was used as a loading control. Expression of VEH treated mice was equal to 1.0. C, Expression of Pro IL-1β and Active IL-1β. Data are the mean ± SEM. A *p* value of <0.05 was considered significant and is indicated by an * in comparison with VEH mice. VEH (n=4); DEHP (n=4); and DHS (n=4).

ii. Impact of exposure on markers of inflammasome activation in heart tissue in females

DEHP treated females had higher levels of pro Caspase-1 and active Caspase-1 than the VEH, although the comparison did not meet significance and the differences were lower than that observed in males (Figure 23C). Expression of pro Caspase-1 protein was 190% higher in males compared with that of females. Meanwhile, DHS treated females resembled the VEH treatment cohort. Active IL-1β was significantly higher in DEHP females while pro IL-1β and active IL-1β in DHS females resembled the VEH (Figure 21C). Pro IL-1β was 120% higher in males and active IL-1 β was 180% elevated in males.

In summary, DEHP exposure induced more pro Caspase-1, active Caspase-1, pro IL-1β, and active IL-1β protein expression than VEH and DHS treated mice, regardless of sex. Surprisingly, male DHS treated mice had lower Caspase-1 and IL-1β protein in the infarct than VEH treated mice. These data suggest that DHS is a strong candidate for a safer alternative to current phthalate- and mellitate-based plasticizers. Moreover, the impact of phthalate exposure is more pronounced in males than females. Together, the findings further indicate that the succinate-based plasticizer, DHS, is less toxic and pro-inflammatory than phthalate-based plasticizer DEHP.

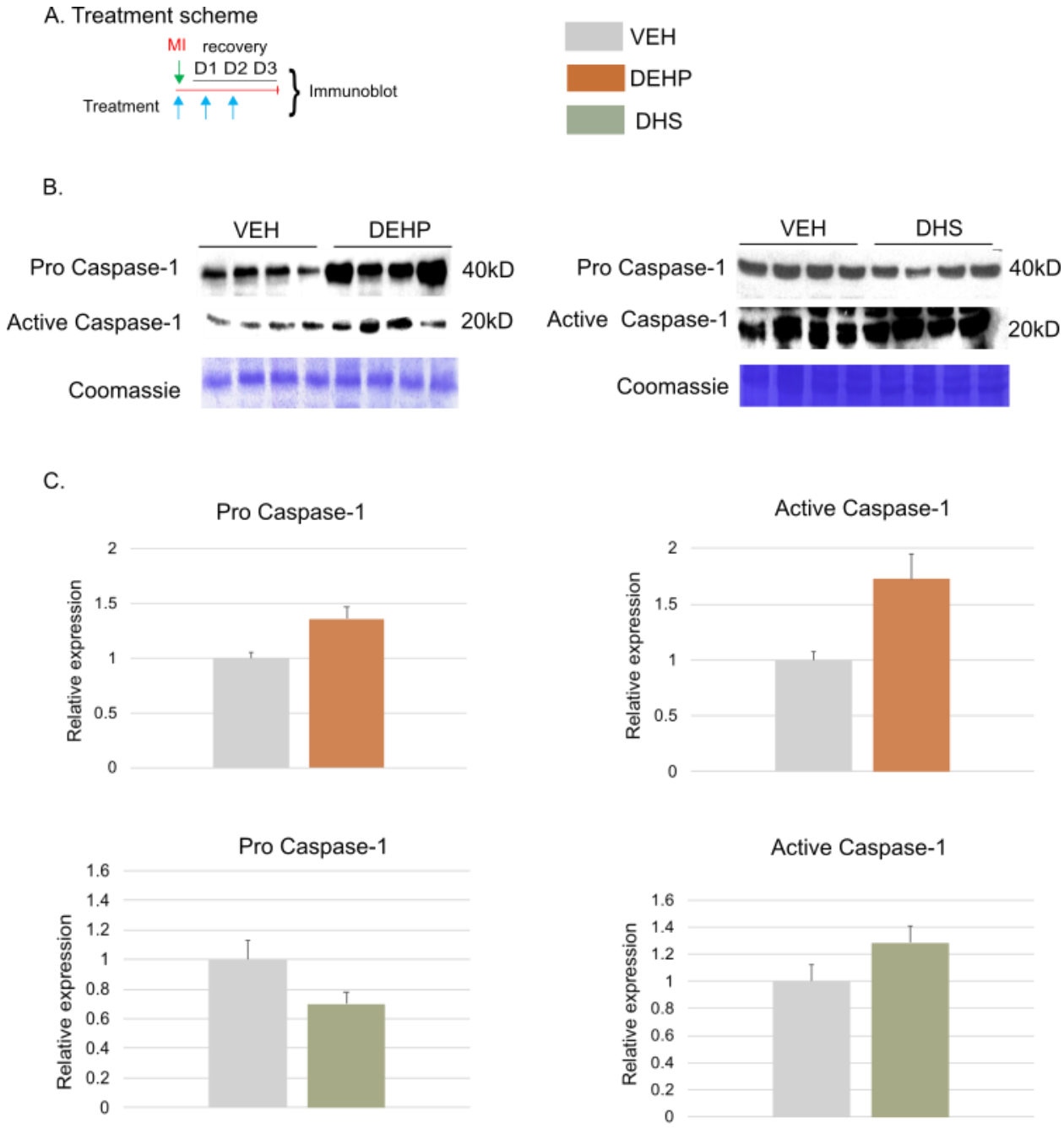

Figure 23. Impact of exposure on Caspase-1, a marker of inflammasome activation in female heart tissue

Impact of phthalate versus non-phthalate plasticizer exposure on Caspase-1 expression in female heart tissue detected by immunoblot. A, Treatment scheme. Surgery was performed on female C57bl/6N mice to create a permanent blockage in the coronary artery. Cohorts were treated with VEH (peanut oil), DEHP, TOTM, DOS, or DHS (25 mg/kg/day). On day 3 post-MI, mice were euthanized, and heart tissue was excised. Protein was isolated and immunoblots were performed. B, Representative blots for expression of Caspase-1. Expression was calculated relative to the stained and scanned Coomassie Blue stained membrane, which was used as a loading control. Expression of VEH treated mice was equal to 1.0. C, Expression of Pro Caspase-1 and Active Caspase-1. Data are the mean ± SEM. A *p* value of <0.05 was considered significant and is indicated by an * in comparison with VEH mice. VEH (n=4); DEHP (n=4); and DHS (n=4).

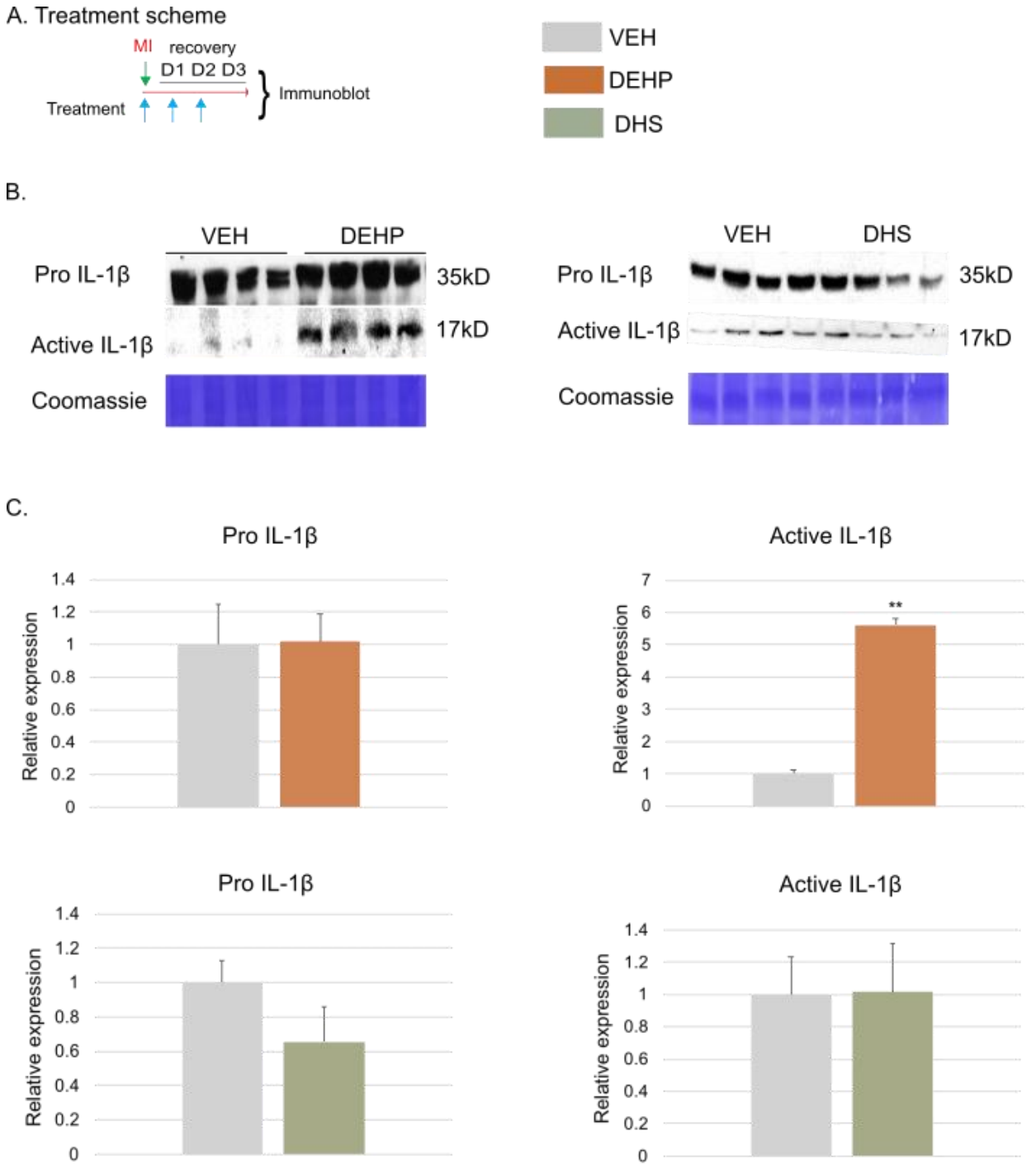

Figure 24. Impact of exposure on IL-1β, a marker of inflammasome activation in female heart tissue

Impact of phthalate versus non-phthalate plasticizer exposure on IL-1β expression in female heart tissue detected by immunoblot. A, Treatment scheme. Surgery was performed on female C57bl/6N mice to create a permanent blockage in the coronary artery. Cohorts were treated with VEH (peanut oil), DEHP, TOTM, DOS, or DHS (25 mg/kg/day). On day 3 post-MI, mice were euthanized, and heart tissue was excised. Protein was isolated and immunoblots were performed. B, Representative blots for expression of IL-1β. Expression was calculated relative to the stained and scanned Coomassie Blue stained membrane, which was used as a loading control. Expression of VEH treated mice was equal to 1.0. C, Expression of Pro IL-1β and Active IL-1β. Data are the mean ± SEM. A *p* value of <0.05 was considered significant and is indicated by an * in comparison with VEH mice. VEH (n=4); DEHP (n=4); and DHS (n=4).

Discussion

A. Phthalate- and mellitate-based plasticizers exacerbate weight loss and reduce cardiac structure and function after an MI in males

The data suggests that post-surgery exposure to phthalate (DEHP) and mellitate (TOTM) plasticizers exacerbates weight loss and LV dysfunction in males. The results indicate that DEHP- and TOTM-treated males experienced the highest percentage of weight loss, and significantly more weight loss than DEHP and TOTM exposed females. In humans, weight loss of >5% after an MI is a significant risk factor for death and cardiovascular complications (174). In addition to increased weight loss, we observed that DEHP and TOTM exposure in males had a tendency towards greater cardiac dilation and significantly reduced cardiovascular function when compared with that of VEH, DOS, or DHS exposed males. Patients that develop LV dysfunction after an MI are at a high risk for a number of adverse outcomes, including recurrent MI, sudden cardiac death, and heart failure (175). Consistent with the lack of any difference in weight loss with treatment in females, no differences in cardiac structure or function post-MI between the different chemical exposure groups in female mice were detected. In contrast to DEHP and TOTM exposure in males, female mice treated with succinate-based plasticizers (DOS and DHS) experienced no changes in weight, cardiac structure, or cardiac function. These results indicate that DEHP and TOTM exposure is more consequential in males and disrupts LV geometric remodeling and indicates sustained LV dysfunction. In support of our results, other studies have shown that DEHP exposure from medical devices disrupts autonomic regulation, heart rate variability, and cardiovascular reactivity (176). Similarly, optical mapping of transmembrane voltage and pacing studies of *ex vivo* rat heart preparations found that phthalate exposure increased atrioventricular node and ventricular effective refractory periods, indicative of disrupted cardiac electrophysiology (63). In other work, TOTM and DEHP exposure led to an increase in inflammation, cardiac dilation, and reduced cardiac function (91).

While previous studies using an *in vivo* mammalian model have shown that lifelong BPA exposure modifies cardiac structure and function in males (177), these are the first studies to directly compare males and females and the first to identify sex-specific differences for phthalate and mellitate exposure on cardiovascular structure and function post-MI. Together, these data imply that DEHP and TOTM exhibit a sex-specific cardiotoxicity, and that exposure can promote complications of MI, including increased weight loss, increased cardiac dilation, and reduced cardiac function. Furthermore, the improved health results of mice exposed to succinate-based plasticizers indicates their potential as less hazardous plasticizers.

B. Acute exposure to TOTM and DEHP leads to greater inflammation post-MI in males

For all mammals, monocytes and macrophages are key innate immune cells that participate in infection control and wound healing (164). Wound healing is equivalent in mammals, follows the same trajectory in solid organs, and depends on the orchestrated infiltration of monocytes and macrophages. Immune cell infiltration into wounds is orchestrated with neutrophils arriving first, followed by monocytes, which then differentiate into macrophages (178). We detected a greater number of neutrophils, monocytes, and macrophages in the post-MI ischemic area of male DEHP and TOTM exposed males compared with the infiltration detected in VEH, DOS, and DHS treated hearts. Accordingly, we found that infarcts from DEHP and TOTM exposed males had increased expression of multiple innate immune cell receptors and the monocyte chemoattractant ligand CCL2. In contrast, DHS exposure resulted in the lowest expression of cell receptors (NLRP3, NLRP6, AIM2, and P2xR7) and CCL2. In concert with the observed increase of infiltrating macrophages, DEHP and TOTM exposure led to increased expression of phagocytosis-related proteins (Mfge8, MMP9, and MerTK). In similar studies, mono-(2-ethylhexyl) phthalate (MEHP) treatment of prepubertal rats increased testis chemokine expression including CCL2 expression, and neutrophil and macrophage infiltration

(179, 180). In a previous study, we observed that DEHP and Bisphenol A exposure elevated levels of monocytes, macrophages, and neutrophils post-surgery in male mice (91). Hence, we conclude that local increases in chemoattractant and immune cell receptors are a primary factor inducing a greater accumulation of inflammatory cells in male exposed mice. While the impact of phthalate exposure on cardiovascular health remains largely unexplored, data from separate tissues support our observation that phthalate exposure induces immune cell accumulation. Thus, phthalate exposure is consistently associated with an elevated immune cell infiltration across a broad variety of tissue subsets.

Consistent with the lack of any change in body weight and echocardiography data in females, the different plasticizers had no influence on immune cell infiltration in female mice. Specifically, neither phthalate, mellitate, nor succinate plasticizers increased the infiltration of monocytes, macrophages, or neutrophils. Similarly, there were no evident differences in expression of chemokine or phagocytosis-related genes. Thus, we found that acute exposure of a clinically relevant dose of DEHP and TOTM resulted in a sex-specific innate immune response associated with increased chemokine ligand and macrophage receptor expression. These are the first data to identify a sex-specific innate immune response to phthalate and mellitate plasticizers.

The greater influx of monocytes, macrophages, and neutrophils is likely detrimental, and suspect that the monocytes and macrophages are dysfunctional. The observed increase in MMP expression in infarct homogenates of DEHP and TOTM exposed males may be a composite of increased myeloid cell infiltration. MMP9 expression is primarily attributed to proinflammatory macrophages, suggesting a link between the increased accumulation of monocyte/macrophages and the increased MMP9 activity in DEHP and TOTM exposed mice. Increased MMP activity is linked to greater dilation post-MI (181). In support of the idea that increased MMP activity in the infarcts was monocyte derived, acute exposure of THP-1 macrophages to DEHP and TOTM *in vitro* increased MMP9 expression. In other work using

THP-1 macrophages, DEHP exposure analogously increased MMP activity (110). Thus, increased MMP activity is a consistent feature of *in vivo* and *in vitro* phthalate and mellitate exposure. Here, these results demonstrate sex-specific DEHP- and TOTM-mediated increases in MMP activity result from acute exposure post-surgery *in vivo* and link this to increased MMP expression to acute exposure in monocyte/macrophages *in vitro* .

C. Macrophages entering the infarct are dysfunctional and proinflammatory in DEHP and TOTM exposed males

Monocyte/macrophage cells entering the infarcts of DEHP or TOTM exposed mice are likely highly inflammatory and promote an excessive immune response that aggravates recovery. Macrophages arrive at the site of tissue injury 24-48h post-injury, serve as phagocytes clearing the wound bed, and initiate the processes that lead to the default outcome of scar tissue formation (182). Immediately after injury, infiltrating macrophages become polarized toward a pro-inflammatory or M1 phenotype. (183). Here, 3 days post-surgery, males exposed to TOTM had a persistently elevated infiltration of CD38+ proinflammatory macrophages and DEHP and TOTM exposed mice had greater expression of M1 markers NOS-2, CCR7, and Egr-1 compared with levels detected in samples from VEH, DOS, and DHS treated males. The transition from inflammatory to repair phenotypes *in vivo* resembles the polarization of monocytes to pro-inflammatory or anti-inflammatory phenotypes *in vitro*. In concert with our *in vivo* data, *in vitro* DEHP and TOTM exposure significantly elevated expression of M1 markers CXCL10 and IL-6 compared to that of the VEH, suggesting that exposure to these chemicals promotes a proinflammatory macrophage population that persists beyond the normal inflammatory phase of wound repair.

During normal wound repair, macrophages participating in the remodeling of injured muscle show a transition from a pro-inflammatory M1 phenotype to the immunoregulatory and anti-inflammatory M2 phenotype after 2 days. Interestingly, in addition to the observed

increased in M1 macrophages, DEHP and TOTM exposure in male mice resulted in a simultaneous increase of M2 anti-inflammatory macrophage polarization markers, Fizzl-1 and IL-10. Similarly, DEHP and TOTM exposure significantly elevated expression of M2 markers CD206 and CD163 *in vitro.* The concept of classically and alternatively activated (M1 and M2) macrophages, often regarded as “good” and “bad” macrophage populations respectively has become less nuanced. Rather, excessive recruitment of both subgroups has been shown to promote inflammation and amplify disease progression (184).Concurrent increases of markers of alternatively activated M2 macrophages as well as activated M1 macrophages suggests a dysfunctional population of macrophages at the site of the wound.

In contrast to male mice, DEHP and TOTM exposure in females did not cause a significantly elevated infiltration of CD38+ M1 macrophages compared to VEH treated mice. DEHP exposure did increase expression of M1 marker NOS-2, however, indicative of an increased inflammatory response, albeit to a lesser extent than that of DEHP males. Interestingly, DOS and DHS treated females displayed an elevated CD206+ M2 infiltration of macrophages, suggesting greater anti-inflammatory response for succinate-based plasticizer exposure. Thus, the data indicates that DEHP and TOTM exposure results in a sex-specific infiltration of dysfunctional inflammatory cells. Meanwhile, the innate immune response and macrophage population of animals exposed to succinate-based plasticizers resembles that of VEH-treated mice.

D. DEHP activates the NLRP3 inflammasome

After an MI, successful reperfusion and revascularization can reduce the size of the infarct and improve clinical outcomes. However, excessive inflammation after an MI, characterized by the release of inflammatory cytokines and chemokines that recruit circulating neutrophils and monocytes to the ischemic area, can impede recovery (185). Inflammasomes mediate the inflammatory responses after vascular injury and act as a sensor for cardiovascular

damage and inflammation. Inflammasomes are large cytoplasmic multiprotein complexes comprising of a sensor protein, inflammatory caspases, and, in some cases, an adaptor protein. Inflammasomes can be activated by a myriad of endogenous and exogenous stimuli, resulting in enzymatic activation of caspase-1, secretion of proinflammatory cytokine IL-1β, and apoptotic or pyroptotic cell death (186). Appropriate inflammasome activation is critical for an effective response to tissue damage, while aberrant or excessive activation can cause uncontrollable tissue responses that impede recovery. Therefore, it is imperative to maintain a fine balance between inflammasome activation and inhibition, especially in the context of recovery from MI.

Damage-associated molecular patterns (DAMPs), which are signals of host cellular distress, are sensed by a wide variety of pattern recognition receptors (PRRs) and cells of innate and adaptive immunity (187). PRRs of the innate immune system can be divided into at least four distinct families: Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors (RLRs), C-type lectin receptors (CLR), and NLRs (188). Activation of these receptors ultimately results in the production of inflammatory cytokines that drive inflammatory responses. At the same time, complete inhibition of PRRs in patients impedes recovery, indicating that a baseline immune response is necessary for effective healing (189).

Here, DEHP exposure significantly increased the expression of NLRP3 in male and female mice. AIM2 expression was also increased in males, although not to the same extent as NLRP3, suggesting some specificity for NLRP3 receptor activation. Activation of the NLRP3 inflammasome leads to the production of the potent proinflammatory cytokine IL-1β. In agreement with this, DEHP exposure increased expression of Caspase-1 and IL-1β RNA and protein in male mice. Similarly, we observed a significant increase in Capsase-1 and IL-1β protein in females exposed to DEHP. However, the fold increase in NLRP3, Caspase-1, and IL-1β was elevated to a lesser extent in females. Furthermore, treatment of macrophages with DEHP *in vitro* resulted in increased Caspase-1 and IL-1β protein released into the media. In other studies, activation of the NLRP3 inflammasome contributed to infarct size through

pyroptosis and was directly linked to recovery post-MI (124). Correspondingly, treatment of THP-1 macrophages increased expression of proinflammatory cytokines, including IL-1β (110). The release of ATP during inflammation or injury stimulates the innate immune system and chronic pain through purinergic receptor P2xR7 (190), which can activate the NLRP3 inflammasome. DEHP exposure also significantly increased the expression of P2xR7 *in vivo* and *in vitro*. Previously, phthalates were shown to activate the NLRP3 inflammasome through the P2xR7 receptor in HepG2 and L-2 cells (191). Meanwhile, DHS exposure did not increase the expression of PRRs, Caspase-1, or IL-1β RNA and protein. In fact, a decrease in Caspase-1 and IL-1β protein relative to the VEH for DHS exposed male mice was observed.

TOTM exposure, like DEHP exposure, resulted in the increased expression of NLRP3,and P2xR7 in male mice. The expression of these receptors was elevated to a lower degree than that of DEHP exposed animals, however.

The abundance and diversity of NLRP3 activators is one the inflammasome's most distinctive features (192). It is therefore assumed that the NLR3 inflammasome likely senses a common secondary activator downstream of these stimuli or responds to cellular stress associated with physiological damage. In fact, production of reactive oxygen species (ROS), potassium efflux, change in cell volume, calcium signaling, and lysosomal disruption have all been proposed to be integral upstream signals required for NLRP3 activation (192). NLRP3's reactivity to a wide variety of

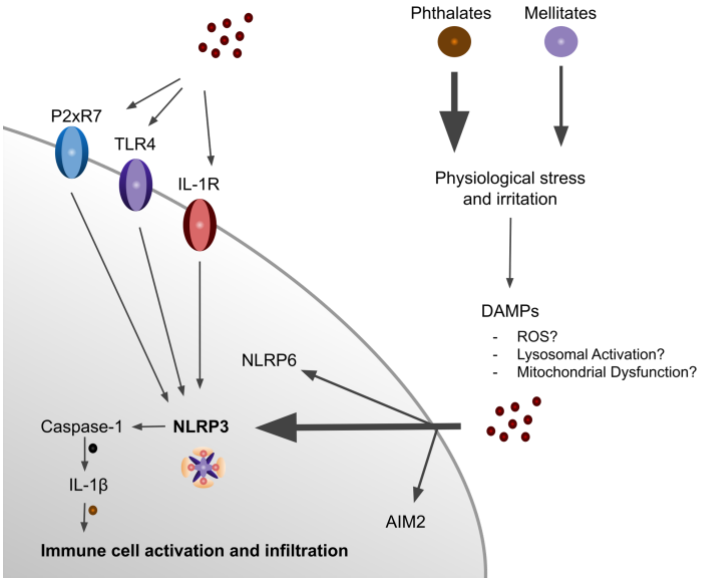

Figure 25. Proposed mechanism for NLRP3 activation
Phthalates and, to a lesser extent, mellitates cause physiological stress and irritation, resulting in damage associated molecular patterns. DAMPs are processed by membrane (P2xR7, TLR4, IL-1R) and cytosolic inflammasome receptors (NLRP3, NLRP6, and AIM2). As a result of its promiscuity, NLRP3 inflammasome activation is the primary transducer of phthalate- and mellitate-dependent stress. Non-NLRP3 receptors work in concert to drive the inflammatory cascade. Inflammasome activation results in Caspase-1 cleavage, IL-1β activation, and, ultimately, immune cell activation and infiltration.

stimuli makes it a potent transducer of cellular distress. In consideration of the data and the current understanding of inflammasome activation, it can be speculated that phthalate and mellitate (to a lesser extent) exposure activates an inflammatory cascade whereby DEHP and TOTM synergize with DAMPs to primarily activate the NLRP3 inflammasome (Figure 25). NLRP3 activation is more pronounced in males, resulting in an excessive inflammatory response characterized by increased expression of Caspase-1 and IL-1β, which drives immune cell activation and infiltration. Meanwhile, DHS exposure does not activate the NLRP3 inflammasome and induce an exaggerated inflammatory response.

E. Mitigation strategies: the potential of succinate-based plasticizers

The most commonly used plasticizers, phthalates, are classed as carcinogenic, mutagenic, and toxic under CLP regulation and the risks associated with phthalate exposure are well documented (193). As a result, phthalates have been subject to increased regulations and there has been increased pressure to replace phthalates with new, non-toxic, safer alternatives. TOTM is the most common phthalate alternative in clinical settings, despite the limited data assessing its migration from PVC, level of exposure in the population, and toxicity (133, 134). Several candidate “green” plasticizers have been designed based on DEHP’s biodegradation pathways to avoid producing toxic or persistent bioproducts. Succinate-based plasticizers are based on renewable raw materials, are inexpensive to produce, demonstrate effective plasticizing ability, and biodegrade most rapidly from candidate alternatives (131). Thorough testing in a variety of contexts is needed to validate these alternatives and to provide the necessary evidence that they are safer than currently used plasticizers. Here, we evaluated the safety of DEHP, TOTM, DOS, and DHS *in vitro* and *in vivo* in the context of surgical recovery post-MI.

In agreement with the increasing body of evidence documenting the health hazards associated with DEHP exposure, we observed that DEHP caused an increase in infiltrating

neutrophils, monocytes, and macrophages into the wound. Male mice exposed to DEHP experienced the greatest increase in cardiac dilation and reduction in cardiac function. Quantification of pro-inflammatory markers *in vitro* and *in vivo* further indicated that DEHP induces a dysfunctional inflammatory phenotype driven by activation of the NLRP3 inflammasome. Similarly, male mice exposed to TOTM experienced increased cardiac dilation, reduced cardiac function, infiltration of innate immune cells, expression of proinflammatory markers, and elevated NLRP3 expression. Elevated levels of circulating pro-inflammatory biomarkers and immune cell infiltration correlate with disease severity and prognosis (194). Together, the data indicates that both DEHP and TOTM exposure poses a significant obstacle to effective recovery from MI. Moreover, the phthalate alternative, TOTM, does not appear to improve recovery. On the contrary, while TOTM is marketed as a less-toxic alternative, trimellitate treated mice experienced similar impairments in recovery to that of DEHP treated mice.

Meanwhile, mice exposed to the succinate-based plasticizers, DOS and DHS, exhibited no changes in weight, cardiac dilation, or cardiac function, regardless of sex. Immune cell infiltration for male and female mice exposed to succinate-based plasticizers resembled VEH treated animals. From the two succinate-derived plasticizers, DHS outperformed DOS in several categories. While the 7 carbon structure used for the production of DHS is from a biobased source, the 8 carbon backbone of DOS is petroleum derived, suggesting a potential source for the variability between the two succinate-based plasticizers (195). DHS exposed males and females exhibited the lowest expression of NLRP3, and pro-inflammatory markers *in vivo* and *in vitro*. While DEHP and TOTM strongly activate the NLRP3 inflammasome to drive a dysfunctional proinflammatory phenotype that impedes recovery, neither DOS nor DHS activate the NLRP3 proinflammatory cascade. Taken together, succinate-based plasticizers allow better healing post-MI and are, thus, promising candidates for the replacement of phthalates. In addition to their performance in the context of recovery, bio-based plasticizers are

environmentally appealing because they are extracted from renewable resources. In consideration of the aforementioned data, we rank the safety of plasticizers from safest to most toxic in the following order: DHS > DOS >>> TOTM > DEHP.

F. Phthalates and mellitates have a sex-specific impact on recovery

The adverse impacts of endocrine disrupting chemicals, including phthalates, are complex and sex specific in several contexts. Current literature indicates that males appear more sensitive to the adverse consequences of phthalate exposure, albeit the bodily systems that are disproportionately affected and the underlying mechanisms governing these differences remain largely unexplored. From the few studies published that examine male versus female plasticizer response, phthalate exposure is associated with a greater risk for cardiometabolic disease (143, 144), neuroendocrine dysfunction (136), and impaired reproductive development in males (139, 140). The sex-specific influence of phthalate exposure on immune responses and recovery from a major surgery *in vivo* has not been explored, however. Moreover, the mechanistic underpinnings of sex-dependent responses to phthalate exposure remain largely elusive. We are the first to classify a sex-specific response to phthalate and mellitate exposure in the context of recovery. We found that male mice exhibit greater cardiac dilation, reduced cardiac function, and increased infiltration of neutrophils, monocytes, and macrophages in response to phthalate and mellitate exposure compared to equally exposed females.

We postulate that the observed increase in immune cell infiltration and hindered recovery in males can be attributed to greater activation of the NLRP3 inflammasome. Recently, it has been shown that NLRP3 is upregulated at the surgical site and drives postoperative mechanical pain-like behaviours in male mice, but not in female mice (196). Similarly, human male patients with aortic aneurism and cancer have been shown to display increased mRNA levels of NRLP3 compared to that of female patients (145, 197). This data suggests that the immune-mediated mechanisms that underlie postoperative responses are sex-specific and

influenced by NLRP3 inflammasome assembly. In support of this notion, emerging evidence has shown that estrogen can act as an inflammatory protective factor to suppress NRLP3-mediated inflammation (146, 198). In fact, several studies strongly indicate that key difference in immune-inflammatory responses between men and women may be driven by sex hormones, whereby estrogen carries anti-inflammatory properties while testosterone promotes inflammation (199). Females have elevated levels of baseline estrogen and progesterone levels whereas males have higher baseline testosterone. Estrogen, progesterone, and testosterone receptors are expressed on both adaptive (T cells and B cells) and innate (macrophages, neutrophils, dendritic cells, and natural killer cells) immune cells (200). Thus, the level of immune cell infiltration and the extent of the innate immune response at an injury site are influenced by sex hormones. Estrogen suppresses neutrophil and macrophage recruitment in female mice (201-203). Furthermore, estrogen drives macrophages toward the M2 phenotype (anti-inflammatory) while testosterone promotes an M1 phenotype (proinflammatory). As a result, males display higher expression of TLR4, NLRP3, and produce more IL-1β than females (204). Additionally, global deletion of NLRP3 decreases IL-1β production in males, but not in females, suggesting that IL-1β production in females occurs independent of NLRP3 (205). Thus, sex hormones may be involved in the sex-dependent NLRP3 inflammasome-mediated response to plasticizer exposure in the context of tissue injury. The exact role of sex hormones on inflammasome activation need to be further elucidated in this context, however.

Many of the differences in immune-mediated responses to injury between males and females are caused by the presence or absence of sex hormones, but besides sex hormones there are other factors that can influence adverse cardiac modeling after an MI, such as sex chromosomes. It has been shown that several genes related to adverse cardiac remodeling, such as macrophage activation, apoptosis, and lipid metabolism are located on the Y chromosome (151).

In conclusion, the greater activation of the NLRP3 inflammasome in males post-MI can likely be attributed to several factors. The NLRP3 pathway is likely influenced by the expression of genes on the Y chromosome and by sex hormones. Our data indicates that the immune-mediated mechanisms that underlie postoperative recovery are likely sex-specific, and, as a result, respond differently to disruption by plasticizer exposure. The protective role of estrogen and absence of Y chromosome genes likely makes females less susceptible to the adverse consequences of phthalate and mellitate exposure.

G. Plasticizers in the Context of Clinical Settings

Plastics maintain an important presence in everyday life and have revolutionized hospital care. Despite their many advantages, concerns continue to be raised about the ubiquitous use of plastics and plasticizers in a clinical setting (141). The consequences and risks associated with phthalate exposure are well documented, nevertheless DEHP continues to be used in medical devices. Due to its low-cost of production, strength, flexibility, and suitability for steam sterilization, DEHP remains the most widely found plasticizer in PVC-based medical products (64). A recent safety assessment by the U.S. Food and Drug Administration expresses concern that certain medical procedures that result in high levels of phthalate exposure may not be safe (165). Elevated phthalate exposure has been observed in patients undergoing invasive medical procedures that use large volumes of plastic materials, including cardiopulmonary bypass, extracorporeal membrane oxygenation, dialysis, and transfusion procedures (176). This is particularly worrisome, since patients who are most susceptible to hospital-based phthalate exposure are often at increased risk for complications. For example, cardiovascular function is highly susceptible to xenobiotic toxicity, and hence, exogenous chemical exposure may contribute to adverse health outcomes in a variety of settings (206). Plastics are an integral part of hospital care and plastic chemical exposure during medical procedures is virtually

unavoidable, yet the direct impact on patient health and recovery remains to be properly addressed.

Concentrations of DEHP leachates from medical equipment are significantly higher than that of food and water and hospital patients experience the most intense exposure (18). To mitigate the complications associated with phthalate exposure and to improve patient outcomes, phthalates need to be replaced with safer, non-toxic alternatives. In our evaluation of plasticizer safety, a clinically relevant dose of succinate-based plasticizers did not pose a significant safety risk to post-surgical recovery, contrary to DEHP and TOTM. Consequently, the replacement of currently used plasticizers with succinate-based plasticizers (DOS and DHS) is a promising intervention to improve patient outcomes for the many operations that employ plastic medical equipment.

H. Limitations

Some limitations should be noted. First, we did not test other cell types beyond the male human THP-1 monocyte cell line, or other female macrophages. Furthermore, we identified a sex specific impact, but can only speculate that it is a byproduct of sex hormone or Y chromosome mediated mechanisms.

Conclusion

Our data suggests that escape of phthalates and the phthalate alternative, TOTM, from medical devices used during cardiac surgery, increases inflammation and impairs cardiac healing post-MI. Moreover, we are the first to evaluate the sex-specific impact of phthalate versus non-phthalate plasticizer exposure in the context of surgical recovery post-MI. The detrimental effects of DEHP and TOTM on recovery and excessive inflammation are more pronounced in males compared to that of females. In response to DEHP and TOTM exposure, males experienced increased weight loss, greater cardiac dilation, and reduced cardiac function. Furthermore, males experienced increased proinflammatory immune cell infiltration in the infarct, greater expression of polarization, inflammatory, and macrophage markers *in vitro* and *in vivo*, and excessive release of Caspase-1 and IL-1β protein *in vitro* and *in vivo.* Meanwhile, the succinate-based plasticizers, DOS and DHS, most closely resembled the VEH treatment group. DHS exposure consistently exhibited the greatest recovery and lowest indication of an excessive inflammatory phenotype. Together, our findings indicate that males are more affected by plasticizer exposure than females in the context of surgical recovery post-MI. Moreover, in our evaluation safety and toxicity of non-phthalate-based plasticizers, DHS ranks first followed by DOS. Meanwhile, replacement of DEHP with TOTM does not ablate the impact of phthalate exposure.

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