Characterizing the Role of Pre-Pubertal Status in Sepsis: Why Do Children Survive When Adults Do Not

Characterizing the role of pre-pubertal status in sepsis:

Why do children survive when adults do not?

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Abstract

Epidemiological data from both historical and contemporary sources suggest that pre-pubertal children possess a relative resistance to death from severe infections and sepsis. To assess the role that pubertal status may play in protection from sepsis mortality, we employed two different experimental strategies. In our first strategy, we demonstrated the significant enhancement of survival in pre-pubertal animals using an experimental mouse model of endotoxemia. Although both pre- and post-pubertal animals exhibited an equally robust initial response to endotoxin, there were significant age-associated differences in cytokine and leukocyte dynamics later on. Prevention or acceleration of puberty by hormonal manipulation resulted in increased or decreased survival respectively, highlighting the importance of the pubertal transition in defining the resistance phenotype. In addition, the adoptive transfer of pre-, but not post-pubertal, peritoneal cells improved the survival of post-pubertal recipient mice, supporting our hypothesis that a pre-pubertal biology may hold the key to improving the survival of adults. In our second strategy, we sought to identify drug candidates for the treatment of sepsis by data-mining publicly available whole blood transcriptomes from septic adults and children. Pathprint, a pathways-based transcriptome analysis platform, was used to highlight key differences between the two age groups. Subsequently, a novel *in-silico* drug development system was used to identify agents that might promote beneficial pathways (i.e. activated in children) or inhibit harmful ones (i.e. activated in adults). This method uses a pathway-drug network (PDN) to probe specific clusters of pathways exhibiting age-associated differences for correlations with drug-based gene signatures built into the network. We validated resultant drug candidates by literature curation, and found that the PDN method produced a substantially higher rate of positives when compared to a purely gene-level ConnectivityMap analysis. Additionally, in direct testing using an endotoxemia model of murine sepsis, 5 of 10 compounds tested significantly improved survival.

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CHAPTER 1: Introduction

1.1. Sepsis: a major public health problem

Despite decades of research and vast improvements in clinical care, sepsis remains a devastating cause of death in the United States and throughout the world. Sepsis is a complex syndrome in which the host response to infection becomes dysregulated, causing life-threatening organ dysfunction and failure despite appropriate antibiotic treatment and supportive care [1]. Estimated to afflict between 1 and 3 million people in the USA annually [2], sepsis still lacks any specific therapeutic interventions beyond supportive care. The resulting lengthy hospital stays cost our healthcare system over $20 billion per year—more than any other medical condition [3]. Although improvements in supportive care strategies have increased survival in the US over the last thirty years [4], sepsis still remains the 11th leading cause of national mortality [5].

Why is sepsis still such a significant public health issue? The rapid identification and treatment of sepsis is complicated by the extreme heterogeneity of the condition and the people it affects [6]. While the risk factors for developing a major infection are well established (e.g. chronic obstructive pulmonary disease, AIDS, certain cancers, immunosuppressive treatments etc.), the risk factors for subsequent organ dysfunction are less understood. Individual patient risk varies greatly by the type of infectious agent involved, the location of infection, the timeliness of supportive care as well as a particular patient's comorbidities, age, sex, and race [4]. An incomplete understanding for the complex pathophysiology that drives sepsis has made the search for a targeted therapy extremely difficult. Despite hundreds of clinical trials for various drug candidates over the past thirty years, at present sepsis treatment is limited to rigorous supportive care.

1.2. The "dysregulated" host response

The precise pathophysiology of sepsis remains poorly understood, but a general consensus is that what begins as a normal host response to infection can, in certain circumstances and individuals, lead to body-wide chaos and a complete inability to return to homeostasis. There are a dizzying number of cellular and molecular players involved in this dysregulated host response, as surveyed next.

The host response to infection can be triggered by a variety of mediators derived either from the pathogen or from the host. These mediators include microbe-derived pathogen associated molecular patterns (PAMPS) (e.g. gram negative bacterial lipopolysaccharide (LPS), viral double-stranded RNA (dsRNA)) and host-derived damage associated molecular patterns (DAMPs) (e.g. high-mobility group box 1 (HMBG1), heat shock proteins, histones). Both PAMPs and DAMPs mobilize the immune system by binding to and signaling through pattern-recognition receptors (PRRs), such as the toll-like receptors (TLRs), nod-like receptors (NLRs) and others, found on and within leukocytes and structural cells like endothelial and epithelial cells. PRR binding by PAMPs and DAMPs activates the cells and promotes the rapid generation and local release of many different pro-inflammatory mediators. These include 1) vasoactive compounds that increase endothelial permeability and local vasodilation (e.g. histamine, nitric oxide (NO), prostaglandins), 2) chemokines that draw blood phagocytes into infected tissue (e.g. interleukin 8 (IL-8), Monocyte Chemoattractant Protein 1 (MCP-1) etc.), 3) adhesion molecules that allow leukocytes to traverse past the endothelial barrier (e.g. integrins, intracellular adhesion molecule I (ICAM 1), vascular adhesion molecule 1 (VCAM1), etc.), 4) cytokines that activate nearby cells (e.g. tumor necrosis factor α (TNF-α), IL-1, IL-6), and 5) antimicrobial compounds that help damage and destroy pathogens (e.g. reactive oxygen species (ROS), reactive nitrogen species (RNS), various proteases). The release of IL-6 and other cytokines has important systemic effects as well, especially in a more serious illness. In particular, these cytokines promote the development of fever and sickness behaviors in the brain like lethargy, weakness, and anorexia, and also drive the production of various acute phase proteins by the liver. These proteins serve many important functions in the inflammatory response such as regulating coagulation (e.g. fibrinogen, pro-thrombin, etc.) and supporting the activation and anti-microbial activities of leukocytes (e.g. complement factors, C-reactive protein, ferritin, etc.).

All together, these events drive the increased recruitment of pathogen-fighting phagocytes into infected tissues and organs, followed by the activation of antigen-presenting cells like dendritic cells and subsequent activation of the B and T cells of adaptive immunity. Normally, these inflammatory responses are beneficial and will resolve after clearance of the pathogen in response to the release of anti-inflammatory cytokines (e.g. IL-10, soluble IL-1 receptor, soluble TNF-α receptor) and pro-resolution

lipids (e.g. resolvins, lipoxins, maresins). However, in septic individuals, the intensity of the inflammatory stimulus, due to either the systemic distribution (bacteremia) or virulence of an infectious agent, becomes excessive, chaotic and prevents a return back to homeostasis.

Our understanding of this dysregulated host response and how it drives sepsis pathophysiology has changed radically over the last four decades, and continues to evolve. In the mid-1980s, concurrent with the discovery and characterization of several important pro-inflammatory cytokines (e.g. IL-1, TNF-α, IL-6), sepsis was deemed to be simply the product of an overly exuberant pro-inflammatory immune response to infection [7,8]. This deleterious inflammatory response, separated from its infectious cause, was termed the systemic inflammatory response syndrome or SIRS [9]. The possibility of an anti-inflammatory "magic bullet" to treat SIRS/sepsis precipitated an explosion of research into drugs that either blocked the initial stimulation of the host response (e.g. anti-endotoxin antibodies, etc.) or blocked the cytokines and other acute phase proteins that were released as a result (e.g. anti-TNF-α antibodies, etc.). Unfortunately, in large-scale clinical trials, these "magic bullets" repeatedly proved to be ineffective or even harmful in the treatment of sepsis [10].

These failures, coupled with a growing understanding of the anti-inflammatory arm of the immune system set the stage for further evolution of the sepsis dogma. With improvements in supportive care, a greater percentage of sepsis patients were actually surviving the initial pro-inflammatory insults, only to then descend into a prolonged state of severe immunosuppression. Global leukocyte anergy and apoptosis greatly increased these patients' risk of developing secondary infections [11]. This compensatory anti-inflammatory response syndrome (CARS) was considered the anti-inflammatory "twin" of SIRS [10]. Despite the promise of therapies to stimulate the immune system back into normal functionality (e.g. granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), intravenous immunoglobin (IVIG), Interferon γ (IFN-γ), etc.), none have yet been proven effective in large-scale clinical trials [12]. It became clear that a bimodal SIRS/CARS view of sepsis was still too simplistic.

The current consensus is decidedly more vague than in the past: sepsis is an extremely complex condition that can take on multiple trajectories depending on the particular individual affected. This

trajectory may skew toward release of pro-inflammatory mediators, anti-inflammatory mediators, or, in most cases, simultaneous release of both without resolution. In an effort to encapsulate the extreme lack of homeostasis that drives the body's organs to fail, the attendees of the Sepsis-3 conference adopted the imprecise term "dysregulated" to describe the host response during sepsis [1]. The most deadly iteration of this anti-homeostatic condition, termed chronic critical illness (CCI), takes the affected individual along a prolonged trajectory of un-resolving inflammation and immunosuppression. Long-term studies have shown that even the patients that survive CCI are typically afflicted with recurrent infections, continuing organ dysfunction, malnutrition, and cognitive decline and exhibit poor long-term survival [13,14].

Much of sepsis research has focused on the effect that the systemic, dysregulated release of inflammatory mediators can have on the pathogenesis and outcomes of human sepsis patients and animal models. For example, the intensity of the mixed (pro- and anti-inflammatory) cytokine response in plasma samples of septic patients is associated with the occurrence of severe complications and increased mortality [15-20]. A similar mixed cytokine response has been demonstrated in mouse models of sepsis, and is also associated with a poor outcome [21-25]. Unfortunately, as of yet, none of this sepsis research has yielded any effective molecular targets for sepsis therapy. However, we do now have a more detailed picture of the ways in which the various components of the dysregulated immune response contribute to the deadliest complications of sepsis, namely organ dysfunction and failure and immunosuppression. These two topics are reviewed next.

1.3. The pathogenesis of organ dysfunction and failure in sepsis

Depending on the individual and the intensity of the response, sepsis can trigger the development of numerous potentially fatal complications that harm the body's main organ systems. Sepsis most commonly affects the respiratory and cardiovascular systems, manifesting as Acute Respiratory Distress Syndrome (ARDS) and shock, but also has profound effects on brain, kidney and liver function. Though the exact mechanisms are unknown, organ dysfunction and failure during sepsis can be linked to four disturbances of homeostasis: 1) breakdown of endothelial barriers, 2) hemodynamic

deregulation, 3) hemostatic deregulation, and 4) mitochondrial dysfunction. All of these disturbances stem back to the dysregulated release of inflammatory mediators in response to infection.

Breakdown of Endothelial Barriers: The endothelial cells lining the vasculature serve as a barrier between the circulating blood and parenchymal cells. In health, the endothelium helps to control fluid homeostasis and regulate leukocyte adhesion and migration into the tissues. The boundaries between adjacent endothelial cells are the main locations where permeability is regulated. Within these border zones are multiple points of connection or "junctions" between adjacent endothelial cells that serve to regulate the entry of fluid, solutes, large proteins (e.g. albumin), and, during local inflammation, leukocytes [26]. Adherens junctions are especially important in regulating endothelial permeability during inflammation [27]. Endothelial permeability is also regulated by a thick, gel-like layer composed of proteoglycans and glycoproteins on the apical side of the endothelial cell called the glycocalyx [28]. The unique chemical composition of this layer helps to inhibit the entry of plasma proteins like albumin [29], protect the endothelial cell from oxidative damage [30], and prevent unnecessary leukocyte adhesion and transmigration [31,32].

During sepsis, the barrier function of the endothelium becomes markedly compromised, leading to an uncontrolled flow of fluid and inflammatory leukocytes into the tissues. Systemic release of inflammatory mediators such as vascular endothelial growth factor (VEGF), TNF-α, platelet activation factor, histamine, thrombin, nitric oxide (NO), and peroxynitrite promote the disassembly of adherens junctions and increase permeability [26]. Many of these same mediators, reactive oxygen species in particular [33-36], cause shedding of the protective glycocalyx layer from the endothelial cells [31,37]. Accumulation of parenchymal and interstitial fluid due to systemic vascular leak can impair organ function by increasing the distance required for oxygen and nutrients to diffuse and by interfering with microvascular perfusion.

Circulating leukocytes migrate into inflamed tissues by responding to chemoattractant agents including bacterial products, DAMPS, and chemokines. Migration from the bloodstream requires the leukocytes to tightly adhere to endothelial cells and then transmigrate between (or through) them. Cytokines such as VEGF, TNF-α, IL-1, IL2, IL-6, and other inflammatory mediators like thrombin, and

histamine cause increased expression of leukocyte adhesion proteins (e.g. endothelial leukocyte adhesion molecule 1, ICAM 1, VCAM 1, etc.) on the endothelial cell surface [37]. Damage to the glycocalyx during sepsis exposes these adhesion molecules and promotes leukocyte migration. Once adhered, migrating cells easily cross into the tissue due to adherens junction disassembly and increased paracellular permeability [31].

Hemodynamic Deregulation: Under normal physiological conditions, when there is a localized area of hypo-perfusion, endothelial cells sense the metabolic and mechanical signals associated with the decrease in blood flow and respond through production of vasodilators such as NO via the enzyme endothelial NO synthase (eNOS). Sepsis affects hemodynamic regulation in a major way. Profuse vascular leak causes systemic loss of blood volume setting into motion severe vasomotor dysfunction. In response to inflammation, inducible NO synthase (iNOS), may be differentially expressed across vascular beds, leading to hyper-perfusion of certain areas and hypo-perfusion of others [38-40]. The inactivation of eNOS, and the interference with NO production by reactive oxygen species further decrease blood flow to these specific areas [31,41]. In areas with excessive NO production, the endothelial and smooth muscle cells can eventually lose their ability to communicate and react in response to changes in the microcirculatory environment. Additionally, the abilities of the glycocalyx to mechanically sense alterations in blood flow are disrupted by inflammatory injury [39]. The result is an overall decrease in functional capillary density and an extreme heterogeneity of perfusion [42]. This increases the diffusion distance for oxygen to different cells and can lead to the presence of dangerously hypoxic zones in organs despite measures of overall blood flow and oxygen saturation being normal.

Hemostatic Deregulation: The role of hemostasis is to keep the blood flowing normally throughout the body, close off damaged blood vessels by creation of blood clots, and remove clots when they are no longer needed. Typically, coagulation is initiated when the endothelial barrier of the vasculature is disrupted and cells like smooth muscle cells become exposed to the blood steam. These cells expose tissue factor (TF), which is an important initiator of coagulation. Coagulation is also triggered by other pathways including interaction with collagen and neutrophil extracellular traps (NETs) [43]. TF binds and activates other coagulation factors, which promote the conversion of pro-thrombin into

thrombin on TF-expressing cells. Thrombin begins to activate platelets that have adhered to the site of injury via receptors binding to collagen and Von Willebrand factor (vWF), and promotes the continued production of thrombin by the platelets. Platelets aggregated at the site of injury begin to produce fibrin clots through the activation of thrombin and its subsequent actions on fibrinogen. As a final step, thrombin-activated plasma transglutamase forms covalent crosslinks between adjacent fibrin chains to create an elastic, yet strong fibrin clot [44]. The ample presence of protease inhibitors in the plasma such as tissue factor pathway inhibitor (TFPI) and antithrombin limits the diffusion of activated coagulation factors to keep the response local [45,46]. In addition, as concentrations rise during coagulation, thrombin binds to thrombomodulin present on endothelial cells and then activates protein C, bound nearby to endothelial protein C receptors (EPCR). Activated protein C (APC) then suppresses thrombin production in a negative feedback loop. Furthermore, resting platelets are kept in a non-adherent state by actions of the endothelium which include the release of prostaglandins and nitric oxide as well as the inactivation of factors that activate platelets like thrombin [44].

During sepsis, there are issues with both the over-activation of pro-coagulation factors and the inhibition of anti-coagulation factors. These factors can lead to the formation of microthrombi, hypoperfusion of organs and disseminated intravascular coagulation. Toll-like receptor activation by endotoxin and other PAMPs/DAMPs, as well as downstream cytokines like IL-6 and TNF-A, can induce the expression of active TF on circulating monocytes and endothelial cells [47,48]. This expression leads to widespread activation of coagulation, formation of fibrin and consumption of clotting factors. In addition, the activated endothelium can result in exposure of sub-endothelial collagen and expression of other pro-thrombotic agents including high molecular weight von Willebrand factor (VWF) and plasminogen activator inhibitor type 1 (PAI-1) [31]. During sepsis, platelets are rapidly activated by thrombin and become increasingly adherent to the activated endothelium and adherent leukocytes [49]. Platelet binding to adherent neutrophils can lead to activation of NETS [50]. While NETS are beneficial in trapping microbes and preventing their spread, in situations like sepsis, large quantities of these NETs are released and can impair microcirculation, damage tissues, and promote inflammation [43]. A faulty APC system also plays a pivotal role in the pathogenesis of disseminated intravascular coagulation (DIC) and associated organ

dysfunction. In septic patients, protein C levels are low due to impaired synthesis and increased consumption [44]. TNF-α and IL-1-induced down-regulation of thrombomodulin and EPCR leads to decreased protein C activation [51,52]. DIC results in consumption of coagulation factors and platelets and presents in patients as thromboembolic diseases (or less apparent microvascular occlusion), causing multiple organ dysfunction (MODS) and excessive bleeding [53].

Mitochondrial Dysfunction: All cell types (except red blood cells) possess mitochondria that generate the majority of adenosine triphosphate (ATP) required for normal cell functioning via oxidative phosphorylation. The substrate for electron transfer and ATP production comes primarily from glucose or the β-oxidation of fat. Oxygen is the terminal electron acceptor of the chain in Complex IV and is reduced to water. However, premature or incomplete reduction of oxygen increases the production of superoxide radicals. Normally, the mitochondria protect themselves from intrinsic damage by ROS with an assortment of antioxidants including superoxide dismutase, glutathione, thioredoxin, etc. [54]. However, during sepsis many aspects of these normal processes can go awry, impairing the body's ability to make enough ATP. This is mainly due to local hypoxia and damage to mitochondria from excessive ROS exposure. Impaired perfusion due to vascular leak and the formation of micro-thrombi throughout the vasculature can lead to localized tissue hypoxia and impairment of oxidative phosphorylation [39]. However ATP shortages can also occur in tissues in the absence of apparent microvasculature abnormalities. This is due to the effects of increased production of ROS on mitochondrial function. These free radicals interfere with mitochondrial respiration by directly damaging mitochondrial proteins and the lipid membrane [55]. It has been found that genes transcribing mitochondrial proteins are down regulated during the inflammatory response [56,57] meaning that the turnover of mitochondria is perturbed as well. Post-mortem analysis of liver and kidney samples from sepsis patients revealed signs of widespread mitochondrial injury [58].

Logically, one would think mitochondrial dysfunction and decreased ATP would cause massive amounts of cell death in key organs, thus precipitating organ dysfunction and failure. Interestingly, cell death in major organs is not a major feature of sepsis [58]. At post-mortem, humans were found to show only a small increase in apoptosis among immune tissues such as spleen and lymphoid organs, and the

gut epithelium. In organs that typically experience dysfunction and failure in sepsis (heart, lung, brain, muscle, and kidney) there was minimal change in cell viability. This suggests that despite a lack of ATP, the cells were somehow coping with the decreased energy supply. The coping strategy appears to rely on enhanced glycolytic metabolism and an overall decrease in metabolic activity to reduce energy requirements. In this new steady state, though the cell cannot function normally, it does not allow ATP levels to drop to points that will trigger cell death [59]. This type of cellular "hibernation" is a well-recognized phenomenon in the human heart and is thought to perhaps be an adaptive process for dealing with prolonged severe inflammation [60]. Though perhaps adaptive in preventing cell death, this metabolic hibernation contributes to major organ dysfunction and failure in sepsis patients.

1.4. The pathogenesis of immunosuppression in sepsis

Immunosuppression increases a patient's vulnerability to either the primary cause of infection, or secondary infections (typically opportunistic in nature), and is associated with increased morbidity and mortality. The main causes for immunosuppression during sepsis are 1) widespread immune cell apoptosis, particularly among lymphocytes and dendritic cells (DCs), and 2) suppressed inflammatory capabilities of both immune and endothelial cells. The impact that sepsis can have on the suppression of the immune system is far-reaching and highly specific according to cell type. This suggests that it may be part of some underlying regulatory program [61].

Apoptosis: Immunosuppression during sepsis is characterized by massive apoptosis among $CD4^+$ and $CD8^+$ T cells [62-64], B cells [62-66], follicular and myeloid dendritic cells [67-70] and in lymphoid tissues [71,72]. This phenomenon has been identified in septic patients of varying ages and infection type and is associated with poor outcomes. Prevention of lymphocyte apoptosis improves survival during experimental sepsis in mice [73-77]. Sepsis-induced apoptosis is mediated via both death receptor and mitochondrial-mediated pathways, and is unlikely to be blocked in humans by targeting only a single apoptotic pathway [78,79].

Suppressed Inflammatory Activity: Immune cells that survive this massive wave of cell death typically show a dysfunctional phenotype. Phagocytic cells of the innate immune system such as

neutrophils, monocytes and macrophages exhibit disrupted functions including impaired clearance of bacteria, decreased pro-inflammatory cytokine expression, increased anti-inflammatory cytokine expression, reduced production of ROS and decreased recruitment of cells to the infected tissues [80-82]. Among the antigen presenting cells, DCs, monocytes and macrophages exhibit lower expression of human leukocyte antigen-antigen D related HLA-DR (also known as major-histocompatibility class II surface receptor), produce increased levels of IL-10, and were unable to promote robust T effector responses [83-87]. What remains of adaptive immunity after apoptosis, is also highly dysfunctional during sepsis. T-helper subtypes 1, 2 and 17 (Th1, Th2, and Th17), are all suppressed in their functions, while regulatory T cell function is sustained [88].

1.5. The search for a sepsis cure continues

The organ failure and immunosuppression seen in sepsis interact to make sepsis a complicated and deadly condition. Nevertheless, improvements in diagnostic protocols [89] and supportive care practices have increased survival among sepsis patients in the developed world. Supportive care improvements for the management of sepsis and septic shock include rapid administration of broad spectrum antimicrobials followed by narrowing of therapy based on cultures or clinical improvement, fluid resuscitation and vasopressor therapy in patients with hypotension and hypo-perfusion, and protective mechanical ventilation in patients with sepsis-related ARDS [90]. However, the prolonged supportive care required by patients with CCI, is extremely costly to the healthcare system and often leaves patients at a high risk for subsequent complications and death [91].

While advances in supportive care are vital, they are no substitute for targeted molecular therapies, a remedy that sepsis is sorely lacking. Failure to develop a drug to treat sepsis reflects, in part, a history of reductionist thinking about the condition. The pathophysiology behind sepsis is far more intricate and dynamic than initially thought and does not just involve the leukocytes, but nearly every cell type, tissue, and organ system in the body. Our view of the host response in sepsis has shifted from a one-note hyper-inflammatory condition, to a dichotomous hyper- and hypo-inflammatory battle between SIRS & CARS, and finally to a generalized cacophony of responses that prevent the body's return to

homeostasis. The inherent complexity of this pathophysiology is compounded further by the affected individual's age, sex, race, comorbidities, location of infection, and type of infection. A failure to represent this diversity in both preclinical animal models as well as in clinical trial design has been a major obstacle in the search for a sepsis therapy. Another significant challenge has been the search for biomarkers to successfully stratify patients in an effort to perform more personalized therapy.

While clinical trials for anti-cytokine, anti-virulence factor, and anti-coagulation drugs were most common in the mid 1990s and early 2000s, in recent years there have been further attempts to use these types of factors to block the process of inflammation. For example in 2004, afelimomab, a monoclonal antibody fragment against TNF-A, was used specifically in patients stratified by high levels of IL-6 at enrollment [92]. There were mild improvements in 28-day mortality among this group of patients, but future studies, most likely of 10,000 or more patients, are required to confirm the validity of these and previous findings [93]. In the 2013 ACCESS trial, eritoran, a lipid A-like molecule that blocks LPS binding to TLR4 was tested but did not show any improvement [94]. Recombinant activated protein C (drotrecogin alfa) promotes fibrinolysis and helps inhibit thrombosis. Though it proved to be beneficial in a 2001 clinical trial, [95] a more recent trial in 2012 saw no benefit [96], resulting in withdrawal of the drug from the market. Heparin treatment to prevent excess coagulopathy (as part of HETRASE trial) also did not improve in-hospital mortality rates [97]. Currently and in the near future, new clinical trials exploring anti-inflammatory and anti-coagulation drugs will include a large-scale trial of low dose hydrocortisone (NCT-01448109) and a large international study of thrombomodulin (ART-123) treatment (NCT-01598831).

Clinical attempts to stimulate immunity in patients suffering from sepsis-induced immunosuppression have become more common in the last decade. In 2003, a study using recombinant G-CSF (filgrastim) to enhance neutrophil function was the first of its kind, but did not lead to improvement in 28-day mortality [98]. Six years later, a small trial using recombinant GM-CSF (sargramostim) was performed among patients stratified by reduced expression of monocyte HLA-DR [99]. Though the effects on mortality were not determined in this study, the treatment was shown immunologically to improve HLA-DR expression and increase cytokine production. Currently, a larger

scale, phase III clinical trial for GM-CSF therapy (NCT-02361528) and a small study exploring the immune-stimulating effects of INF-y (NCT-01649921) are ongoing.

1.6. A new approach to identifying sepsis therapeutics

The lack of progress in identifying new therapeutics for sepsis suggests that we should seek out a new perspective. Specifically, to better understand the regulatory machinery necessary to promote systemic balance and resolution during sepsis, it would be useful to study a population that exhibits natural protection. Interestingly, epidemiologic studies assessing SIRS brought on by infectious and non-infectious causes, point to human *children* as a demographic with significant resistance to morbidity and mortality (Table 1.1). In studies with more detailed age-stratification, this difference in morbidity and mortality is most striking in a subset of pediatric patients: *pre-pubertal children*. In particular, during the 1918 pandemic, children between the ages 5 and 14 (age of puberty onset then) showed decreased morbidity and mortality during pandemic influenza [100,101]. Similar data exist for mortality from other severe infections such as the 1957 pandemic flu [102], tuberculosis [100,103], Ebola [104,105], yellow fever [106], pneumonia [107], chicken pox [108,109], and sepsis [110-112].

Understanding the mechanisms driving this "pre-pubertal resilience" may provide insight into adult-specific changes in immune control that occur during the pubertal transition. Recent research in our lab has demonstrated that "pre-pubertal resistance" can be modeled using a murine system. In particular, compared to post-pubertal mice, pre-pubertal mice exhibited decreased morbidity and mortality in an H1N1 influenza infection model ($p<0.05$ by Kaplan-Meier). The importance of sex steroids in this puberty-dependent susceptibility to influenza was evidenced by the improved survival of mice subject to ovariectomy before the onset of puberty, and the subsequent reversal of this resistance by estrogen replacement. Delaying puberty by the administration of a gonadotropin releasing hormone (GnRH) agonist (leuprolide) also improved survival of female mice following influenza infection (Suber et al. manuscript in preparation). Huang and colleagues reported similar findings following studies of H1N1 influenza in pre- and post-pubertal ferrets [113].

Table 1.1. Childhood resistance to infectious and non-infectious injury
This table shows the difference in mortality between children and adults for a variety of infectious diseases and types of injury. The age range identified as "child" or "adult" varied across the studies. When age was more narrowly stratified for children and adults, an average mortality rate was calculated based on the age ranges of 5-12 and 20-60 respectively. * CFR = Case fatality rate; DP100K = Deaths per 100,000; DHR = Deaths to hospitalization ratio

	Disorder	Child vs. Adult Difference	Child Age Range	Adult Age Range	Metric*	Reference
HISTORIC DTA	1918 Pandemic Flu	176.2 vs. 786.5	5-14	20-34	DP100K	[114]
	Tuberculosis	30.3 vs. 206.9	5-14	20-34	DP100K	[114]
	Measles	0.05 vs. 0.5	5-15	>20	CFR	[115]
	Yellow Fever	144 vs. 759	6-15	21-60	DP100K	[106]
	Typhoid Fever	5 vs. 25	5-15	>20	CFR	[115]
	Plague	7 vs. 28	6-10	>16	DR	[116]
MODERN DATA	Ebola	57 vs. 81 60 vs. 72.5	5-15 5-15	20-60 >16	CFR	[104] [105]
	H1N1 2009	0.01 vs. 0.08 1.7 vs. 5.0	5-14 0-17	25-64 18-64	DHR DP100K	[117] [118]
	Group A Strep Sepsis	2.6 vs. 18	<13	19-96	CFR	[119]
	Staphylococcal Sepsis	2 vs. 25	<16	>16	CFR	[120] [121]
	Sepsis	0.9 vs. 14.5	5-14	25-54	DP100K	[110]
	Sepsis (With co-morbidities)	16.0 vs. 27.6	5-14	20-59	CFR	[112]
	Sepsis (Without co-morbidities	6.3 vs., 12.8	5-14	20-59	CFR	[112]
	Severe Malaria	6.1 vs. 26.7	≤10	21-50	CFR	[122]
	Trauma (MOF)	17 vs. 35	<16	>16	CFR	[123]
	Acute Chest Syndrome (Sickle Cell)	1.1.vs. 4.3	<20	>20	CFR	[124]
	Candidemia	10.1 vs. 30.2 15.8 vs. 30.6	<16 <18	≥16 >18	CFR CFR	[125] [126]
	Invasive Pneumococcus Infection	3.8 vs. 21.5	<13	14-106	CFR	[127]
	Chicken Pox	1.3 vs. 21.3 0.4 vs. 1.6	5-14 5-14	≥20 15-44	CFR CFR	[108] [109]
	Pneumonia	2.5 vs. 9.4	5-14	20-64	CFR	[107]

The biological basis for childhood resistance to SIRS-related morbidity and mortality has rarely been studied. Developing a better understanding of the inherent differences between the immune responses of children and adults could provide important new insights into the susceptibility of adults. Although aging-associated changes in basal inflammation and oxidative stress likely account for some of these differences, findings in our lab suggest that pubertal hormones may orchestrate important immunological changes earlier in adulthood. Whereas an expansive literature exists detailing the specific immunological effects of sex steroids in adult men and women [128-130] the immunological changes resulting from the initial interaction with these hormones during puberty, have rarely been studied.

In this thesis we set out to 1) characterize the effect that pubertal status has on sepsis pathogenesis and mortality using a mouse model of endotoxemia and 2) use data mining techniques to compare transcriptomic profiles from pre- and post-pubertal humans.

1.7. Benefits and caveats of murine models of sepsis

No interventional therapy based on successful results in a mouse model of sepsis has been similarly successful in human clinical trials. Beyond technical issues in clinical trial execution such as timing of therapy, selection of patients that would benefit from the therapies and appropriate dosing regimens, mouse models for pre-clinical sepsis research have significant limitations, often viewed as being so flawed as to be useless. These limitations include both the experimental parameters of the common sepsis models used as well as basic biological differences in the transcriptional and physiological responses of humans and mice.

One clear limitation is that murine models of sepsis are hardly representative of human sepsis. The mice typically used in sepsis studies are inbred, male, young, healthy, and given minimal to no supportive care (e.g. antibiotics, vasopressors, fluid resuscitation, ventilation etc.). It is perhaps no surprise that these animal subjects would differ so strongly in their physiology from human sepsis patients who are of both sexes, over the age of 65, often suffering from co-morbidities, and given aggressive supportive care [131,132]. In addition to the characteristics of the mice themselves, the methods used to create a sepsis-like response in mice are fairly limited in their ability to mimic human sepsis. The

three most commonly used animal models of sepsis are the administration of an inflammation-inducing toxin (e.g. LPS, zymosan etc.), the administration of bacteria into various locations in the body (e.g. peritoneal cavity, lungs etc.), and the surgical disruption of the protective barrier of the cecum to cause an endogenous polymicrobial infection. This third option, called cecal ligation and puncture (CLP), is considered by many to be the "gold standard" in murine sepsis models. However, none of these three models can fully recapitulate the diverse physiological responses seen in human sepsis [133].

Beyond issues with creating more representative models of sepsis in mice, there are fundamentally significant species differences between mice and humans. In the past ten years, there have been multiple attempts to compare the transcriptional regulation in mice and humans, but the results are contentious. For example, the Mouse ENCODE Consortium [134] and Immunologic Genome Consortium [135] compared organ and cell-type specific transcriptomes from healthy mice to that in healthy humans. While the Immunologic Genome Consortium found striking similarities in the transcriptional profiles of various hematopoietic cell types in mice and humans, the ENCODE consortium found that mouse transcriptional profiles across different body organs were more alike than the matching organ in humans. Even more pertinent to the study of sepsis were the findings by the Inflammation and Host Response to Injury Large Scale Collaborative Research Program, which conducted multiple studies on the genomic response to systemic inflammation in humans and murine models. A publication by this group in 2013 shook the field of sepsis research when it found no correlation between the transcriptional networks of human and murine leukocytes following burns, trauma, and endotoxin exposure [136].

However, many still believe there is a strong case for continuing the use of mouse models in sepsis research. For example, studies that reanalyzed the controversial data published by Seok et al. in 2013, found that there were important relationships between the two species when they limited their comparative analysis to the ~2300 homologous genes with significant expression changes after trauma, burns or endotoxin in mice and humans [137]. The key going forward with mouse research is to be forthright about limitations, and devise ways to improve the model. Mouse models can be made more applicable to human sepsis by conducting experiments with aged, outbred mice of both genders that are receiving some sort of supportive care regimen (e.g. fluid resuscitation, antibiotic treatment, vasopressor

treatment etc.). Some have suggested performing these experiments in mice that are also afflicted with a common human comorbidity such as heart disease, obesity, or diabetes. In addition, experts have suggested moving away from simplistic toxin injection models towards bacterial infections of the lungs, urinary tract and abdomen, which tend to be the most common locations for infections in human sepsis patients [131,132]. By adding some of the complexities of human patients into animal experiments, the hope is to more closely replicate the transcriptional responses occurring in septic humans.

In this thesis, we chose to use an endotoxin injection model of sepsis to compare the responses of pre- and post-pubertal mice. This "endotoxemia" model focuses entirely on the role that the host response plays in driving the clinical features of sepsis. It involves a one-time, high-dose injection of lipopolysaccharide (LPS)—the bioactive part of gram-negative bacterial endotoxin—into the intra-peritoneal cavity and subsequent transport into systemic circulation. LPS is a potent TLR4 agonist that sets into motion a powerful host response without live bacteria. After LPS administration in mice, there is a rapid onset of systemic clinical signs including reduced motor activity, lethargy, shivering, pilo-erection, and hypothermia [138-140]. Endotoxemia produces a hypo-dynamic cardiac state characterized by decreased cardiac output and decreased peripheral vascular resistance, decreased total white blood cell counts with marked reduction in lymphocytes and neutrophils and a rapid, but transient increase in systemic cytokine levels [140,141]. There are both pros and cons to using the endotoxemia model to study sepsis in mice.

While some of the manifestations of acute endotoxemia in mice mirror those in human sepsis, there remain key differences in the temporal kinetics and the magnitude of these changes. For example, while endotoxemic mice exhibit the hypo-dynamic cardiovascular (e.g. low systemic resistance, low cardiac output) and hypo-metabolic states (e.g. hypoglycemia, hypoinsulinemia) of late-phase human sepsis they do not experience the initial hyper-dynamic cardiovascular and hyper-metabolic states experienced by humans in early-phase sepsis. Likewise, though both mice and humans exhibit a powerful, simultaneous release of pro- and anti-inflammatory mediators, the intensity and rapidity of the response in endotoxemia is greater than the prolonged response seen in human sepsis. In general, the clinical course and progression of disease in rodent LPS models is much faster in mice than humans with sepsis, and even faster than humans treated with low dose endotoxin [141]. Some have used the phase of

endotoxin resistance that follows sub-lethal endotoxemia as a model for the immunosuppressive features of protracted sepsis. Compared with humans, lab animals appear relatively insensitive to LPS and require high doses to produce a shock like state. The dose required to produce similar cytokine responses in mice was 250 times that in humans [138]. It is thought that there may be one or more factors present in murine serum that are absent or present in lower concentration in human sera and suppress the production of pro-inflammatory cytokines by murine or human cells. For example, mice contain an iron-binding acute phase protein called hemopexin that may contribute to the difference in LPS sensitivity between mice and humans [142,143].

Though endotoxemia is an over-simplified murine model of sepsis, we chose to use this model for several reasons. Considering the exploratory nature of this study, we wanted to characterize the model using a cost-effective, simple procedure that could easily be standardized across differently sized animals and repeated reliably. The age-associated differences between pre- and post-pubertal survival from infectious and non-infectious injury are already evident in human populations. Before any other sepsis-associated research is done, our findings should be tested in more relevant models of sepsis such as CLP, which better models the cardiovascular, metabolic, and inflammatory phases of human sepsis. If any particular pathways emerge as being the driving force behind pre-pubertal resistance, these should also be tested in aged mice, potentially with comorbidities in a number of different models of sepsis.

1.8. Data-mining and *in silico* analysis of sepsis transcriptomes

The second approach we took in this thesis was to compare transcriptome changes in the resistant child vs., susceptible adult populations. Considering the lack of certainty regarding the cells and molecules driving sepsis outcomes, broad transcriptomic analyses and other 'omics approaches are appealing. However, transcriptome research in sepsis has exhibited a unique set of challenges when designing and executing experiments. Obstacles include the strongly debated limitations of animal models of sepsis and conflicting results from several studies focused on gene level changes [144].

The gene expression differences between adults and children during sepsis have never been studied. Genome-wide transcriptome analysis is a powerful tool to expedite the process of discovering

these differences. However, the time and financial costs required to run a prospective clinical study comparing whole blood transcriptomes from human adult and pediatric sepsis patients are extreme. Though this type of transcriptomic study would be more feasible to perform using mice, attempting to glean information that is relevant to humans from these analyses is fraught with difficulty. Fortunately, well-documented primary data from past microarray studies are readily accessible in public databases like ArrayExpress and GEO. Thus, data from multiple studies can be integrated and analyzed *in silico* to compare the responses of septic adults and pre-pubertal children.

Sepsis is a highly heterogeneous and dynamic condition, adding substantial complication to the design and execution of gene expression analysis in human subjects [145]. Firstly, choosing a homogenous tissue sample for study across many septic patients is problematic. The reaction is systemic and the node of infection may not be accessible for biopsy and is often different across different individuals. For this reason, whole blood or various leukocyte fractions from the blood are most frequently used for transcriptomic studies. Whole blood is particularly useful because it is easy to collect and requires minimal processing, which may introduce transcriptional artifacts. However, it is unclear whether the transcriptomes of whole blood accurately reflect the most important changes in gene expression during sepsis [146,147].

The expression profiles of whole blood essentially represent a weighted sum of the patterns of gene expression for each blood cell type [148]. Patients with sepsis exhibit enormous fluctuations and person-to-person differences in the leukocyte composition of the blood. Along with dynamic fluctuations in percent leukocyte composition, dramatic shifts in the transcriptional program of leukocytes can occur within just a few hours of an inflammatory challenge [56,149]. Additionally, there is the added complexity of comparing gene expression in individuals across age, gender, ethnicity, co-morbidities, and medical status at the time of sample collection. All of these factors lead to huge variability in sepsis transcriptomes across multiple studies and even within individual studies [150].

We first identified public datasets of transcriptome profiling performed on whole blood samples in the high vs. low survival groups (children or adults, respectively). However to avoid problems with extreme variability within and across the different studies, we decided to use a pathways-based approach

rather than gene level analysis alone. The bioinformatics analysis used Pathprint [151], a tool that allows comparison of gene expression at the pathway level across multiple array platforms. In this method, sample data is converted into discrete sets of activity states (low, high or average pathway activity). By comparing pathways instead of individual genes, this method can be used across multiple microarray platforms and species and can detect differences in biological activity between groups, even when the individual causative genes exhibit high variance.

Methods that use gene expression signatures to identify drug candidates have also been developed (e.g. the Connectivity Map [152,153]). Because these methods rely on gene-level instead of pathway-level analysis, we experimented with the use of a new Pathprint-based method to identify possible drug candidates—the Pathway Drug Network (PDN). After identifying age-associated differences in pathway activity using Pathprint, these pathways were used to build upon the pre-existing correlation of the expression of >16,000 gene signatures, including disease signatures from the Comparative Toxicogenomics Database (CTD), PharmGKB and GeneSigDB, pathway signatures from Wikipathways, KEGG, Netpath and Reactome, and drug signatures from CTD, PharmGKB and Connectivity Map, across >50,000 individual microarrays. The network nodes most closely correlated with the sepsis pathway signatures were used to identify drugs that were positively or negatively linked to high-survival (child) or high-mortality (adult) signatures. We validated the top drug leads by analyzing prior data collected in pre-clinical models of sepsis and also by direct testing for improved survival in a mouse model of fatal endotoxemia.

1.9. Summary

Sepsis is a mysterious and deadly syndrome. This is due to the complex pathophysiology and incompletely characterized mechanisms by which it affects the body. Considering the chain of failures in the identification of therapeutic targets, a new perspective is needed. We chose to explore new possibilities for sepsis research by studying a subset of the human population with a natural protection from sepsis mortality: children, namely pre-pubertal children. Our study was broken into two experimental strategies 1) characterization of the differences in response to illness using an endotoxemia

mouse model and 2) data-mining of human sepsis data and use of that data to identify putative drug targets.

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CHAPTER 2: Characterizing the Pre- and Post-Pubertal Responses to an Endotoxemia Model of Sepsis

2.1. Abstract

Epidemiological data show that pre-pubertal children possess a relative resistance to death from severe infections—particularly those characterized by "cytokine storm" and profound immune dysfunction (e.g. the 1918 pandemic flu, measles, bacteremia). To assess the role that pubertal status may have on an animal's ability to appropriately regulate inflammation, we employed an endotoxemia model of sterile sepsis in mice. Following acute endotoxin injection (i.p. 23 μg/g), pre-pubertal mice (PND 25) exhibited significantly better survival than post-pubertal mice (PND 35) over a 72-hour period (76.3% vs. 28.6%, $p<0.0001$, $n\geq50$). Serum endotoxin concentrations were similar in both age groups at 2- and 20-hour time points, suggesting that the difference in mortality was not due to differential endotoxin absorption. Improved survival among pre-pubertal animals did not correlate with a decreased inflammatory response. At 2 hours, pre- and post-pubertal mice exhibited comparable responses in the majority of inflammatory parameters assessed, except higher absolute white blood cell counts (8.5 ± 3.3 K/μl vs. 6.0 ± 3.1 K/μl; $p=0.001$) and increased serum levels of GM-CSF (714.2 ± 232.8 pg/ml vs. 485.8 ± 161.4 pg/ml; $p=0.0005$) in pre-pubertal mice. Age-associated differences in the response to endotoxemia were more evident at a later 20-hour time point. In comparison to pre-pubertal mice, post-pubertal mice showed continued elevation of multiple cytokines (IFN-γ, IL-5, IL-6, IL-13, IL-15, and IL-17), growth factors (LIF and VEGF), and chemokines (eotaxin, MCP-1, and MIP-2). The older mice also exhibited monocytic (18.0% ± 7.4% vs. 7.3% ± 2.3% CD11B+ cells; $p=0.001$), rather than granulocytic (21.8% ± 5.4% vs. 40.3% ± 12.4% CD11B+ cells; $p=0.001$), influx of cells into the peritoneal cavity. Mechanistically, the prevention or acceleration of puberty using hormonal treatments (leuprolide or estrogen) led to increased (80% vs. 35%; $n = 20$, $p<0.0001$) or decreased (60% vs. 26.7%; $n = 45$, $p=0.002$) survival from endotoxemia, respectively. In addition, the adoptive transfer of pre-pubertal peritoneal cells improved the survival of post-pubertal recipient mice, while post-pubertal peritoneal cells or vehicle did not (78.3% vs. 25.0% vs. 36.4%; $n \geq 37$, $p<0.0001$). The data establish a model for childhood resistance to mortality from endotoxin, demonstrate that estrogen is responsible for increased susceptibility after puberty and identify peritoneal cells as mediators of pre-pubertal resistance.

2.2. Introduction

Despite decades of research, sepsis remains a major cause of death in the United States and throughout the world. Sepsis is a complex syndrome in which the host response to infection becomes dysregulated, causing life-threatening organ dysfunction and failure despite appropriate antibiotic treatment and supportive care [1]. Normally, rapid recognition of invading pathogens and subsequent activation of a robust pro-inflammatory program are the key to surviving an infection. Once the pathogens are cleared, the immune cells naturally shift to a pro-resolution state that dampens the inflammatory response and promotes the body's return to homeostasis. However, during sepsis, the intensity of the infectious stimuli or a patient's unique susceptibility (e.g. age, comorbidities etc.) can lead to a harmfully exaggerated pro-inflammatory response known as the Systemic Inflammatory Response Syndrome (SIRS) [2]. SIRS can trigger numerous potentially fatal complications including shock, disseminated intravascular coagulopathy, and multiple organ failure [3,4]. Additionally, sepsis patients who survive the initial "hyper-inflammatory" phase do not necessarily transition back to homeostasis. Instead, affected patients are often plunged into a hypo-immune phase known as the Compensatory Anti-inflammatory Response Syndrome (CARS), in which leukocyte anergy and apoptosis increase the risk of secondary infection and death [5-8].

Unfortunately, drugs designed to target the early, pro-inflammatory response by blocking specific cytokines (e.g. TNF-A) and pattern recognition receptors (e.g. TLR4), have been unsuccessful in clinical trials [9,10]. Others meant to stimulate the immune system, specifically during the late hypo-inflammatory phase (e.g. GM-CSF; IFN-γ), have shown some promise in small-scale studies, but rely on patients being in a particular "phase" of sepsis [6], and have not progressed to wider use. Thus, despite enormous efforts, currently the only approved treatment for sepsis remains antibiotics (if bacterial) and supportive care.

The inherent difficulty in treating sepsis is that it is a condition defined by complexity and variability. Our understanding of this dysregulated host response and how it drives sepsis pathophysiology has changed radically over the last four decades, and continues to evolve. Despite the attempted division of sepsis pathology into early hyper-inflammatory and later hypo-inflammatory phases, actual patients fall within a very wide spectrum between these two polarities [11]. Key to

developing improved therapies for sepsis is an understanding of the natural regulatory machinery that serves to promote resolution and a return to homeostasis.

To discover the regulatory systems that can promote balance and successful resolution during sepsis, it would be useful to study a population that exhibits a natural resistance to sepsis mortality. Interestingly, epidemiologic studies point to human children as a demographic that meets this criterion. Compared to adults, pediatric populations show decreased mortality from a variety of conditions that typically involve immune system dysfunction as part of their pathology (Table 1.1.). In studies that have a detailed breakdown by age, this difference in mortality is particularly apparent in a specific subset of pediatric patients: *pre-pubertal children*. For example in mortality data for 1918 pandemic influenza, children between the ages 5 and 14 (age of puberty onset then) showed decreased morbidity and mortality [12,13]. Similar data exist for mortality from other severe infections such as the 1957 pandemic flu [14], tuberculosis [12,15], Ebola [16,17], yellow fever [18], pneumonia [19], chicken pox [20,21], and sepsis [22-24].

Understanding the mechanisms driving this "pre-pubertal resilience" may provide insight into adult-specific changes in immune control that begin during the pubertal transition. The biological basis for childhood resistance to SIRS-related morbidity and mortality has rarely been studied. In this work, we established a simple murine model of pre-pubertal resistance to sepsis using an endotoxin injection protocol. We used this model to characterize the immune responses of the pre- and post-pubertal age groups as well as to explore possible mechanisms driving the differences between the two groups.

2.3. Materials and Methods

Animals

All C57Bl/6 and CD1 mice were obtained from Charles River (Wilmington, MA), housed in micro-isolator FST cages, and fed standard chow (5058) *ad libitum*. After delivery, mice were allowed at minimum, a three-day acclimatization period prior to usage in experiments. Female mice were used in all experiments. Pre-pubertal (Pre-P) mice typically arrived in the animal facility on post-natal day (PND) 21 and were used for experiments on PND 24-26. Post-pubertal (Post-P) mice typically arrived in the animal facility on PND 30 and were used for experiments on PND 33-35. Mice were cared for according to the

Guide for the Care and Use of Laboratory Animals (NIH) and all animal protocols were approved by the Harvard Center for Comparative Medicine.

Endotoxemia Model of Sterile Sepsis

Female pre- and post-pubertal mice were given intra-peritoneal injections of *E. coli* lipopolysaccharide (LPS) (L3755, Sigma; Lots: 123M4096V; 066M4118V) diluted in sterile water (Hospira; NDC0409-4887-20) followed by a prophylactic fluid resuscitation of 0.9% sodium chloride (Hospira; NDC0409-4888-20) delivered subcutaneously in the scruff of the neck, equal to 2.5% of the animal's body weight. The concentrations of LPS used to elicit acute endotoxemia differed depending on the potency of the particular lot of product. Lot 123M4096V achieved effective mortality in a range of 23-25μg/g while Lot 066M4118V did so at a dosage of 30-32μg/g. Dose response experiments were performed whenever a new lot of LPS was used to determine the 80-90% lethal dose. To avoid diurnal differences in metabolism and immune function [25,26], all LPS injections were administered between 11:00AM and 2:00PM. After injections, mice were monitored twice daily for weight-loss, mortality and signs of significant morbidity including stressed posture, lack of movement and labored breathing. Mice determined to be moribund were euthanized via injection (i.p.) with a 1:4 dilution of Fatal Plus, a pentobarbital sodium solution, (Vortech; NDC0298-9373-68) in PBS. Typically, experiments ended after 72 hours. For each time course study, in addition to the mouse subjects sacrificed for sample collection at 0, 2 and 20 hours, n≥5 mice from each age group were set aside in order to evaluate survival and determine whether the "pre-pubertal resistance" criteria was being met in each experiment.

In studies involving estrogen pre-treatment, pre-pubertal mice were treated with daily subcutaneous 100μl injections of 17β-Estradiol at 100μg/ml (Cayman; 10006315) or vehicle (0.4% DMSO (ATCC-4X)) suspended in corn oil for three days prior and once on the day of endotoxin injection. In studies involving leuprolide pre-treatment, pre-pubertal mice were treated with daily subcutaneous 100μl injections of leuprolide (Tocris Bioscience-2873) at 250μg/ml or vehicle (0.9% sodium chloride) starting on PND 24 and continuing until PND 35 before endotoxin injection. For this particular experiment *Salmonella enterica* LPS (Sigma; L2262; Lot: 081M4034) was used at 32μg/g.

Blood and Serum Analyses of Endotoxemic Mice

During endotoxemia time course studies, mice were euthanized 0, 2, or 20 hours after LPS injection by inhaled isoflurane (Piramal Health Care; NDC 66794-013-25) overdose. Blood was collected via cardiac puncture and transferred into lavender-top BD Microtainer K_2EDTA (365974) tubes and gold-top BD Microtainer SST (365967) tubes and inverted to mix. Anti-coagulated EDTA-treated blood was used for red and white blood cell counts and differentials assessed using the Hemavet 950 (Drew Scientific). Gold top tubes were centrifuged for 10 min and the isolated serum frozen at -20°C. Serum cytokines were quantified via multiplex analysis (Mouse Cytokine Array/Chemokine array 32-plex) performed by EVE Technologies (Calgary, Alberta, Canada). Endotoxin was quantified using two different methods: 1) Pierce LAL Chromogenic Endotoxin Quantitation Kit (ThermoFisher Scientific-88282) and 2) Pyrogent™ 5000 Kinetic Turbidimetric LAL Assay (Lonza; N383). Serum levels of injury markers aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and urea were quantified using commercial kits. These included Infinity™ AST Liquid Stable Reagent (Thermo Scientific), Lactate Dehyrdrogenase (Liquid) Reagent Set (Pointe Scientific, Inc.), and Liquid Urea Nitrogen (BUN) Reagent Set (Pointe Scientific Inc.). Serum analysis of metabolic factors including free fatty acids (FFA), triglycerides (TG), and cholesterol (CL) were performed using commercial kits. These include Wako HR series NEFA-HR (Wako Diagnostics), Infinity™ Cholesterol Liquid Stable Reagent (Thermo Scientific), and Infinity™ Triglycerides Liquid Stable Reagent (Thermo Scientific). Serum glucose was assessed using the Easy Step Blood Glucose Monitoring System (Easy Step). To measure blood glucose, mice were securely held and a razor blade used to elicit a small drop of blood from the tip of the tail. This drop of blood was put into contact with the test strip, allowing movement of the blood into the analyzer by capillary action and the glucose concentration calculated. Blood flow from the animal's tail was stopped by gentle application of pressure with gauze and Kwik-Stop Styptic Powder (Miracle Care).

Peritoneal Lavage

At 0, 2, or 20 hours following LPS injection, mice were euthanized and subjected to peritoneal lavage. Briefly, surgical scissors and forceps were used to make an incision in the skin and reveal the peritoneal wall. A syringe outfitted with a 27G needle was used to inject 5mL of PBS (4°C) into the

peritoneal cavity. The fluid-filled peritoneal cavity of each mouse was gently mixed by massaging for one minute. The lavage fluid was then drawn out of the peritoneal cavity using a syringe outfitted with a 22G needle. Lavage fluid was centrifuged at 300G for 10 minutes and resuspended in the appropriate assay buffer.

Immuno-phenotyping of Peritoneal Cells

For immuno-phenotyping analyses by flow cytometry, isolated peritoneal cells were resuspended in cell staining buffer (Biolegend; 420201), incubated for ten minutes at 4°C with Tru-Stain fcX™ (anti-mouse CD16/32) antibody (Biolegend; 101320) to prevent non-specific binding, and finally incubated with multiple combinations of fluorescent antibodies for 15 minutes at 4°C (Supplemental Table 2.1). Cells were collected on a BD FACSCantoII™ using the BD FACSDIVA Software. Data were further analyzed using Flow-Jo®.

Adoptive Transfer Studies

Naïve peritoneal cells were collected from pre-pubertal mice or post-pubertal mice by peritoneal lavage as described above. Lavage fluid was centrifuged at 300G for 10 minutes and resuspended in PBS (4°C) at a concentration of 2 million total cells/ml. Recipient post-pubertal mice were administered either 1mL of pre- or post-pubertal peritoneal cell suspensions or 1mL of PBS (i.p.). Following 30-60 minutes of incubation within the peritoneal cavity, the recipient mice were then subjected to the endotoxemia protocol described above. In some studies, specific cell types were separated from the pre-pubertal peritoneal cell suspensions prior to adoptive transfer using the Miltenyi MACS® system. Briefly, following lavage and centrifugation, peritoneal cells were resuspended in MACS® buffer (PBS pH 7.2; 0.5% BSA, 2mM EDTA) at 1 million cells/ml and incubated (15 minutes; 4°C) with either biotinylated F480 antibody (Biolegend; 123106) or a cocktail of biotinylated CD3 and CD19 antibodies (Biolegend; 100243; Biolegend; 115503). This methodology allowed for positive or negative selection of macrophages and T cells/ B cells respectively. After incubation, cells were washed twice and then resuspended in 80µl MAC Buffer. 20µl of anti-biotin microbeads (Miltenyi; 130-090-485) were added to the cell suspension and incubated for 15 minutes on ice. Cells were washed once and resuspended in 500µl MAC buffer before application to the LS Column (Miltenyi; 120-042-401) within the magnetic field of the QuadroMACS

Separator. The column was washed three times with MAC buffer and the untagged cells collected in the flow through. The column was then removed from the magnet and the tagged cells were washed from the column. Separated cells and flow-through cells were each centrifuged at 300G for 10 minutes and resuspended in PBS for adoptive transfer. Separated macrophages were administered at 1.5 million cells/ml, the T and B cell mix at 0.5 million cells/ml, recombined cell mix at 2 million cells /ml, or un-separated cell mix at 2 million cells/ml.

Statistical Analyses

All statistical analyses were performed using Prism 7 (GraphPad). Descriptions of the statistical tests used can be found in the figure legends.

2.4. Results

Pre-P mice exhibit resistance to mortality from endotoxemia

Using an optimized endotoxemia protocol with C57BL/6 mice, we found that pre-pubertal mice exhibited significantly greater survival than post-pubertal mice over a 72-hour period (6 experiments; $n \geq 56$; $p < 0.0001$) (Figure 2.1 A). Average pre-pubertal survival was 76.3%, while post-pubertal survival was only 28.6%. Serum concentrations of endotoxin at both early (2 hour) and late (20 hour) time points were similar in pre- and post-pubertal animals (Figure 2.1 B). This suggests that both age groups were exposed to the same effective concentration throughout the experiment. Along with improved survival, pre-pubertal mice exhibited significantly higher percent weight loss compared to post-pubertal mice (Figure 2.1 C).

Pre-P mice exhibit greater variability in WBC counts and differentials

White blood cell (WBC) counts and differentials were similar at baseline (0 hours) in pre- and post-pubertal animals. Two hours after LPS injection, the total WBC-count of pre-pubertal animals spiked above that of post-pubertal animals (8.5 ± 3.3 K/µl vs. 6.0 ± 3.1 K/µl; $p=0.001$) before decreasing at twenty hours to levels matching those of post-pubertal animals (4.7 ± 2.2 K/µl vs. 4.7 ± 2.1 K/µl) (Figure 2.2 A). At 2 hours there was no change in either pre- or post-pubertal differentials from baseline, despite the increases in total WBC count. However, at the 20-hour time point there were some changes (Figure

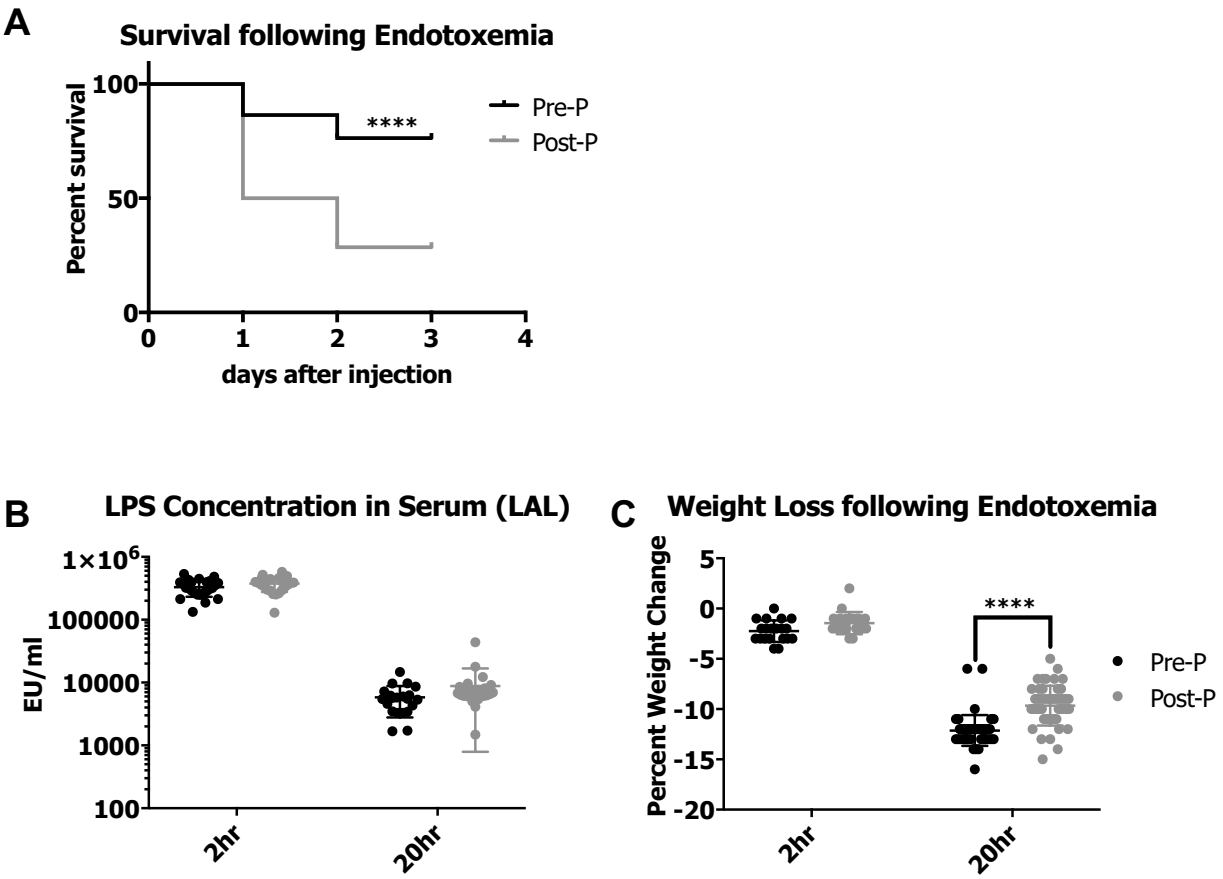

Figure 2.1. Pre-pubertal mice show increased resistance to mortality from endotoxemia. A) Pre and post-pubertal mice were administered 23µg/g LPS via intra-peritoneal injection, followed by prophylactic fluid resuscitation with a subcutaneous injection of saline. Mice were monitored twice daily and moribund individuals euthanized. B) Endotoxin concentrations in serum samples collected at 2- and 20-hour time-points were quantified using LAL. C) Mouse weight was recorded prior to LPS injection and then at 2- and 20-hour time points. Data is shown as the percent change in weight in comparison to time 0. Significant differences between Pre-P or Post-P mice are labeled with **** ($p<0.0001$). Percent survival was compared using a log rank Mantel Cox test, while LPS concentration and weight loss were compared using Two-way ANOVA followed by Tukey's test for multiple comparisons.

2.2 B-G). In both age groups there was a significant, but equal, increase in the percentage of blood neutrophils, eosinophils, and monocytes. This increase in percent monocytes was slightly greater in pre-pubertal animals (7.3 ± 2.5 % vs. 5.4 ± 1.6 %; $p<0.0001$) (Figure 2.2 E). In addition, while both age groups showed significant decrease in blood lymphocytes, pre-pubertal animals exhibited a slightly lower percent composition (47.8 ± 9.9 % vs. 53.4 ± 12.6 %; $p=0.02$) (Figure 2.2 F). At 20 hours, while both age groups experienced profound thrombocytopenia (Figure 2.2 C), only pre-pubertal animals showed a decline in RBC count (6.5 ± 1.3 M/µl vs. 7.9 ± 1.0 M/µl; $p<0.0001$) (Figure 2.2 B).

Pre-P mice exhibit robust early cytokine release followed by later dampening of expression

In both animal models and human patients, sepsis severity and mortality risk are tightly associated with elevated expression of both pro- and anti-inflammatory cytokines. To compare cytokine expression in pre- and post-pubertal mice following endotoxemia, serum samples from both mouse age groups at 2 and 20-hour time points were subjected to multiplex cytokine analysis (Figure 2.3 & Supplemental Figure 2.10). At the 2-hour time point, pre- and post-pubertal mice showed similar expression of all cytokines except GM-CSF (714.2 ± 232.8 pg/ml vs. 485.8 ± 161.4 pg/ml; $p=0.0005$) (Figure 2.3 A). At the 20hr time point, post-pubertal animals exhibited higher expression of many cytokines (IFN-γ, IL-5, IL-6, IL-13, IL-15, and IL-17) (Figure 2.3 B-G), growth factors (LIF and VEGF) (Figure 2.3 H, I), and chemokines (eotaxin, MCP-1, and MIP-2) (Figure 2.3 J-L) in comparison to their pre-pubertal counterparts. Overall, the temporal trends in cytokine expression were substantially different in pre- and post-pubertal animals. Specifically, while cytokine expression was similar early in endotoxemia, pre-pubertal animals exhibited an overall greater degree of dampening of cytokine expression as time passed.

Pre- and Post-P mice show few differences in serum injury markers and metabolites

Death from sepsis in both humans and mouse models is thought to be due to injury and failure of important organ systems such as the liver and kidneys [27,28] In order to identify any age-associated differences in the accumulation of injury to these organs, we measured serum levels of well-known injury markers: lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and urea. At the 2-hour time point, pre-pubertal animals showed higher serum LDH (292.2 ± 166.4 U/L vs. 136.3 ± 76.3 U/L;

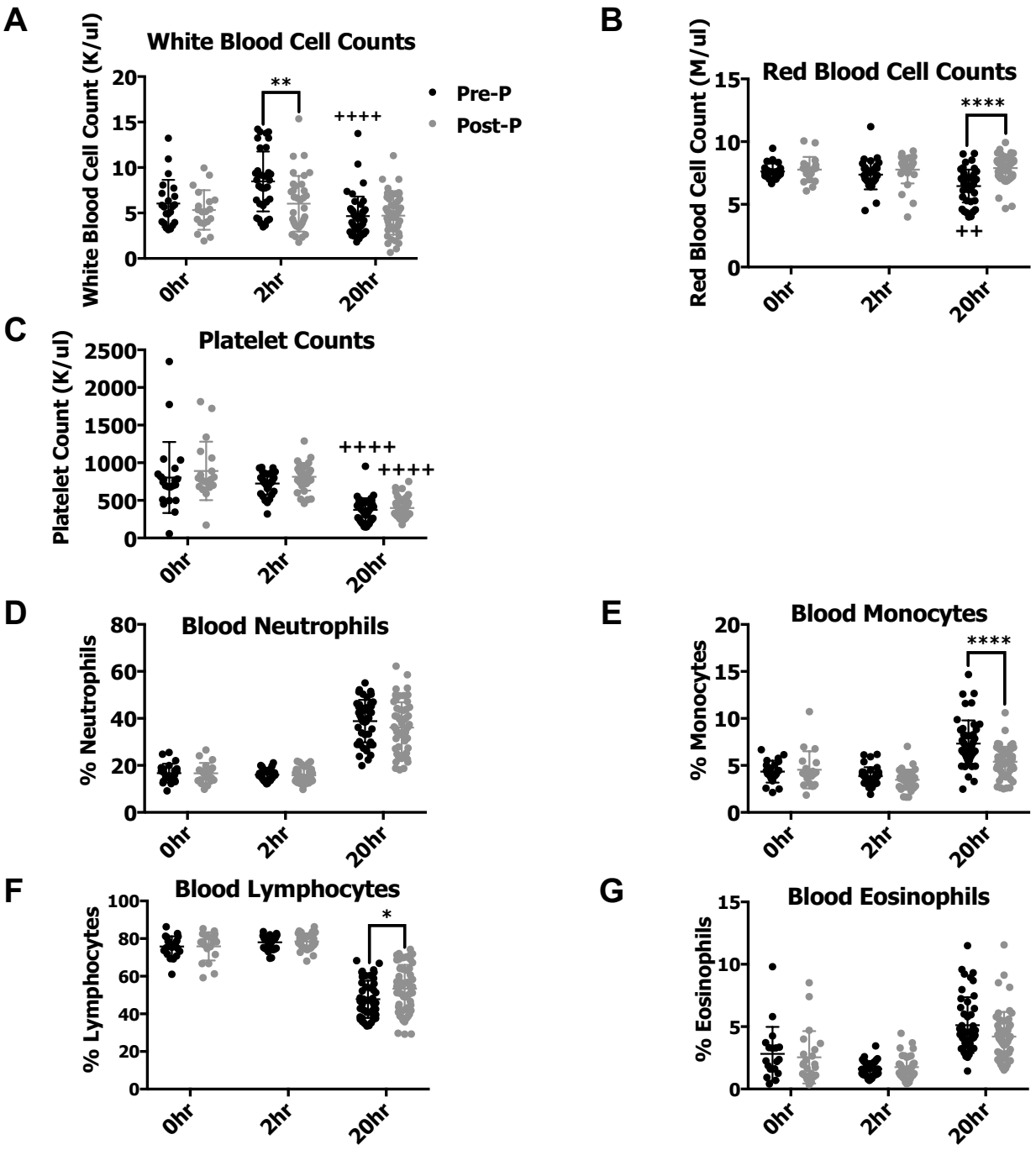

Figure 2.2. Pre and post-pubertal white blood cell composition during endotoxemia
All data for A) total white blood cell count, B) red blood cell count, C) platelet count, D) % Neutrophils, E) % Monocytes, F) % Lymphocytes and G) % Eosinophils were obtained using the Hemavet 950 blood analyzer (Drew Scientific). N≥19 mice at 0hr, N≥35 mice at 2hr, and N≥46 mice at 20hr per age group. Significant changes in concentration between 2 and 20 hours for either pre- or post-pubertal mice are labeled with ++++ ($p<0.0001$) or ++ ($p<0.01$). Significant differences in concentration between Pre-p or Post-p mice are labeled with **** ($p<0.0001$), ** ($p<0.01$), or * ($p<0.05$). All comparisons were made using Two-way ANOVA followed by Tukey's test for multiple comparisons.

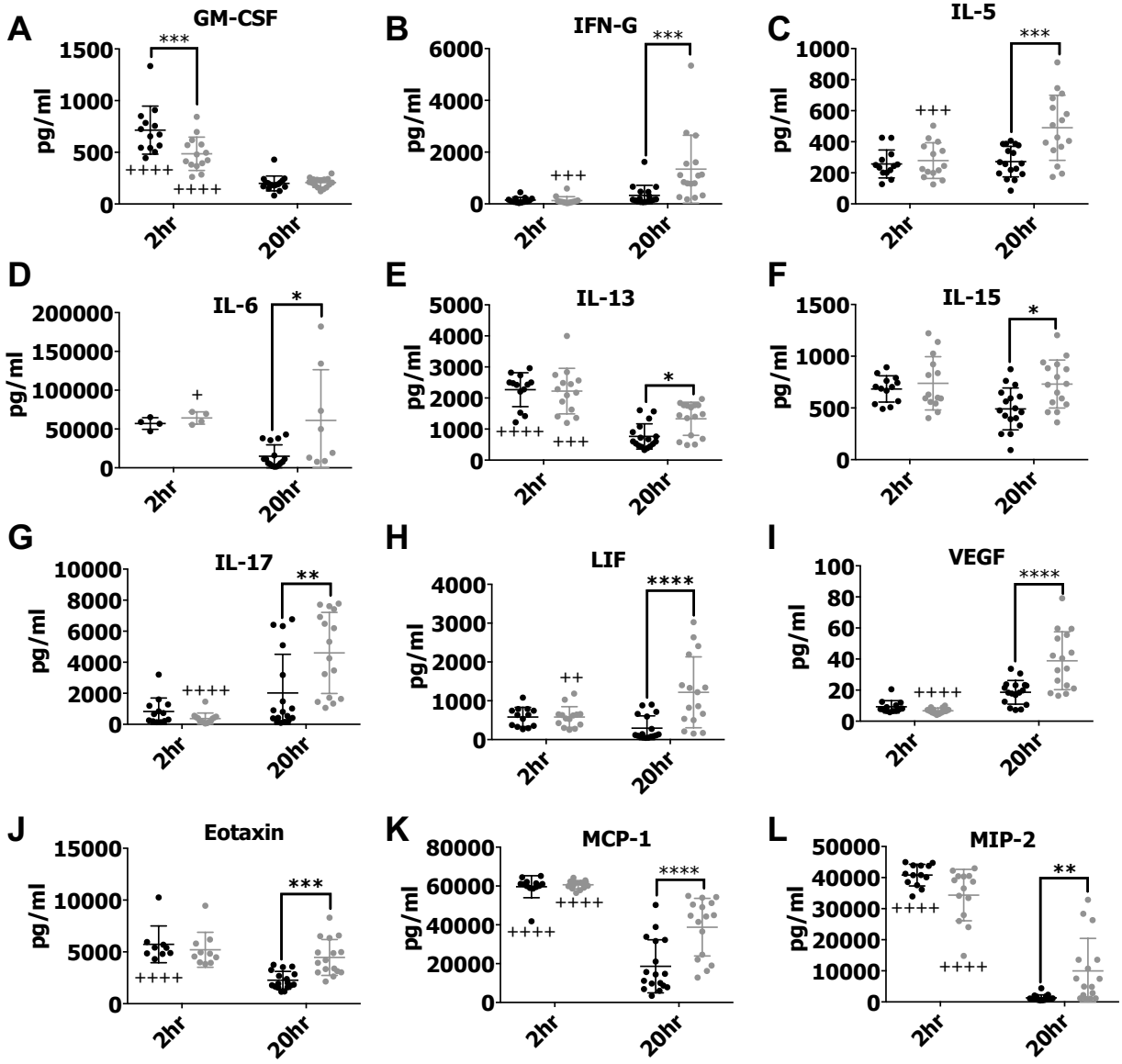

Figure 2.3. Post-pubertal serum cytokine expression shows lack of resolution.
Serum samples from pre- and post-pubertal mice were subjected to a 32-plex cytokine assay. Pre-pubertal data are in black and post-pubertal data are in grey. Data points above or below the detectable limit were not included. Significant changes in concentration between 2 and 20 hours for either pre- or post-pubertal mice are labeled with ++++ ($p<0.0001$), +++ ($p<0.001$), ++ ($p<0.01$), or + ($p<0.05$). Significant differences in concentration between pre- and post-pubertal mice are labeled with **** ($p<0.0001$), *** ($p<0.001$), ** ($p<0.01$), or * ($p<0.05$). All comparisons were made using Two-way ANOVA followed by Tukey's test for multiple comparisons.

p=0.01), an overall marker of cellular damage, and lower serum AST (37.3 ± 20.4 U/L vs. 66.1 ± 15.8 U/L; p=0.04, a marker of liver damage, in comparison to post-pubertal animals (Figure 2.4 A, C). Serum urea, a marker of kidney damage, was not significantly different between the two age groups at 2 hours however there was a significant increase in both age groups between 2 and 20 hours (Figure 2.4 B). At the 20-hour time point, pre- and post-pubertal animals demonstrated no differences in any of the three injury markers quantified.

Metabolic dysfunction is also an important driving factor in sepsis outcomes [29-32]. To elucidate possible age-associated differences in metabolic function, we compared the concentrations of several serum metabolites in pre- and post-pubertal animals. Pre- and post-pubertal mice show similar blood glucose levels at rest (171.3 ± 17.9 mg/dl vs. 162.8 ± 16.6 mg/dl), and similar temporal patterns following endotoxin treatment (Figure 2.5 A). For both age groups, there was a spike in blood glucose 15 minutes after endotoxin administration (~25% increase) followed by a steady decline such that within 6 hours, blood glucose levels were nearly half that of the resting values. Hypoglycemia continued to worsen until the 20-hour time point. In both pre- and post-pubertal animals, the lipid metabolites triglyceride, free fatty acids, and cholesterol decreased in concentration 2 hours following the initiation of endotoxemia and then increased by 20 hours to near pre-endotoxemia levels (Figure 2.5 B-D). Thus there were no age-associated differences in serum metabolites.

Pre- and post-pubertal mice exhibit different naïve peritoneal cell compositions

Before treatment with endotoxin, pre- and post-pubertal mice exhibited mostly similar peritoneal cell profiles, as assessed by flow cytometric phenotyping (Figure 2.6 A-G). Though there were slightly higher percentages of $F480^+$ macrophages in pre-pubertal mice (Figure 2.6 A) and $CD3^+$ T cells in post-pubertal mice (Figure 2.6 D) ($N \geq 23$), neither of these differences was significant. However, there were significantly higher percentages of $CD19^+$ B cells in the peritoneal cavity of post-pubertal mice (Figure 2.6 F). The percentages of monocytes/neutrophils ($Ly6C^+$) as well as mast cells were equal in both age groups (Figure 2.6 B, C). Post-pubertal animals exhibited significantly greater percentages of $CD8^-CD4^+$ but not $CD8^+CD4^-$ cells, suggesting any age-associated difference in peritoneal T cell composition is due specifically to greater numbers of $CD4^+$ helper T cells in post-pubertal animals (Figure 2.6 E). Post-

pubertal animals showed significantly increased percentages of both $IgM^{-}IgD^{+}$ and $IgM^{+}IgD^{-}$ cells (Figure 2.6 G), suggesting that the age-associated difference in B cells numbers is due to increased numbers of both immature and mature forms.

Pre-pubertal mice exhibit a greater influx of granulocytes into the peritoneal cavity

In both age groups, LPS injection caused an influx of leukocytes into the peritoneal cavity. In general, among $CD11B^{+}$ cells (myeloid lineage), pre-pubertal mice exhibited a higher percentage of granulocytic $Ly6C^{+}Ly6G^{+}$ cells and a lower percentage of monocytic $Ly6C^{+}Ly6G^{-}$ cells, when compared to post-pubertal mice (Figure 2.7 A). These differences were significant at the 20-hour time point. Between 0 and 2 hours, there were substantial increases in granulocytic cells within the peritoneal cavities of both pre- and post-pubertal animals (~20%). However, while these percentages continued to increase from 2 to 20 hours in pre-pubertal animals (25.5% ± 7.3% vs. 40.3% ±12.4%), they did not change in post-pubertal animals (22.9% ± 4.1% vs. 21.8% ±5.4%) (Figure 2.7 B). Contrastingly, while there were no significant changes in the peritoneal influx of monocytic cells for either age between 0 and 2 hours, post-pubertal animals exhibited a significant increase in this cell type from 2 to 20 hours (8.7% ± 4.8% vs. 18.0% ± 7.4%), while pre-pubertal animals did not (5.4% ± 0.4% vs. 7.3% ± 2.3%) (Figure 2.7 C).

Hormonal treatments alter survival from endotoxemia

We next investigated how sex hormones and pubertal status influence the differential mortality of pre- and post-pubertal animals in response to endotoxemia. Specifically, we tested the postulate that the increased estrogen production that accompanies puberty onset mediates the greater susceptibility of post-pubertal animals. Pre-treatment of pre-pubertal mice with estrogen prior to endotoxemia significantly expedited vaginal opening (Supplemental Figure 2.11) and increased mortality from endotoxemia compared to vehicle-treated mice (4 experiments; N=45; 60% vs. 26.7%; p=0.002) (Figure 2.8 A). In a complementary strategy, we prevented onset of puberty by pre-treatment with the GnRH agonist leuprolide. This drug interacts with and desensitizes the GnRH receptor in the brain and prevents release of pubertal gonadotropins (e.g. luteinizing hormone). This intervention led to improved survival compared to age-matched controls (3 experiments; N=20; 80% vs. 35%; p<0.0001) (Figure 2.8 B).

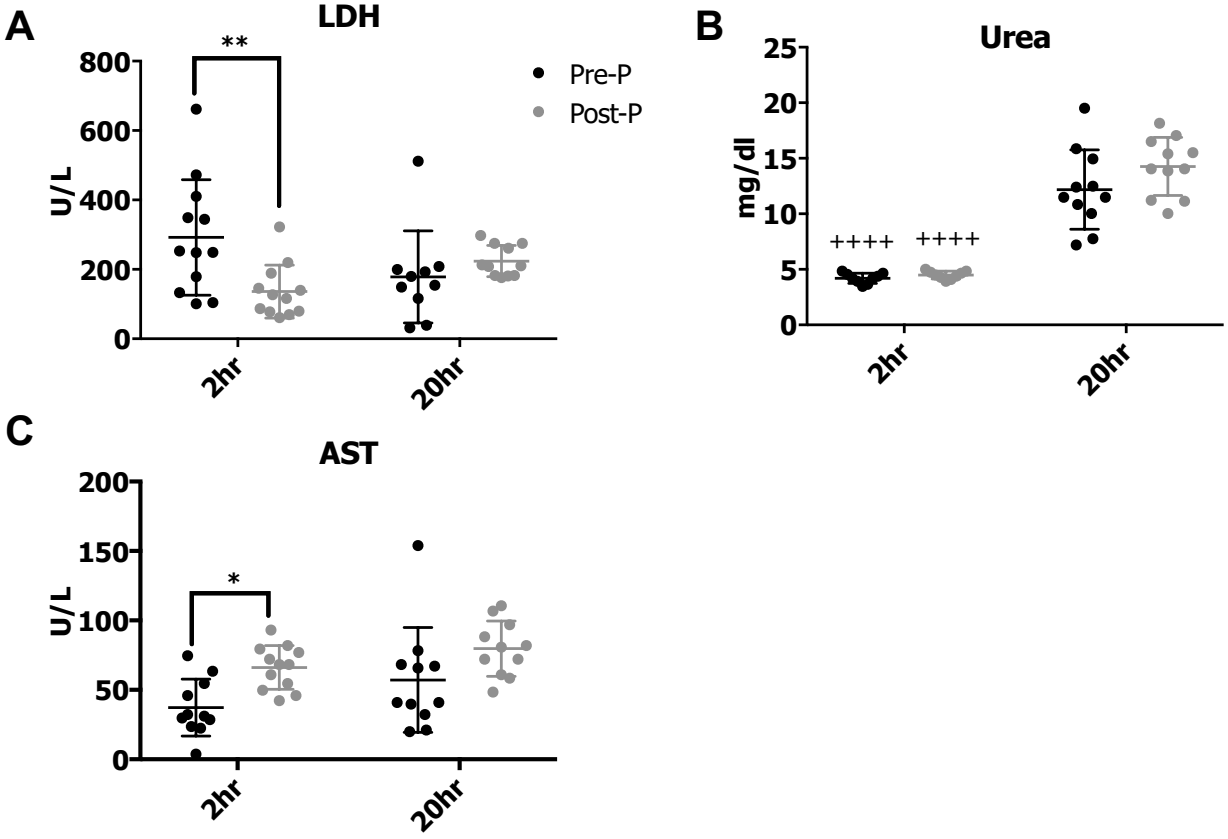

Figure 2.4. Serum injury markers in endotoxemic pre- and post-pubertal mice
A) Lactate dehydrogenase (LDH), B) Urea, and C) Aspartate Aminotransferase (AST) were quantified in pre- and post-pubertal serum samples collected 2 and 20 hours following endotoxemia. Significant changes in concentration between 2 and 20 hours for either Pre-P or Post-P mice are labeled with ++++ ($p<0.0001$). Significant differences in concentration between Pre-P or Post-P mice are labeled with ** ($p<0.01$) or * ($p<0.05$). All comparisons were made using Two-way ANOVA followed by Tukey's test for multiple comparisons.

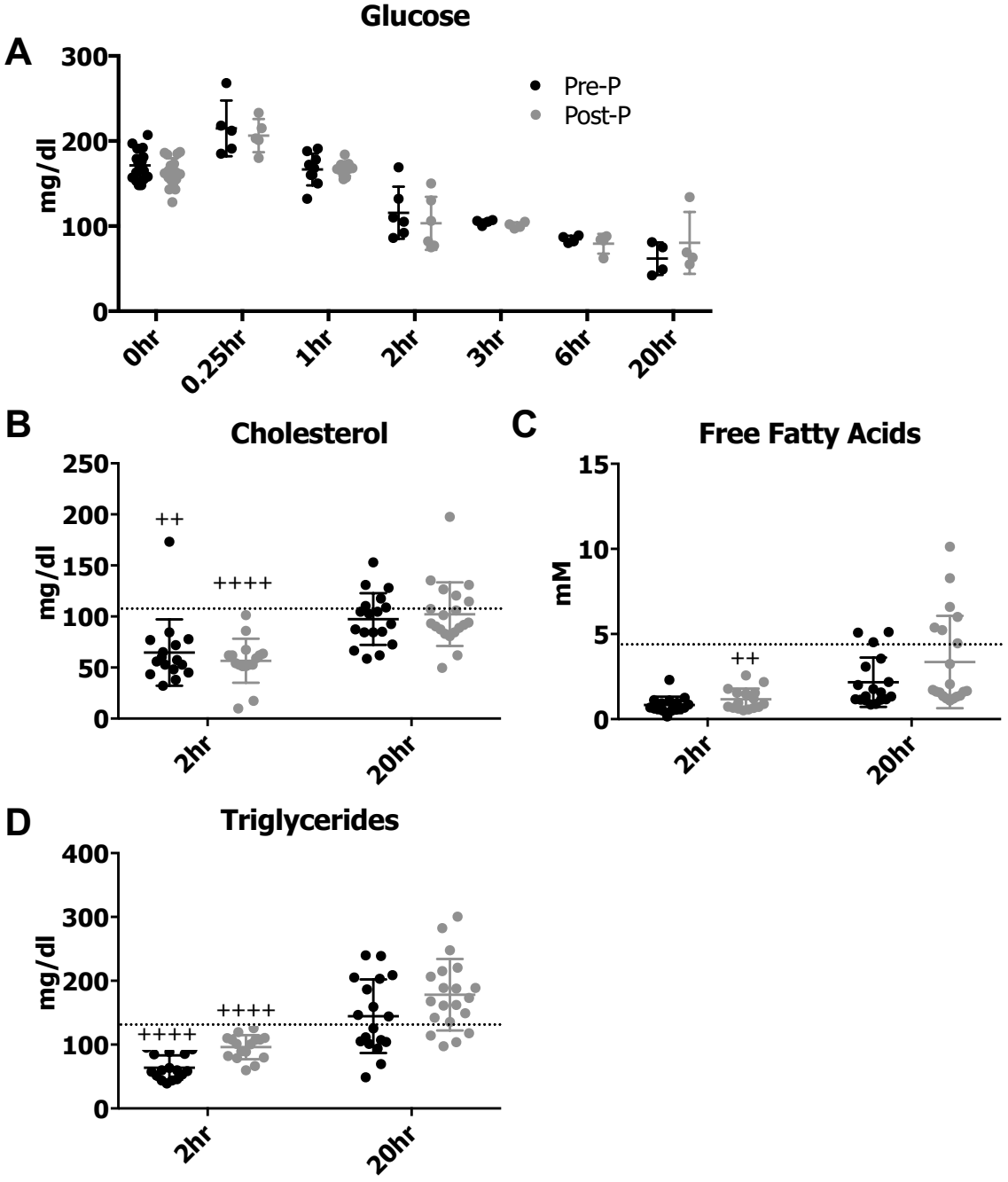

Figure 2.5. Serum metabolic factors are similar in endotoxemic pre- and post-pubertal mice. A) Glucose was quantified in whole blood using the Easy Step Blood Glucose Monitoring System at multiple time points following LPS administration. For each time point, N≥4 mice. B) Cholesterol (CL), C) Free Fatty Acids (FFA), and D) Triglycerides (TG) were quantified in serum samples isolated from pre- and post-pubertal mice at 2 and 20 hour time points. N≥16 mice per group. The dotted line indicates average baseline (0hr) level for each of the metabolites. Significant changes in the concentration of each metabolite over time (between 2 and 20 hours) in either pre- or post-pubertal mice are labeled with ++++ ($p<0.0001$) or ++ ($p<0.01$). All comparisons were made using Two-way ANOVA followed by Tukey's test for multiple comparisons.

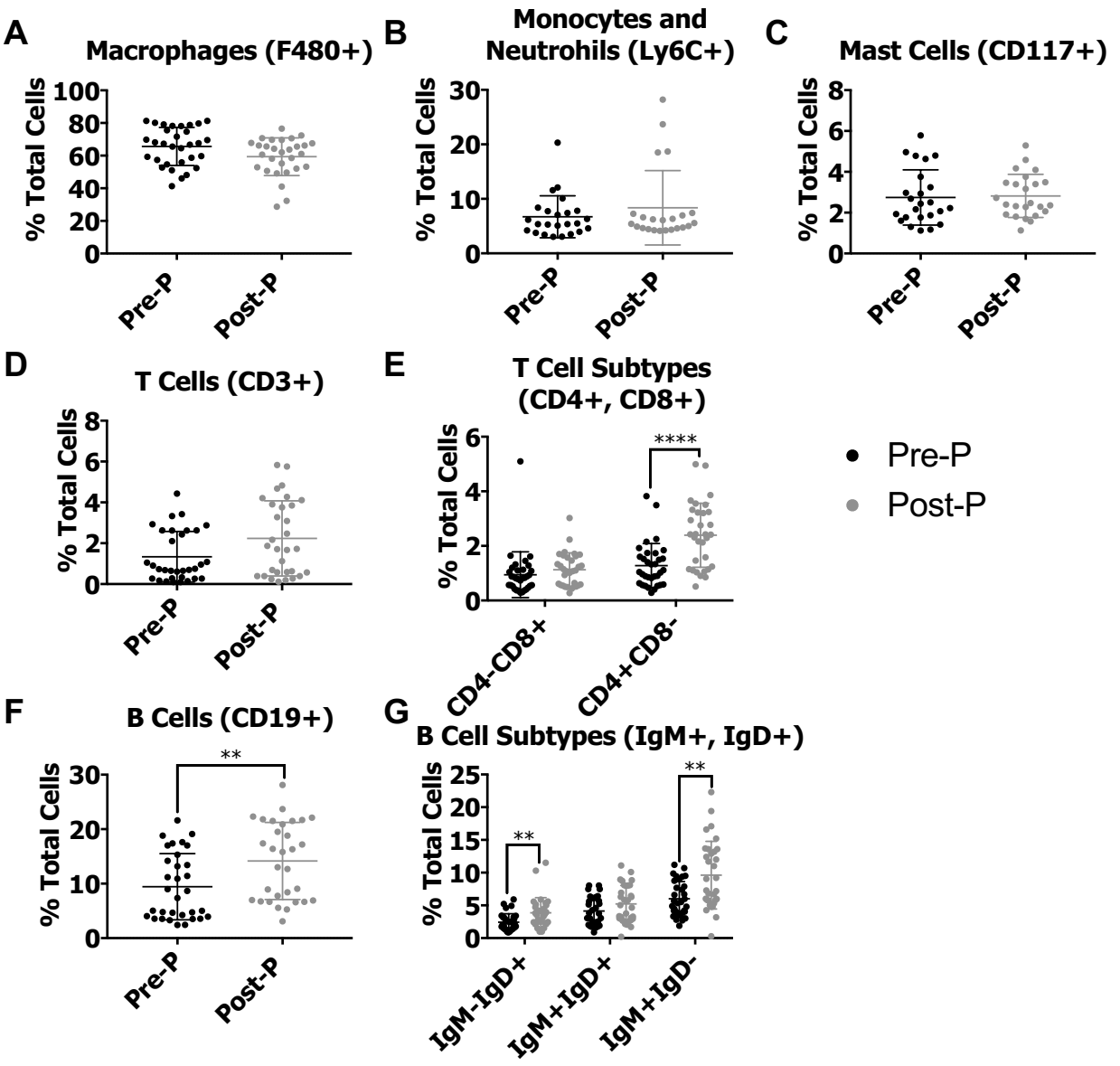

Figure 2.6. Pre- and Post-pubertal mice show different naïve peritoneal cell profiles
Naïve peritoneal cells from pre- and post-pubertal mice were collected through peritoneal lavage. Cells were blocked with Tru-Stain fcX^{TM} to prevent non-specific binding and then treated with different combinations of antibodies for subsequent immuno-phenotypic analysis by flow cytometry. These included A) F480+ Macrophages, B) Ly6C+ Monocytes and Neutrophils, C) CD117+ Mast Cells, D) CD3+ T cells and associated E) CD4+ and CD8+ subsets and F) CD19+ B cells and associated G) IgM and IgD+ subsets. N≥23 individuals per age group. Significant differences in concentration between pre- and post-pubertal mice are labeled with ** ($p<0.01$). All comparisons were made using the Mann Whitney test.

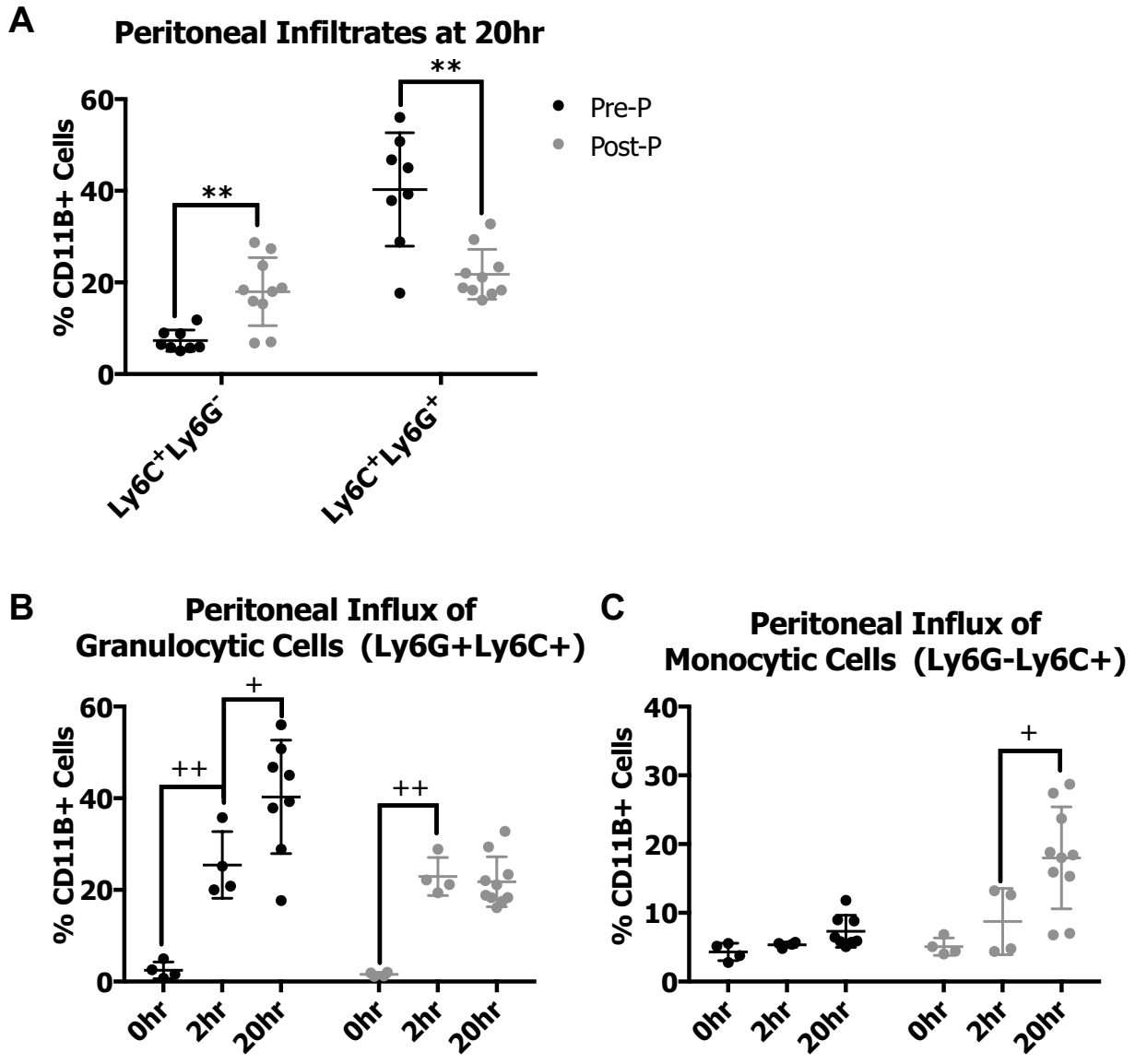

Figure 2.7. Pre- and post-pubertal mice show differential recruitment of cells into the peritoneal cavity

At 2 and 20 hours following endotoxemia, peritoneal cells from pre- and post-pubertal mice were collected through peritoneal lavage. Cells were blocked with Tru-Stain fcX™ to prevent non-specific binding and then treated with different combinations of antibodies for subsequent immuno-phenotypic analysis by flow cytometry. A) Age-associated differences in the peritoneal migration of granulocytic $CD11B^+Ly6C^+L6G^+$ and monocytic $CD11B^+Ly6C^+Ly6G^-$ cells were most apparent at the 20hr time point. B) Temporally, the patterns of B) granulocytic and C) monocytic influx into the peritoneal cavity were different between the two age groups. N≥4 individuals per age group. Significant differences in cell concentration between pre- and post-pubertal mice are labeled with ** ($p<0.01$) or * ($p<0.05$). Significant changes in the percent composition of each cell type over time in either pre- or post-pubertal mice are labeled with ++ ($p<0.01$) or + ($p<0.05$). All comparisons were made using Two-way ANOVA followed by Tukey's test for multiple comparisons.

Adoptive Transfer of pre-pubertal peritoneal cells improves survival from endotoxemia

We wanted to determine whether adoptive transfer of pre-pubertal cells could improve resistance of post-pubertal animals to endotoxemia-induced mortality. Adoptive transfer of naïve peritoneal cells from pre-pubertal mice into recipient post-pubertal mice, markedly increased survival after endotoxemia, compared to transfer of either post-pubertal cells or vehicle (4 experiments; $N \geq 37$; 78.3% vs. 25.0% vs. 36.4%; $p < 0.0001$) (Figure 2.9 A). These findings indicate that the "pre-pubertal resistance" can be transferred to a post-pubertal animal.

Considering the minor differences in naïve peritoneal cell composition between the two age groups, we sought to determine whether one or more subpopulation(s) contributed specifically to the adoptive transfer findings. We used a magnetic bead-based system (Miltenyi MACS) to perform positive and negative selection of different cell types from the pre-pubertal peritoneal cells and then used the separated cells for adoptive transfer. Adoptive transfer with positively or negatively-selected macrophages, or combined B and T cells, was unable to confer the same survival benefit as treatment with the non-separated peritoneal cell mix. However, even when separated cells were recombined to reconstitute the original mixed peritoneal cell population prior to adoptive transfer, no survival benefit was gained (Figure 2.9 B). The lack of survival among post-pubertal mice given an adoptive transfer of re-combined pre-pubertal cells indicates that the separation method was interfering with the functionality of the cells (see Discussion).

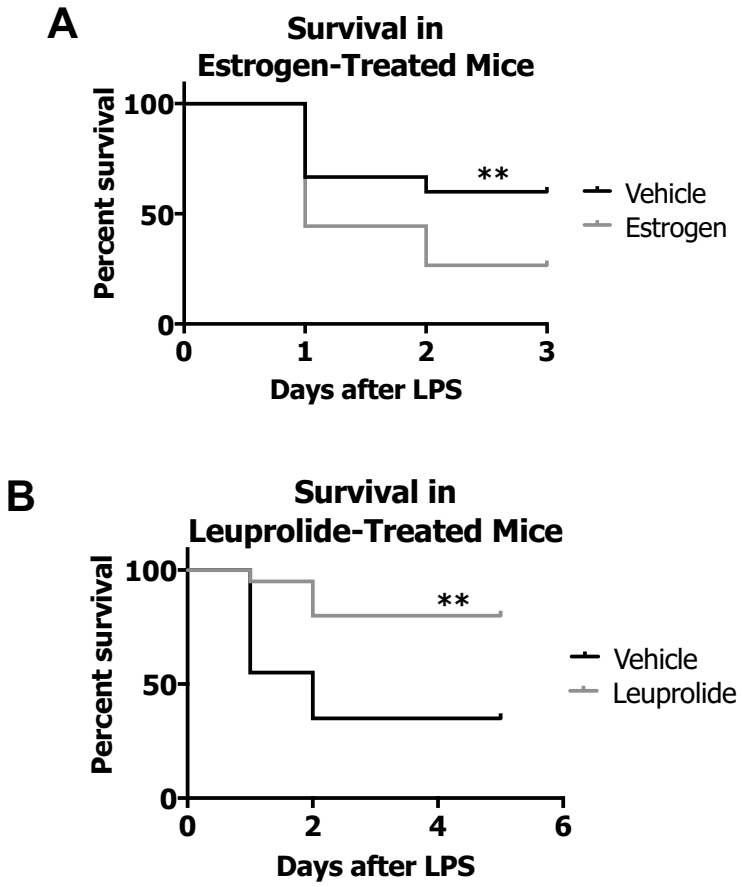

Figure 2.8. Delay or expedition of puberty by hormonal treatment alters mortality from endotoxemia

A) Pre-pubertal mice were pre-treated with daily subcutaneous injections of 17β-Estradiol at 100μg/ml (or vehicle (0.4% DMSO) suspended in corn oil for three days prior and once on the day of *E.coli* endotoxin injection. B) Pre-pubertal mice were treated with daily subcutaneous injections of leuprolide at 250μg/ml or vehicle (0.9% sodium chloride) starting on PND 24 and continuing until PND 35 before *S. enterica* endotoxin injection. Significant differences in survival between pre- and post-pubertal mice are labeled ** ($p<0.01$). Percent survival was compared using a log rank Mantel Cox test.

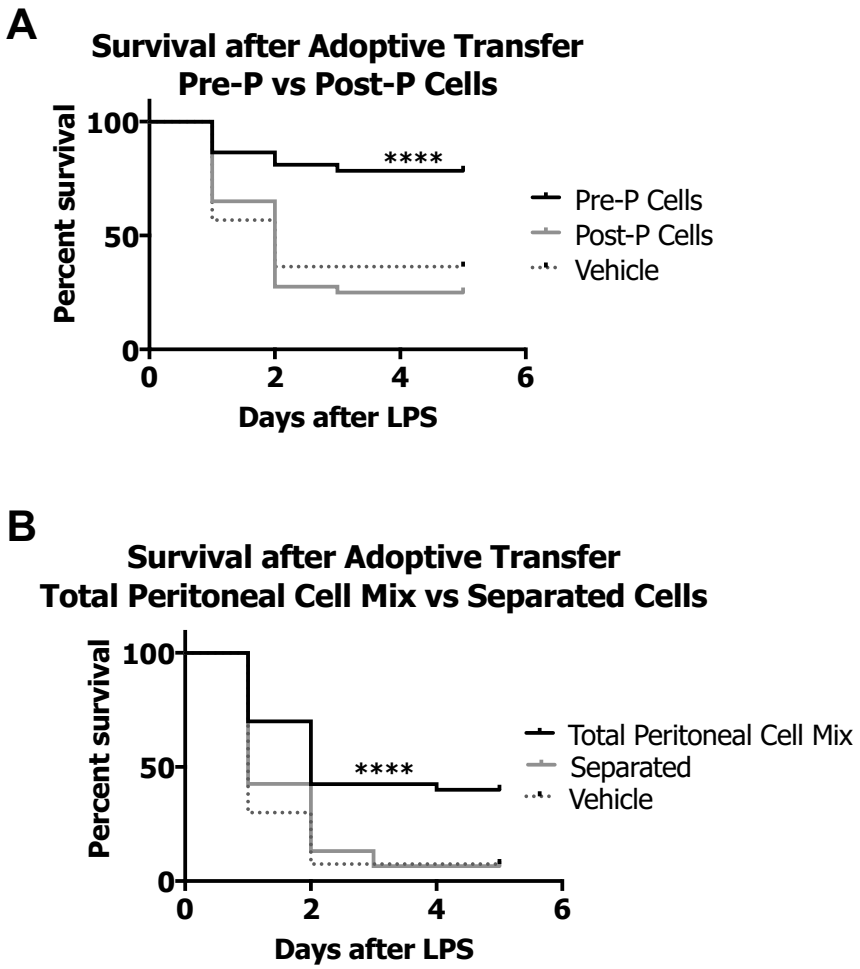

Figure 2.9. Adoptive transfer of pre-pubertal cells improves post-pubertal survival
Naïve peritoneal cells were collected from pre- or post-pubertal mice by peritoneal lavage. A) Recipient post-pubertal mice were administered either 1mL of pre-pubertal (Pre-P) or post-pubertal (Post-P) peritoneal cell suspensions, or 1mL of the vehicle, PBS (i.p.). Following incubation within the peritoneal cavity, the recipient mice were then subjected to endotoxemia. Significant differences in survival between mice administered Pre-P cells vs. those receiving Post-P cells or Vehicle were **** ($p<0.0001$). B) In some studies, specific cell types were separated from the pre-pubertal peritoneal cell suspensions prior to adoptive transfer using the Miltenyi MACS® system. Despite multiple different separation strategies, these separation attempts always interfered with the protective effect of adoptive transfer. Significant differences in survival between mice administered the un-separated, total pre-pubertal peritoneal cell mix vs. those receiving Separated cells or Vehicle were **** ($p<0.0001$). Percent survival was compared using a log rank Mantel Cox test.

2.5. Discussion

Epidemiological data indicate that human children, particularly in the pre-pubertal age-range, exhibit a striking but understudied resistance to mortality from sepsis. In this study, we used a mouse model of endotoxemia to characterize the phenomenon of childhood resistance to sepsis mortality and to begin to explore its underlying mechanisms. Pre-pubertal mice showed a highly significant resistance to mortality from endotoxemia in comparison to post-pubertal mice, despite having equally robust initial responses in cytokine production and leukocyte dynamics. Age-associated differences in the response to endotoxemia were most evident at a later 20-hour time point, when post-pubertal mice showed continued elevation of cytokines and monocytic, rather than granulocytic influx of cells into the peritoneal cavity. Blocking puberty using leuprolide or acceleration of puberty using estrogen led to increased or decreased survival from endotoxemia, respectively. In addition, the adoptive transfer of pre-pubertal, but not post-pubertal, peritoneal cells improved the survival of post-pubertal recipient mice, suggesting that specific pre-pubertal cell type(s), rather than pre-formed mediators or cells elsewhere (e.g. endothelium) might be functionally sufficient to mediate protection from mortality.

The endotoxemia model of sepsis has important limitations, including a dominant focus on the dangerously vigorous host response that it induces. Extrapolations from this model can favor the identification of treatments that dampen exuberant inflammation but ultimately do not improve survival in more complex human sepsis. However, in our study the improved survival of pre-pubertal mice did not appear to be a result of an inadequate or dampened initial response to immune stimulation by endotoxin. At two hours, pre- and post-pubertal animals exhibited equally robust inflammatory responses to endotoxin in both serum cytokine production and leukocyte recruitment. In fact, pre-pubertal animals showed some signs of having an even more intense response. For example, at the two-hour time point, pre-pubertal animals experienced a temporary surge in their white blood cell count and expressed elevated levels of serum GM-CSF. The lack of substantial age-associated differences in inflammation at this time point, as well as the detection of equal endotoxin concentrations in the sera of both age groups, suggest that the survival benefit of pre-puberty is not based on a weaker initial response. However, this conclusion, as well as other findings in our study would benefit from testing in a more complex and

realistic model of sepsis, for which there are several contenders, each with their own advantages and limitations [28,33].

Twenty hours after endotoxin administration, key differences between pre- and of post-pubertal mice became apparent in both the regulation of cytokine expression and peritoneal leukocyte composition. Post-pubertal animals exhibited continued elevation of a number of cytokines, chemokines and growth factors commonly associated with poor outcomes in sepsis (Figure 2.3). Surprisingly most of the canonical sepsis cytokines (e.g. IL-10, IL-1A, IL-1B, TNF-A) were expressed equally in both age groups (Supplemental Figure 2.10). While IL-6 did show a slightly increased expression level in post-pubertal animals, interpretation of this data is constrained by scant data points reflecting saturation (too high signals) of the multiplex analysis methodology. Interestingly, the majority of differentially regulated cytokines were associated with T cell biology. These cytokines included Th1 (IFN-γ), Th2 (IL-5 & IL-13) and Th17 (IL-7) type cytokines, as well as IL-15, a T cell growth factor expressed by mononuclear cells. Post-pubertal mice also exhibited increased expression of three chemokines: eotaxin 1, monocyte chemotactic protein 1 (MCP-1), and macrophage inflammatory protein 2 (MIP-). These three chemokines target the recruitment of eosinophils, inflammatory monocytes and neutrophils respectively. However, there was no clear connection between the differential expression of these chemokines and the composition of leukocytes found in the blood and peritoneal cavity. Finally, the growth factors leukemia inhibitory factor (LIF) and vascular endothelial growth factor (VEGF) also showed increased expression in post-pubertal serum at 20 hours. LIF is a highly pleiotropic cytokine in the IL-6 family that is commonly identified in the serum of critically ill humans and animal models and has both documented pro- and anti-inflammatory functions [34-37]. Among other functions, VEGF is extremely important in the regulation of vascular permeability. Overexpression of this factor, paired with the finding of diminished weight loss in post-pubertal animals (Figure 2.1 C), suggests that vascular leak and edema may have contributed to poor outcomes in this group [28,38,39].

In addition to cytokine expression, there were substantial changes to the cellular composition of the peritoneal cavity. While both pre- and post-pubertal mice had a substantial influx of granulocytic $CD11B^{+}$ cells between 0 and 2 hours following endotoxemia, only pre-pubertal animals showed continued

transmigration through to 20 hours. In contrast, only post-pubertal animals showed a marked increased in the percent composition of monocytic CD11B+ cells between 2 and 20 hours. The basis for this preferential recruitment of monocytic cells into the peritoneal cavity is unclear, considering that post-pubertal animals express high levels of both neutrophilic and monocytic chemokines. The incongruence between the elevation of neutrophilic chemokines and the decreased numbers of peritoneal neutrophils in post-pubertal animals may be due to impaired neutrophil chemotaxis. Neutrophil dysfunction, a common feature of sepsis in both human patients and mouse models, typically results in the troublesome adhesion and sequestration of activated neutrophils in the intravascular spaces and peripheral, unaffected tissues (e.g. lung, liver, kidney etc.). The mediators released by these activated neutrophils contribute to harmful systemic inflammation, endothelial dysfunction, hypotension, and coagulation [40,41]. Thus, an increased presence of activated neutrophils in non-peritoneal tissues could have contributed to the increased mortality seen among post-pubertal animals and is worthy of further investigation.

In comparison to the peritoneal cavity, age-associated changes in the cellular composition of the blood during endotoxemia were minute. In both age groups, between the 2 and 20-hour time points, the percent composition of neutrophils, eosinophils, and monocytes in the blood increased, while the percent composition of lymphocytes decreased. These changes, particularly the neutrophil and lymphocyte dynamics, are a common feature in mouse models of endotoxemia [42]. Though these trends were similar in both age groups, the increase in monocytes and decrease in lymphocytes were slightly greater in pre-pubertal animals. One might hypothesize that post-pubertal mice exhibited a comparatively lower percentage of monocytes in the blood at 20 hours, because of increased monocytic migration into the peritoneal cavity. In addition, the slightly elevated composition of lymphocytes in the blood of post-pubertal animals at 20 hours, was most likely due to dampened apoptosis [43-45] and might relate to the increased production of T-cell associated cytokines seen in serum samples from these mice. The severe thrombocytopenia observed in both age groups is a common finding in sepsis and reflects the elevated usage of platelets by a hyper-activated coagulation system. Interestingly, despite anemia being a predictor of poor outcome in human sepsis [46-48], only pre-pubertal animals exhibited a substantial drop in red blood cell counts (with matching decreases in hematocrit and hemoglobin (data not shown)) at the

20-hour time point. More detailed phenotypic analyses of these changes in red and white blood cells are needed to refine any conclusions about differences between the two age groups.

This work indicates that estrogen, and its elevation during puberty, plays a role in driving the differences between pre- and post-pubertal responses to endotoxemia. Hormonal acceleration of puberty through estrogen pre-treatment increased mortality while the inhibition of puberty with leuprolide decreased mortality. The importance of estrogen and the pubertal transition is also evident in another model of pre-pubertal resistance explored by our lab using H1N1 influenza infection. Mice subjected to ovariectomy before the onset of puberty had increased survival from infection—a finding that was reversed with estrogen replacement. In addition, delaying puberty by the administration of leuprolide improved survival of female mice following infection (Suber et al. manuscript in preparation). Despite the apparent importance of estrogen in driving the loss of resistance after puberty, pre-treatment of post-pubertal mice with the estrogen-receptor antagonist fulvestrant before endotoxemia did not reliably improve their survival compared to vehicle-treated controls (Supplemental Figure 2.16). One possibility is that exposure to increased estrogen during PNDs ~27-35 before fulvestrant was started, was sufficient to cause an irreversible estrogen-dependent change in susceptibility. It is also worth noting that increases in estrogen occur in both sexes at puberty. Although these studies used only female mice, the epidemiology and other experimental data (Suber et al. manuscript in preparation) show pre-pubertal resistance to mortality in both sexes.

In addition to the contribution of estrogen to the control of pre-pubertal resistance, we established other clues toward possible downstream mechanisms. Specifically, pre-pubertal resistance can be conferred to post-pubertal animals by adoptive transfer of pre-pubertal peritoneal cells. The particular biological activity of these cells in driving pre-pubertal resistance remains unknown. We consider the two most numerous cell types as possible mediators: B-cells and macrophages. Efforts to test macrophages (or B cells) purified by magnetic bead separation failed, but the reasoning was that the methodology itself abolished the protective function of pre-pubertal cells. Alternative methods (e.g., flow cytometric sorting, use of mice missing specific lineages such as macrophages [49,50] or B cells [51] ($Ighm^{tm1Cgn}$, Jackson Labs)) may allow identification of the responsible cell type(s) in future studies.

In parallel with the cell separation and adoptive transfer work, we used the mixed peritoneal cells from pre- and post-pubertal mice to compare their activity *in vitro*. However, in general, these data did not reveal any conclusive age-associated differences. Peritoneal cells from pre-pubertal animals expressed slightly higher levels of cytokines in response to *in vitro* LPS treatment (Supplemental Figure 2.12) as well as showed increased rates of apoptotic death (Supplemental Figure 2.13). Naïve peritoneal cells exhibited similar ROS production in the Fc Oxyburst® Assay (Supplemental Figure 2.14) and similar metabolic responses as evidenced by assessment of energy flux following LPS treatment using the Seahorse XF^{e} 96 reader (Supplemental Figure 2.15). These findings suggest that either pre-pubertal cells differ in a parameter of immunological function that has not yet been identified or that detection of functional differences between pre- and post-pubertal cells is only apparent in the context of the *in vivo* peritoneal environment.

Our findings suggest that a change occurring at puberty, mediated by increased estrogen, causes a permanent adjustment in the regulation of the innate immune system, which results in greater susceptibility to death from endotoxemia. Although aging-associated changes in basal inflammation and oxidative stress may account for some of these differences, our findings suggest that the estrogen released during puberty is critical. The data establish a model for childhood resistance to mortality from endotoxin, and identify peritoneal cells as mediators of pre-pubertal resistance that merit additional characterization.

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CHAPTER 3: Pathway-centered Analysis of the Relative Resistance of Children to Sepsis Mortality

3.1. Abstract

Sepsis remains a major cause of morbidity and mortality for which new therapies are urgently needed. Epidemiological data reveal that pre-pubertal children enjoy a relative natural resistance to mortality from severe infections like sepsis. To exploit this natural experiment, we used a data-mining approach to collect publicly available whole-blood transcriptome datasets from septic adults and children and compared gene expression patterns. We used a total of 12 datasets consisting of a study population that included 167 adults (55% Male; Mean age=59) and 95 children (64% Male; Mean age=8). To address the technical challenges of comparing gene expression across platforms, we used summary pathway activity for the gene expression patterns of the two age groups using Pathprint. This approach is robust to batch effect and can summarize gene expression at the pathway level across multiple array platforms. We identified key differences in pathway activity between the two age groups, particularly in the regulation of inflammation. We then applied *in-silico* drug repurposing by creating a pathway-drug network (PDN) to identify agents that might promote beneficial pathways (i.e. activated in children) or inhibit harmful ones (i.e. activated in adults). The PDN relies on the expression correlation between each of 16,150 drug, disease and pathway gene signatures collected from the Comparative Toxicogenomics Database (CTD), PharmGKB, GeneSigDB, Wikipathways, KEGG, Netpath, Reactome, and Connectivity Map, across 58,475 publicly available human microarrays (Affymetrix HGU133 Plus2). We used this PDN to interrogate specific clusters identified in our adult vs. child Pathprinted data for correlations between the activity of pathways of interest and the drug-based gene signatures built into the network. We validated resultant drug candidates by literature curation, and found that the Pathprint-PDN method had a substantially higher rate of positives (54%, $p<0.01$) compared to gene expression based tools (e.g., LINCS (12%)). In direct experimental testing using an endotoxemia model of murine sepsis, 5 of the top 10 compounds found by correlation, improved survival. Analyzing differences in the transcriptomic response of septic children vs. adults through a pathway-centric PDN method is an effective new way to identify drug leads for the treatment of sepsis and provide insight into the pathways that may determine survival.

3.2. Introduction

Sepsis is a major cause of global morbidity and mortality for which there remains no targeted therapy [1-3]. Central to sepsis pathophysiology is a dysregulated host inflammatory response [4-6] suggesting that host-directed immunomodulators could be of therapeutic benefit [7]. There is little agreement or certainty about which particular cells or molecules are critical to defining sepsis outcomes [8]. As a result, transcriptome analyses and 'systems biology' approaches have been eagerly embraced as novel ways to identify drugs targets for sepsis [9-12]. However, the hope that 'omics-based approaches might guide the selection of promising therapeutics to target sepsis has not yet been realized. This is despite the fact that tools like the Connectivity Map [13,14] and LINCS [15], which use gene expression signatures to identify drug candidates, have been available for over a decade. Obstacles to progress include discordant results across human studies focused on gene level changes [16], as well as the strongly debated limitations of animal models of sepsis for these types of analyses [17,18]. We address these problems by exploiting a natural phenomenon in humans that allows for the comparison of two groups with widely disparate rates of survival from sepsis—children and adults. Using novel pathway-centered bioinformatics tools to optimize data-mining across multiple platforms, we were able to identify key differences in the responses of both age groups to sepsis as well as identify potential therapeutics.

The comparison of data from children and adults arose from a striking finding, which at first glance seems unrelated to the problem of sepsis. Despite similar rates of infection during the 1918 influenza pandemic, children aged 5-14 experienced a remarkably lower rate of mortality compared to adults, dubbed the 'honeymoon period' [19]. Puberty (~age 14 in the early 1900s) marked the age-range in which mortality began to increase, suggesting that sex hormones could influence changes in fatality rates. Importantly, the 'honeymoon period' is not limited to 1918 influenza-related resistance to mortality. Historical mortality rates are much lower in children after various high fatality challenges, spanning from bubonic plague to measles. Contemporary data for trauma, the recent Ebola outbreaks, and other severe infections (Table 1.1) confirm this resistance. These data include lower case-fatality rates for sepsis in children, either when linked to specific pathogens (e.g. candidemia, Group A streptococcal sepsis, staphylococcal sepsis etc.; (Table 1.1)) or when analyzed as a broad diagnostic category [20-22].

To better understand the basis for this childhood resistance, we began by identifying public datasets of transcriptome profiling performed on blood leukocyte samples in the high vs. low survival groups (children or adults respectively.) The bioinformatics analysis used Pathprint [23,24], (https://bioconductor.org/packages/release/bioc/html/pathprint.html), a tool that is robust to batch effect and allows for comparison of gene expression at the pathway activity level across multiple array platforms. After identifying differences in pathway activity, we applied a novel method to build upon the correlation of the expression of >16,000 gene signatures, including disease signatures from the Comparative Toxicogenomics Database (CTD), PharmGKB and GeneSigDB, pathway signatures from Wikipathways, KEGG, Netpath and Reactome, and drug signatures from CTD, PharmGKB and Connectivity Map, across >50,000 individual microarrays–the Pathway Drug Network (PDN). The network neighborhood of the sepsis pathway signatures was used to identify drugs that were positively or negatively linked to high-survival (child) or high-mortality (adult) signatures. We assessed the validity of top drug leads by analyzing prior data collected in pre-clinical models of sepsis and also by direct testing for improved survival in a mouse model of fatal endotoxemia..

3.3. Materials and Methods

Data-mining

The Gene Expression Omnibus (GEO) and ArrayExpress databases [25,26] were queried to identify microarray transcriptome datasets from sepsis whole blood samples. The criteria for inclusion were 1) the availability of annotation data for the age of subjects and 2) the use of microarray platforms supported by the Pathprint tool. Patient samples from the ages 5 - 11 comprised the children's group. This was similar to the 5 -14 age range that showed greater survival in the 1918 influenza pandemic [19], but adjusted to reflect the earlier onset of puberty in modern times [27,28]. Samples from individuals 18 years of age or older constituted the adult group (details of demographics and datasets in Table 3.1).

The main workflow began with curation by downloading datasets of interest along with their associated metadata. Metadata was filtered, standardized and the relevant annotations of interest extracted (i.e. age, sepsis status, gender, data locations). The curated metadata from each study was

then combined to create a covariate table that was used to download each sample's expression data using the GEOQuery library [29]. To analyze data from multiple array platforms, differential activation of pathways was assessed using Pathprint. To identify pathways with minimal intra-group variation and maximal inter-group variation, two filtering criteria were set: 1) to maximize homogeneity within an age group based on minimizing the standard deviation (a cutoff of SD<0.475 in the Pathprint score was used); 2) to maximize differences between group comparisons using t-tests (Pathprint scores between groups were only included if $p<10^{-10}$). Heatmaps to visualize differences in pathway activation were generated using the pheatmap package (cran.r project.org/web/packages/pheatmap/index.html). Gene-level analysis was performed on a subset of datasets that were all obtained using the same array (U133 Plus 2, GPL570). This was done following initial quality control and normalization (using the arrayQualityMetrics and RMA packages, respectively [30,31]). Similar gene-level analysis was performed using another strategy to identify DEGs across multiple platforms and datasets, the Gene Expression Barcode [32], using the fRMA package [33]. Filtering by binary entropy measures (<0.295 for intra-group binary entropy and >0.3 for inter-group binary entropy) was used to identify genes with maximal expression differences between age groups. The top up- and down-regulated genes in the adult vs. child comparisons were used to query the Library of Network-Based Cellular Signatures (LINCS) database [15], and compounds anti-correlated to the adult profile were evaluated for published evidence of efficacy in sepsis models as described below.

Selection of Pathprint Results for Analysis by PDN

Four different clusters of pathways, generated through Pathprint analysis, were identified based on similar patterns of relative expression: cluster A) expression up in adults, down in children; cluster B) expression down in adults, up in children; cluster C) expression unregulated (not significantly changed) in adults, down in children; cluster D) expression unregulated in adults, up in children. Pathways from each cluster that showed the greatest difference between the two comparison groups were selected for further analysis by PDN. This selection was based on the percentage (N) of samples that satisfied the criteria (N=80% for clusters A-C; N=70% for cluster D). For example, the pathways selected from cluster A were up-regulated in at ≥80% of samples in adults AND down-regulated in ≥80% of samples in children.

PDN

Curation of gene-sets for base network creation

A set of drug, disease and pathway gene-sets were curated from the following resources:

1. Comparative Toxicogenomics Database (2,452 chemical/drug and 609 disease gene-sets):

The Comparative Toxicogenomics Database (CTD) [34] includes curated data describing cross-species interactions between chemicals and genes/proteins as well associations between chemicals, genes, and diseases. The data were retrieved from the CTD, MDI Biological Laboratory, Salisbury Cove, Maine, and NC State University, Raleigh, North Carolina (http://ctdbase.org/). [Nov 05 2012 retrieval]

2. The Pharmacogenomics Knowledgebase (178 chemical/drug gene-sets, 78 disease gene-sets):

The Pharmacogenomics Knowledgebase (PharmGKB) [35] is a pharmacogenomics knowledge resource that encompasses clinical information including dosing guidelines and drug labels, potentially clinically actionable gene-drug associations and genotype-phenotype relationships. Data (updated 11/6/12) were downloaded from the PharmGKB website (www.pharmgkb.org).

3. Connectivity Map (12,200 chemical/drug gene-sets):

The Connectivity Map (CMap) [13] is a collection of genome-wide transcriptional expression data from cultured human cells treated with bioactive small molecules. CMap contains 6,100 expression profiles representing 1,309 compounds. The data can be retrieved from http://www.broadinstitute.org/cmap. The rank matrix available on the website (contains 22,283 gene probes and 6,100 samples) was used to build unique gene signatures for each perturbation (drug treatment). Probe sets were ranked in descending order of the ratio of the treatment-to-control values. The probe that was most up-regulated relative to the control was designated as top rank (#1), while the probe that was most down-regulated relative to the control was designated as bottom rank (#22,283). Separate up-and down-regulated gene signatures in response to each drug were compiled using the top and bottom 1% of ranked genes respectively. These gene signatures served as a proxy for the transcriptional impact of each drug and allowed for the addition of CMap nodes to the PDN.

4. Pathprint (633 gene-sets):

Gene-sets from the pathways used by the Pathway Fingerprint (Pathprint) [23] were taken from the R package Pathprint (compbio.sph.harvard.edu/hidelab/pathprint/Pathprint.html) and (bioconductor.org/packages/pathprint/). The pathway list contains gene-sets derived from a range of databases (Reactome, KEGG, Wikipathways, Netpath; see Pathway Fingerprint for more information), and modules derived from a functional gene interaction network known as static modules [36].

The gene-sets derived from each of the resources described above were combined to create a library of 16,150 unique gene signatures.

Base Network Construction

A base network was constructed based on the expression correlation between each of the 16,150 gene signatures, across 58,475 publicly available human microarrays (Affymetrix HGU133 Plus2) obtained from GEO. The 2,120 experiments used to create this network consist of the same set of microarrays that make up the GPL570 expression background in the Pathprint package (see Bioconductor package for full list). For each microarray, the genes were ranked by expression level, from #1 (low expression) to T (high expression), where T is the total number of genes in the array. The expression score, En(G), for a gene signature, G, of size n, represented in an array by genes g_1, g_2 ...g_n, is defined by the mean squared rank of the member genes, $En(G)=n^{-1}\times\sum R^2_i$, where R_i is the rank of gene g_i in a pathway containing n genes. The network edges are represented by the partial correlation between each gene signature expression score, which is the correlation coefficient between two gene signature expression scores after accounting for the influence of the other gene signatures. The partial correlation was calculated using the R package GeneNet, which makes use of shrinkage estimators of partial correlation for fast and statistically efficient processing of the data (cran.r-project.org/web/packages/GeneNet/index.html).

The significance of each of the connecting edges was assessed by fitting a mixture model to the partial correlations, where the null model is estimated from the data. The calculation used the R package, fdrtool (http://cran.r-project.org/web/packages/fdrtool/index.html) to generate two-sided p-values for the test of non-zero correlation for each edge, corresponding posterior probabilities for edges, and q-

values [37]. The PDN method creates a network that is dynamic and can be extended to cover any number of additional signatures. The network was benchmarked using curated case-control interactions.

Determining CMap drugs associated with query cluster pathways or an individual gene signature

Once the base network was constructed, we interrogated it with a set of query pathways from pre-defined Pathprint clusters A-D. When pathways from a particular cluster (e.g. Cluster A) were inserted as nodes into the base network, new edges were drawn that highlighted specific correlations between the original base network nodes and added cluster pathway nodes. To assure that the new network was specific to correlations associated with a given cluster of pathways, we generated targeted sub-networks by removing base network nodes if they did not connect to at least three or more of the pathways in the cluster. We then ranked the significance of the remaining nodes by aggregating the p-values associated with each of the network edges by Fisher's method. For all non-CMap nodes, p-values were simply aggregated across the entire pathway cluster into a single p-value. For CMap nodes, p-values were first aggregated across the separate CMap up- and down-regulated gene signatures for each drug and secondly across the entire pathway cluster. An overall positive correlation between a cluster pathway and a pair of CMap nodes was determined by combining the p-values calculated for positive correlation with the up-regulated CMap drug signature and negative correlation with the corresponding down-regulated CMap drug signature. Overall negative correlation between a cluster pathway and a pair of CMap nodes was established in a similar way. Therefore, pairs of p-values for positive and negative correlation were calculated between each CMap drug in the sub-network and the pathway cluster. The p-values for positive and negative correlation were then ranked and combined into a simple association score: Score = rank(negativeRank - positiveRank) / (nDrugs/2) - 1, where negativeRank is the rank of the negative p-value, positiveRank is the rank of the positive p-value, and nDrugs is the number of drugs tested. Any CMap drug with a p-value of > 0.1 for both positive and negative association was given a score of 0. Thus, a negative score means that the drug opposes the activity of the cluster pathways, a positive score means that that drug enhances the activity of the cluster pathways. A score of 0 means no significant interaction. The highest negative or positive scoring drugs were prioritized for testing based whether reversing or mimicking a cluster would promote the higher survival phenotype. To compare PDN

functionality at the pathway- vs. gene-level, we also queried the PDN directly with gene signatures derived from standard limma analysis of adult and child transcriptomes. The top 500 up- and down-regulated probes from a comparison of children vs. adults using datasets limited to a single array platform (GPL570) were matched to 427 and 405 up- and down-regulated genes. These gene signatures were incorporated into the PDN and ranked for positive or negative association with CMap drug signatures by a similar approach as the Pathprint cluster pathways.

Validation of drug leads

To evaluate therapeutic leads identified through this method, a literature search using PubMed was performed. We used specific terms (i.e., keywords: survival, mortality, sepsis, endotoxin) to identify studies that tested a particular drug, or a closely related compound, for *in vivo* benefit in animal models of sepsis. Compounds were scored as follows: positive (prior studies showing survival benefit were identified); both (prior studies showing both benefit and harm to survival were identified); negative (no relevant studies were identified). The efficacy of the Pathprint-to-PDN methodology was compared to several other transcriptome-to-drug discovery approaches (described in results and Figure 3.2 legend) as well as to drugs randomly chosen from the CMap database. Prism software (GraphPad) was used to compare the frequency of prior studies showing benefit for drug leads across the multiple transcriptome-to-drug methodologies using Fisher's exact test.

Therapeutic leads were directly tested for survival benefit using a murine model of endotoxemia. Female C57bl/6 mice (5 weeks old, Charles River, Wilmington, DE) were injected with a high lethality dose (e.g. 38-40 μg/g) of *E. coli* LPS (L3755, Sigma; Lot: 123M4096V) between hours 5 and 7 of the light period in the animal facility (12-2PM). In order to mitigate fluid loss and dehydration, each mouse was also given a subcutaneous injection of sterile saline (equal to 2.5% of body weight). To test the effects of drug leads, compounds were injected 24 hours before and on the day of LPS administration, using routes and doses specified in Supplemental Table 3.4. Analysis of mortality included counting deceased mice as well as humane euthanasia of mice with severe, pre-terminal morbidity (scored by evaluation of appearance, movement, and response to touch). Survival data was analyzed using Prism (GraphPad) for statistical significance using a log-rank (Mantel-Cox) test.

3.4. Results

Pathprint highlights key pathways differentiating the adult and child responses to sepsis

A total of 12 datasets reporting transcriptome profiling of whole blood samples from sepsis patients were identified for analysis. The study population included 167 adults and 95 children, composed of 55% and 64% males, and mean ages of 59 and 8, respectively (Table 3.1). The Pathprint analysis tool was used to compare activity of pathways in adults and children with sepsis. Substantial differences in active or depressed pathways were identified, as illustrated in Figure 3.1. We focused on four pathway clusters A-D (defined in the methods.) After applying thresholds to select the clusters with the greatest age-associated differences, the pathways shown in Table 3.2 were used for further analysis by PDN. Supplemental Table 3.5 and Supplemental Files 1-3 provide additional details of the Pathprint scoring for all significantly different pathways.

PDN produces higher rates of positively validated drug leads.

The PDN methodology is an alternative approach to drug discovery that tests whether an experimental gene signature is correlated or anti-correlated to the gene signature associated with drug treatment. By measuring the correlation or anti-correlation between pathway, drug, and disease signatures over more than fifty thousand experiments, one can hypothesize whether the action that regulates, or is regulated by, those two signatures, (i.e. from the drug and from the experimental phenotype (e.g. better survival), may be linked and/or have a similar action (or opposing action in the case of negative correlation).

Drug-pathway networks incorporating each of the four Pathprint clusters (A-D) were created as described in the methods section. This and other drug discovery methodologies generate enormous quantities of possible drug leads. This necessitates efficient validation methods. Considering the large number of previous studies that have evaluated compounds for possible benefit in sepsis using animal models, we reasoned that one metric for evaluating the results from the PDN would be how often the identified drug leads corresponded to agents already shown to have positive (or negative) effects experimentally. Hence, we conducted extensive literature curation for each compound or closely related

Table 3.1. Demographic information on datasets used for data-mining
The GEO and ArrayExpress databases were queried to identify microarray transcriptome datasets from whole blood samples of septic adults and children. Samples from patients aged 18-91 comprised the adult's group and patients aged 5-11 comprised the children's group. The table above specifies the study GEO experimental accession number (GSE#), age category, age mean, age range, the number of male or female patients, the timing of sample acquisition in the sepsis course, the GEO microarray platform number (GPL#) and the reference used to access the original study.

Study GSE#	Age Category	Age mean	Age Range	M	F	Total	When Sampled	Array GPL#	Reference
28750	Adult	60	38-82	6	4	10	≤ 24h	570	[38]
13015	Adult	55	40-81	11	18	29	Time of Diagnosis	6947	[39]
10474	Adult	58	18-83	18	16	34	≤ 48h	571	[40]
40586	Adult	59	37-75	8	7	15	≤ 48h	6244	[41]
57065	Adult	63	29-84	19	9	28	~30 min. onset shock	570	[42]
33341	Adult	58	24-91	31	20	51	Time of Diagnosis	571	[43]
Summary	Adults:	59	18-91	93	74	167			
4607	Child	8	9-11	12	6	18	≤ 24h	570	[44]
9692	Child	7	5-9	6	2	8	≤ 24h	570	[44]
26440	Child	8	5-11	18	10	28	≤ 24h	570	[45]
26378	Child	8	5-10	18	10	28	≤ 24h	570	[45]
13904	Child	7	5-10	5	6	11	≤ 24h	570	[46]
40586	Child	8	7-8	2	0	2	≤ 48h	6244	[41]
Summary	Children:	8	5-11	61	34	95			

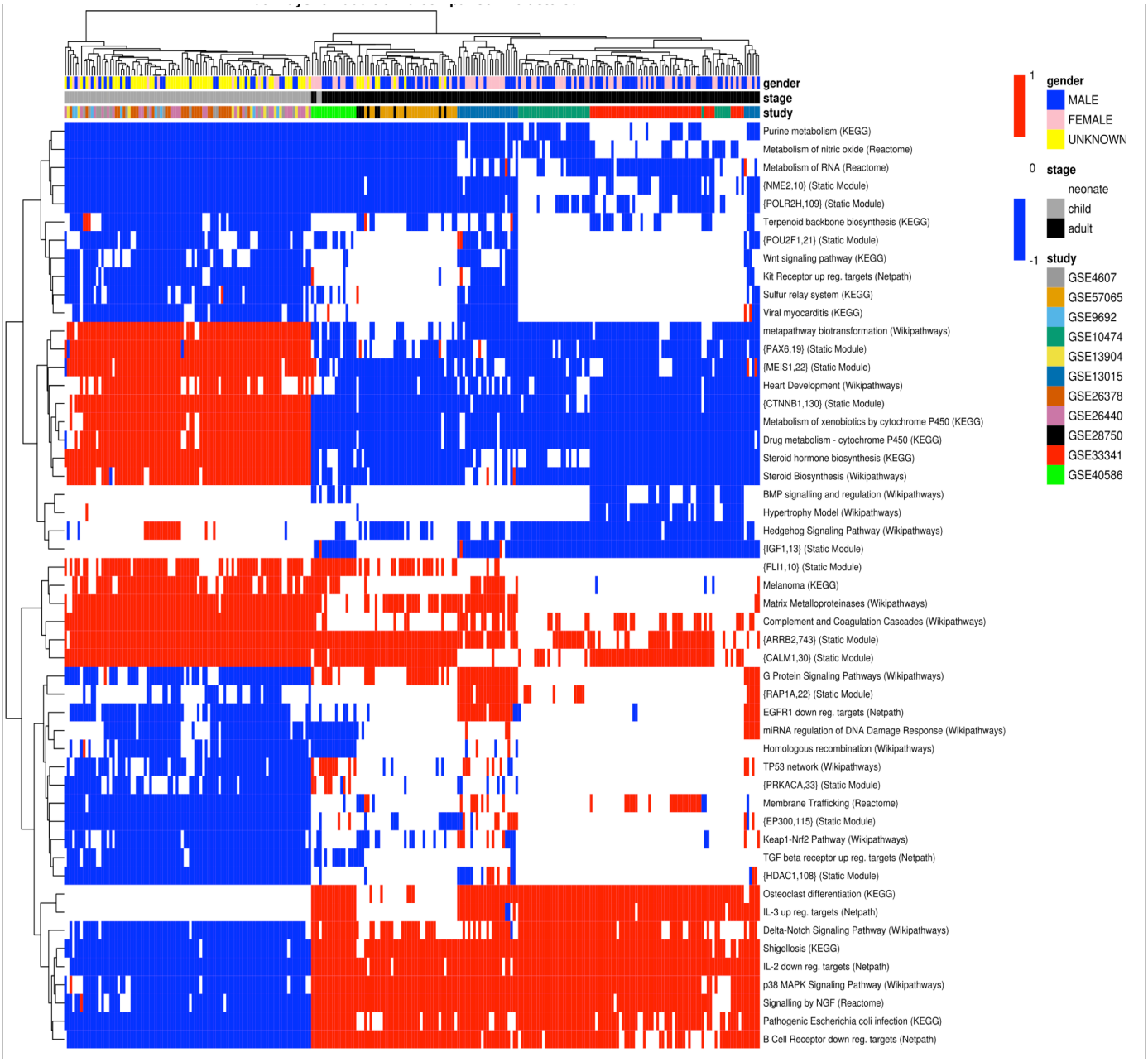

Figure 3.1. Sample heat-map generated from adult vs. child comparison using Pathprint
Pathprint analysis was used to analyze adult and child transcriptomes at the pathway level. To minimize intra-group variation and maximize inter-group variation, two filtering criteria were set to generate the data displayed above: 1) to maximize homogeneity within an age group based on minimizing the standard deviation, a cutoff of SD<0.475 in the Pathprint score was used; 2) to maximize differences between group comparisons using t-tests, Pathprint scores between groups were only included if $p<10^{-10}$. The heatmap above was generated using the pheatmap package.

Table 3.2. Pathprint clusters chosen for drug-candidate analysis using PDN
Four different clusters of pathways, generated through Pathprint analysis, were identified based on their relative activation (+ Pathprint score) or inhibition (- Pathprint score) in adults and children. The clusters were defined as follows: cluster A): pathway expression up in adults, down in children; cluster B) pathway expression down in adults, up in children; cluster C) pathway expression unregulated in adults, down in children; cluster D) pathway expression unregulated in adults, up in children. From each cluster, pathways showing the greatest divergence between the two age groups were selected for further analysis by PDN. More detailed descriptions can be found in Supplemental Table 3.5.

Cluster A (Up in Adults, Down in Children)	**Children Pathprint Score**	**Adults Pathprint Score**	**Children-Adults Difference**	**p-value**
IL-2 down reg. targets (Netpath)	-0.94	0.97	-1.91	1.87E-88
Shigellosis (KEGG)	-0.93	0.96	-1.88	2.04E-88
Endocytosis (KEGG)	-0.82	0.99	-1.82	1.95E-56
B Cell Receptor down reg. targets (Netpath)	-0.95	0.84	-1.79	1.69E-109
Signaling by NGF (Reactome)	-0.83	0.95	-1.78	9.11E-66
Pathogenic Escherichia coli infection (KEGG)	-0.96	0.82	-1.78	1.51E-113
Pentose Phosphate Pathway (Wikipathways)	-0.79	0.99	-1.78	1.80E-50
EGFR1 Signaling Pathway (Wikipathways)	-0.78	0.99	-1.77	6.46E-57
p38 MAPK Signaling Pathway (Wikipathways)	-0.80	0.95	-1.75	2.91E-62
{HCLS1,17} (Static Module)	-0.96	0.63	-1.59	1.28E-64

Table 3.2 (continued)

Cluster B (Down in Adults, Up in Children)	Children Pathprint Score	Adults Pathprint Score	Children-Adults Difference	p-value
{CTNNB1,130} (Static Module)	0.93	-0.95	1.87	2.71E-91
Metabolism of xenobiotics by cytochrome P450 (KEGG)	0.86	-0.98	1.84	2.55E-70
Drug metabolism - cytochrome P450 (KEGG)	0.84	-0.96	1.81	7.02E-66
Steroid hormone biosynthesis (KEGG)	0.97	-0.81	1.78	2.79E-128
Steroid Biosynthesis (Wikipathways)	0.87	-0.89	1.77	3.90E-84

Cluster C (Unchanged Adults, Down in Children)	Children Pathprint Score	Adults Pathprint Score	Children-Adults Difference	p-value
{EP300,115} (Static Module)	-0.99	-0.02	-0.97	4.30E-75
{HDAC1,108} (Static Module)	-0.99	-0.02	-0.97	2.46E-91
Keap1-Nrf2 Pathway (Wikipathways)	-0.89	-0.07	-0.82	5.94E-48
Kit Receptor up reg. targets (Netpath)	-0.92	-0.12	-0.80	1.92E-52
Sulfur relay system (KEGG)	-0.85	-0.18	-0.67	1.00E-29
TGF beta receptor up reg. targets (Netpath)	-0.94	-0.09	-0.85	2.87E-67
Viral myocarditis (KEGG)	-0.84	-0.15	-0.69	2.03E-32

Cluster D (Unchanged Adults, Up in Children)	Children Pathprint Score	Adults Pathprint Score	Children-Adults Difference	p-value
{FLI1,10} (Static Module)	0.72	0.23	0.48	7.52E-15
Melanoma (KEGG)	0.77	0.12	0.65	8.98E-26
Serotonin Transporter Activity (Wikipathways)	0.72	0.22	0.49	1.73E-14
Statin Pathway (Wikipathways)	0.96	-0.08	1.04	4.30E-64

agents (e.g. ibuprofen for NSAIDs) and scored the presence of prior publications showing benefit or harm in animal models of sepsis.

The validation efficacy of drug lists derived from Pathprint-to-PDN analysis were compared to three other gene-level drug discovery approaches as well as to a control approach—drugs selected at random from the entire list of CMap compounds. The first, a gene-level approach, used PDN to analyze differentially expressed genes (DEGs) generated from a standard limma analysis of children vs. adult transcriptomes. We found a substantially higher rate of positives in the list produced by a pathway-level approach (i.e. 54% compared to 27% for the gene-level approach and 16% for randomly selected drugs). We also analyzed the top up- and down-regulated DEGs from standard limma analysis (Supplemental Figure 3.4) or from the BarCode method [32] (Supplemental File 4). These up- and down-regulated DEGs were used to query the LINCS database [47], a greatly expanded version of the Connectivity Map [13]. The lists of compounds expected to reverse the gene signature associated with higher sepsis mortality (i.e., up and down-regulated in adults compared to children) were also curated to assess the frequency of prior positive results in the literature. The percentage of positive drug leads achieved by the Pathprint-to-PDN methodology was significantly higher than with each of the four other methods ($p<0.02$ by Fisher's exact test). The percent positives for each of the five categories of drug leads are summarized in Figure 3.2, and details of the lists and references are in Supplemental File 5.

PDN-derived therapeutic leads improve survival in murine endotoxemia

To more directly investigate the utility of this approach, we tested ten of the compounds generated by the Pathprint-to-PDN method (Supplemental Table 3.6) for their effects on survival in an endotoxin shock model. Mice were pre-treated with the compounds as described in the methods, followed by intra-peritoneal administration of endotoxin. As shown in Figure 3.3, five of the ten compounds tested improved survival in this model. In all, eight of the compounds tested had not been previously reported in sepsis studies, and three of these eight compounds showed benefit in our endotoxemia. We also tested two compounds likely to be effective based on prior publications (topotecan, a water-soluble analog of camptothecin and amitriptyline similar to desipramine) and found decreased mortality.

Table 3.3. Curation of drug lists by PubMed literature search

A literature search using PubMed was performed to compare the number of therapeutic leads generated by pathway- or gene-level interrogation of the PDN and found to confer a survival benefit in *in vivo* mouse models of sepsis. These two methods were compared to random selection of drugs from the total CMap list. Compounds were scored as follows: positive (prior studies showing survival benefit were identified: dark grey +); both (prior studies showing both benefit and harm to survival were identified: light Grey +/-); negative (no relevant studies were identified: no color, no entry; prior studies showing only harm to survival were identified: no color, -). More information is provided in Supplemental File 5.

Cluster	Pathprint to PDN	Prior Data for Survival Benefit?		DEGs to PDN	Prior Data for Survival Benefit?		Random	Prior Data for Survival Benefit?
A	fenoprofen	+/-		0297417-0002B			urapidil	
A	glibenclamide	+		indometacin	+/-		trifluoperazine	
A	asiaticoside	+		SB-202190	+		metaraminol	+
A	topiramate			acetohexamide	+		nomegestrol	
A	suramin	+/-		STOCK1N-35215			coralyne	
A	hyoscyamine	+		emetine			citiolone	
A	pancuronium			tacrine			octopamine	
A	N-acetyl-L-leucine			thioridazine			sulfapyridine	
A	mefenamicacid	+		suloctidil			butoconazole	
A	apigenin	+		biotin			0175029-0000	
B	camptothecin	+		cyclopenthiazide			tracazolate	
B	lincomycin	+		mebhydrolin	+/-		tomatidine	
B	ganciclovir			triprolidine	+/-		tetroquinone	
B	fursultiamine			colchicine			repaglinide	
B	tocainide	+		cinchonine			tiletamine	
B	GW-8510			methoxamine			amikacin	+
B	tanespimycin	+		tanespimycin	+		butirosin	
B	carbenoxolone	+		fluorometholone	+/-		meptazinol	+
B	tacrolimus	+		nicardipine	+		tolnaftate	
B	conessine			quinpirole			fasudil	+
C	khellin			cicloheximide	-		enilconazole	
C	eldeline			colchicinic acid			sulfanilamide	
C	sulfathiazole			meteneprost	-		theophylline	
C	geldanamycin	+		puromycin			spiramycin	
C	cefoxitin			digoxin			omeprazole	
C	procaine	+		naftidrofuryl			rolitetracycline	
C	procyclidine			terfenadine			dexpropranolol	+
C	monorden	+		gelsemine			piribedil	
C	hexetidine			sulindac	+/-		sulfathiazole	
C	piperacetazine			drofenine			iobenguane	

Table 3.3 (continued)

Cluster	Pathprint to Drugs	Prior Data for Survival Benefit?	DEGs to Drugs	Prior Data for Survival Benefit?	Random	Prior Data for Survival Benefit?
A & C	desipramine	+	tioguanine		dicycloverine	
A & C	cyclosporine	+	methylergometrine		PF-0053978-00	
A & C	nifenazone	+/-	methotrexate		dipivefrine	
D	etacrynicacid		dexamethasone	+/-	tomatidine	
D	noscapine		tolazoline		bicuculline	+
D	tanespimycin	+	3-aminobenzamide	+	ethosuximide	
D	mebhydrolin		epitiostanol		meclozine	
D	vincamine		benzthiazide		alimemazine	
D	altretamine		0179445-0000		monensin	
D	enalapril	+	lidocaine	+	Prestwick-691	
D	coralyne	+	alexidine		oxaprozin	+/-
D	napelline		dihydroergocristine		amiodarone	
D	clindamycin	+	nifurtimox		ampicillin	

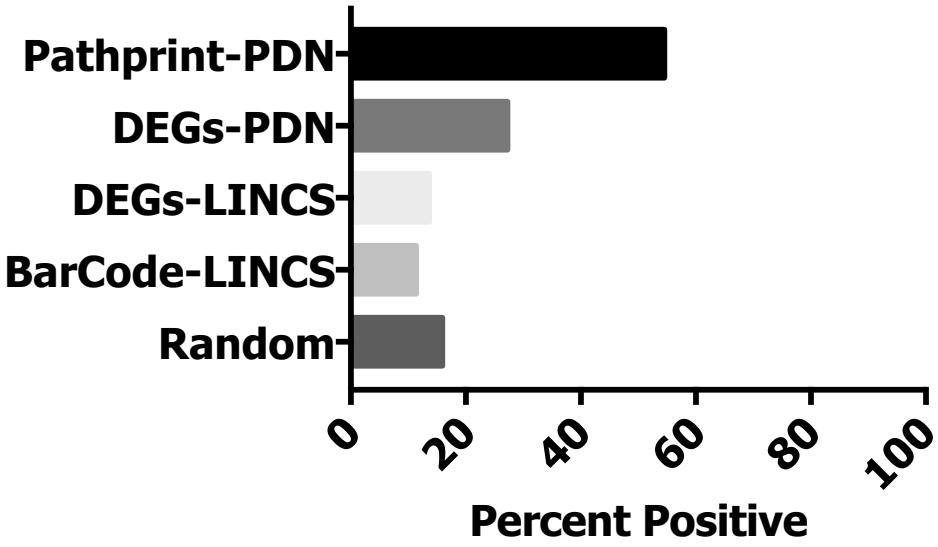

Figure 3.2. Comparison of several methods of drug candidate identification
Five methods of transcriptome analysis & drug candidate identification were compared in their ability to successfully produce drug targets that that showed a survival benefit in an animal model of sepsis in at least one prior study. 1) Pathprint-PDN: Comparison of pathways by Pathprint and drug candidate analysis by pathway-drug network (PDN); 2) DEGs-PDN: Comparison of differentially expressed genes (DEGs) by standard methods and drug candidate analysis by PDN; 3) Random: Drugs chosen at random from the CMap database; 4) DEGs-LINCS: Comparison of DEGs generated by standard methods and drug candidate analysis using LINCS database and 5) BarCode-LINCS: Comparison of DEGs generated by BarCode method and drug candidate analysis using LINCS database. Prism software (GraphPad) was used to compare the frequency of prior studies showing benefit for drug leads Fisher's exact tests.

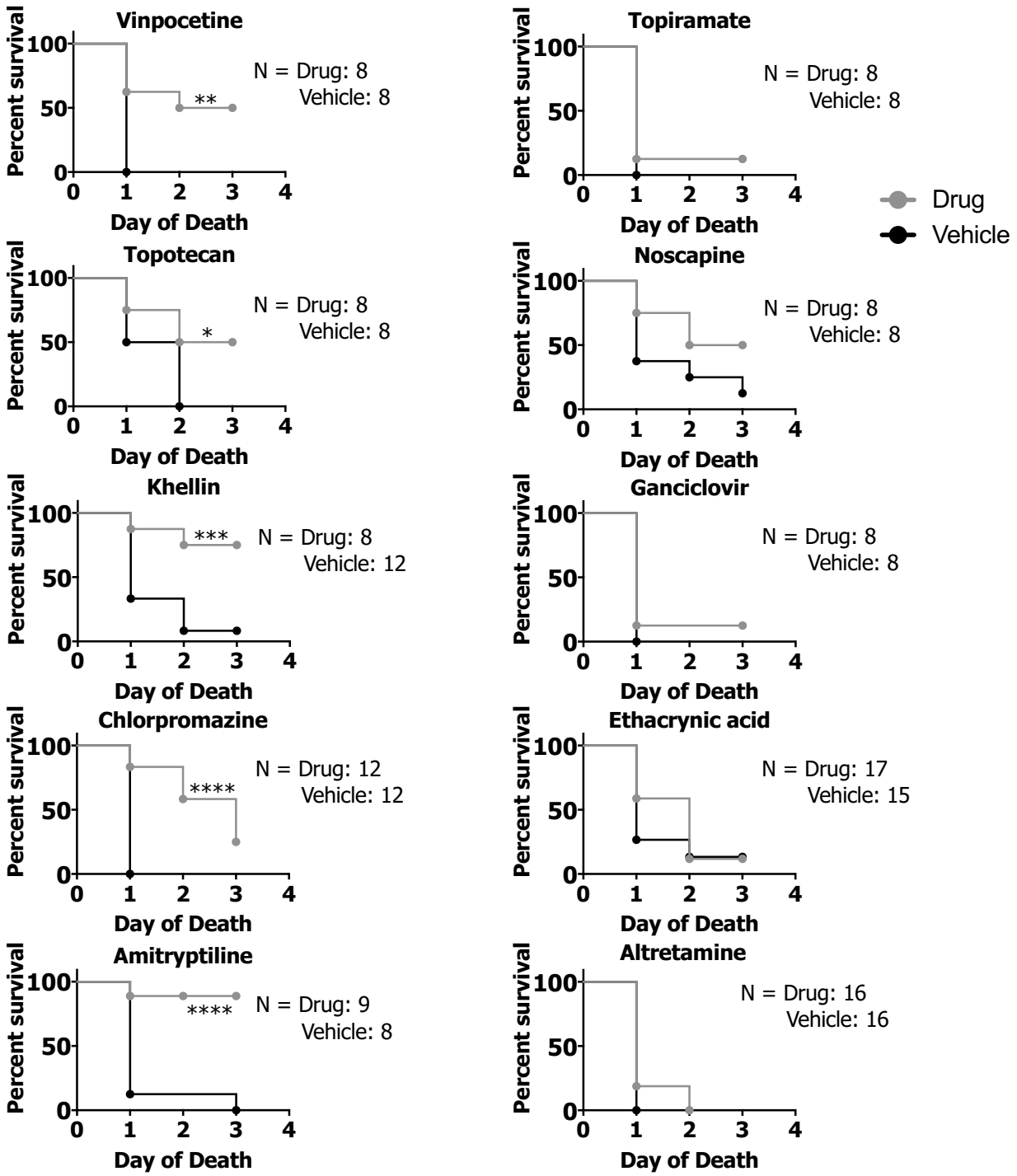

Figure 3.3. Validation of select PDN drug candidates in an *in vivo* endotoxemia model
Therapeutic leads generated using Pathprint-PDN were directly tested for survival benefit using a murine model of endotoxemia. Select compounds were injected 24 hours before and on the day of LPS administration, using routes and doses specified in Supplemental Table 3.4. C57bl/6 female mice were injected with a high-lethality dose of *E. coli* LPS. Significant differences in concentration between pre- and post-pubertal mice are labeled with **** ($p<0.0001$), *** ($p<0.001$, ** ($p<0.01$), or * ($p<0.05$). Percent survival was compared using a log rank Mantel Cox test.

3.5. Discussion

In this study, we sought to identify drug candidates for the treatment of sepsis by combining two novel approaches. Firstly, we focused on a remarkable natural experiment—the relative resistance to mortality in children versus adults with sepsis. By data-mining publicly available whole blood transcriptomes, we were able to identify key differences in pathway regulation between the two age groups. Continuing with a pathway-centric approach, we used pathway-based correlation to build an *in-silico* drug development system to find drugs that might promote beneficial pathways (i.e. activated in children) or inhibit harmful ones (i.e. activated in adults). Evaluation of the resulting drug list by both literature curation and direct experimentation showed substantial enrichment for promising candidates.

By starting with human transcriptome data in our comparison of children vs. adults, we increased the potential value of subsequent 'omics-based analyses. The limitations of using animal models (especially mice) in pre-clinical sepsis studies are well-recognized Mice typically lack many of the common features of human sepsis patients (e.g. age, comorbidities, drug treatments, supportive care, etc.) [48,49] and exhibit highly species-specific transcriptomes after injury or sepsis [17]. In addition, no model of sepsis in mice (e.g. endotoxemia, bacterial pneumonia, cecal ligation and puncture (CLP), etc.) can completely replicate the physiological responses seen in human sepsis [50]. The data-mining strategy used in this study reduces reliance on these animal models of sepsis as the main source of genetic information for the generation of a drug candidate list.

Unfortunately, due to the absence of any effective drugs for human sepsis (excluding antibiotics), it was not possible to validate our method by curating human data. Hence, we instead relied on outcomes in mice for both the *in vivo* testing (Figure 3.3) as well as the curation results (Table 3.3), which compiled drug treatment effects in studies mostly performed in murine models of sepsis. While imperfect, the "reverse-translational" methodology used in this work [48] attempts to exploit the many remaining similarities in the murine and human responses to injury [51]. By limiting our study to pathways identified as important in humans, we diminish the risks of identifying murine-specific biology and trying to apply it to humans. Further assessment of the efficacy of the identified drug 'hits' will need to be conducted in larger animal models and ultimately human patients. Despite the limitations, this approach

offers a substantial improvement in ascertaining drugs that merit further evaluation in pre-clinical assays. The child-adult difference in resistance to mortality may also prove useful as a starting point for drug discovery in other severe infections and disorders (Table 1.1).

In addition to the drug discovery goal of this work, the differences in pathway activation between adults and children also provide clues to the mechanisms driving childhood resistance to mortality. The initial pathway clusters generated through Pathprint, were selected using relatively stringent criteria to maximize differences. Using this approach, the pathways that were down-regulated in children in comparison to adults (Clusters A & C, see Table 3.2) involved response to infections (e.g. Shigellosis, Pathogenic *Escherichia coli* infection, viral myocarditis), canonical inflammatory and oxidative stress signaling pathways (e.g. IL-2 down-regulated targets, B cell Receptor down-regulated targets, p38 MAPK Signaling Pathway, Keap1-Nrf2 Pathway, TGF beta receptor up-regulated targets), pathways involved in growth and cell proliferation (e.g. Signaling by NGF, EGFR1 Signaling Pathway, Kit Receptor up-regulated targets), and pathways involved in chromatin modification. These pathways suggest a chronic regulation of the inflammatory response in adults in comparison to children. In general, there were fewer pathways that met our criteria for significant up-regulation in children in comparison to adults (Clusters B & D), and these were far fewer related to inflammatory/immune responses. These pathways include lipid biosynthesis and regulation (e.g. Steroid hormone biosynthesis, Steroid Biosynthesis, Statin pathway, cytochrome P450 activity), as well as proto-oncogenic genes and cancer (e.g. {CTNNB1, 130} [Static Module], {FLI1, 10} [Static Module], Melanoma [KEGG]). Using somewhat less stringent criteria, we identified the top 50 pathways (out of 633, ~top 8%) that were up-regulated in adults but down-regulated in children or vice versa. The inflammatory (adult) vs. metabolic (child) difference is also evident in this comparison (see Supplemental Files 2 & 3). We speculate that these differences reflect hormonal status, a postulate for future investigation.

We were able to carry out the comparisons reported here in part, due to the large number of datasets available that report whole blood transcriptomes in sepsis. This reflects the systemic nature of the condition, the accepted scientific importance of leukocytes in sepsis pathogenesis, and the relative ease of obtaining blood samples. However, it is unclear whether the transcriptomes of whole blood

accurately reflect the most important changes in gene expression during sepsis. The expression profiles of whole blood essentially represent a weighted sum of the patterns of gene expression for each blood cell type and patients with sepsis exhibit heterogeneity in the leukocyte composition of the blood. No white blood cell count data was available in the data annotations for these studies, making it impossible to control for these differences between individuals. In addition, the analysis of whole blood does not address potentially important contributions from endothelial, epithelial, tissue-resident immune and parenchymal cell types [52].

The drug development strategy applied here has more general applicability beyond sepsis as well. Classical approaches to understanding drug-disease relationships rely on experimental assays to relate cell states and perturbations to the etiology of different diseases, but cannot sample all possible interactions. Fully-connected approaches such as the Molecular Signature Map [53] quantify interactions based on overlapping gene membership. While this method successfully integrates our knowledge of gene lists, it fails to address the issue of how drug, pathway and disease signatures influence each other. As of yet, there has been no attempt to understand the flow of information between processes in the cell by separating out the systematic contribution of gene overlap from the overall correlation in expression. We have used data from the most highly represented platforms in the Gene Expression Omnibus (GEO) to determine the correlation of the expression of over 16,000 drugs, diseases and pathway gene signatures in humans. PDN analysis provides, for the first time, the ability to interpret the cell as a whole from the perspective of the relationships between the processes that are occurring within it. The network brings together disease signatures from the Comparative Toxicogenomics Database (CTD), PharmGKB and GeneSigDB, pathway signatures from Wikipathways, KEGG, Netpath and Reactome, and drugs signatures from CTD, PharmGKB and Connectivity Map. The recent finding that the human and mouse responses to trauma and injury are significantly different [17] has cast doubt on the rationale behind the traditional usage of model species in drug development pipelines. However, by developing independent networks for both human and mouse, a goal for future studies, the relationships between these mammalian systems could actually be assessed for their consistency. We hope that the ability to assay

the cross-species coherence in drug-disease relationships prior to undergoing clinical trials will have profound implications for future drug development.

The standard CMap drug discovery pipeline tests if an experimentally derived up- or down-regulated gene signature is also up- or down-regulated in a set of drug perturbation expression data. Broadly, this is equivalent to querying whether the transcriptional impact of the experiment is similar, or opposite to the transcriptional impact of a drug in CMap. The PDN is an alternative approach that tests if an experimental gene signature is correlated or anti-correlated to the gene signature associated with drug treatment. Importantly, the correlation is measured not just in the setting of the transcriptome data from a single experiment, but across many experiments (over 50,000 arrays from over 2,000 experiments). The rationale for this alternative approach is to look for the correlation or anti-correlation of two signatures across many experiments rather than assessing their similarity in a single test. If correlation is detected, we can hypothesize that the action that regulates, or is regulated by, those two signatures, (i.e. from the drug and from the experimental phenotype (e.g. better survival), may be linked and/or have a similar action (or opposing action in the case of negative correlation).

Limitations of this approach include some of the well-recognized problems in meta-analysis of microarray data in general [54] and in sepsis specifically. The Pathprint approach overcomes some of the problems in merging data from different platforms. However it cannot integrate all platforms, so some sepsis studies could not be included. The study relied on very useful, but imperfect databases. For example, the extensive reliance of CMap (and LINCS) on transcriptomic data derived from cancer cell lines may skew results. Additionally, the overall success rate of drugs identified by 'reversal of signature' methods is unknown, but supported by our individual successes. A further limitation of existing, pair-wise approaches to determine drug-disease relationships, including that which was presented here, is that no mechanistic data can be inferred. The integration of pathway and experimental gene signatures in the PDN only allows for the identification of tightly connected sub-networks around each drug-disease connection, and gives no clue to the directionality of the relationship. However, it does allow for both negative and positive connections to be identified, significantly distinguishing this approach from existing overlap-based *in silico* methods. These features improve the identification of drugs with synergistic

effects or sets of drugs with independent mechanisms of action on a disease. Both of these factors are vitally important in overcoming polygenic drug resistance.

The ultimate aim of this work was to discover novel drug candidates for the treatment of sepsis by data-mining and comparing whole blood transcriptomes from two populations with naturally high (adults) or low (children) susceptibility to death from sepsis. Pathways with age-specific activation were identified through Pathprint and successfully used to interrogate the pathway-drug network (PDN), which allowed for the identification of medications that could promote beneficial pathways during sepsis (i.e. activated in children) or inhibit harmful ones (i.e. activated in adults). Validation by literature curation and direct experimentation in endotoxemic mice indicated that the resulting drug list contained many promising therapeutic candidates. These findings suggest that a pathway-centric approach to drug discovery may prove useful in identifying novel therapeutics for sepsis and other complex medical conditions.

3.6. Acknowledgements

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CHAPTER 4: Conclusions and Perspectives

4.1. Overview of Findings

Epidemiological data show that human children exhibit an unexplored resistance to mortality from sepsis. This resistance is particularly striking in children before they undergo puberty. We used a mouse model of endotoxemia to characterize the phenomenon of pre-pubertal resistance to sepsis mortality and begin to investigate its underlying mechanisms. Pre-pubertal mice were highly resistant to mortality from endotoxemia compared to post-pubertal mice. This improvement in survival occurred despite equally robust initial responses in cytokine production and leukocyte dynamics. Age-associated differences in the response to endotoxemia were only evident later, when post-pubertal mice showed continued elevation of cytokines and monocytic, rather than granulocytic, influx of cells into the peritoneal cavity. Mechanistically, during endotoxemia hormonal pre-treatments that prevented or expedited puberty resulted in increased or decreased survival respectively. In addition, the adoptive transfer of pre-pubertal, but not post-pubertal, peritoneal cells improved the survival of post-pubertal recipient mice, suggesting that a particular pre-pubertal cell type might hold the key to the observed protection from mortality.

In addition to these characterization studies in mice, we sought to identify drug candidates for the treatment of sepsis by data-mining publicly available whole blood transcriptomes from septic adults and children. Analysis using Pathprint revealed key differences in pathway regulation between the two age groups, particularly in the regulation of immune function. We then used a new *in-silico* drug development system to create a pathway-drug network (PDN) to identify drugs that might promote beneficial pathways (i.e. activated in children) or inhibit harmful ones (i.e. activated in adults). Validation by literature curation and direct experimentation in endotoxemic post-pubertal mice suggested that the resulting drug list was substantially enriched for promising candidates.

From the data obtained, as described in Chapters 2 and 3, it is apparent that pre-pubertal individuals (both mice and human) exhibit differential responses to sepsis. In the following sections, I will discuss in greater detail the results of this work and their limitations as well as and the next logical steps in research strategy.

4.2. The pubertal divide: a critical analysis

Though the epidemiological analysis and the experimental data obtained in this thesis support the idea that pre-pubertal resistance to mortality exists, there are several possible confounding factors that need discussion. Two important elements to consider in our interpretation of the epidemiology and data-mining analysis are that 1) adults are more likely to have comorbidities and 2) elderly adults are more likely to have age-associated immune dysfunction. Underlying comorbidities (e.g. COPD, cancer, renal or liver disease, HIV infection etc.) are extremely important in driving sepsis outcome. In some studies, over half of adult sepsis patients have at least one comorbid medical condition [1,2]. Although conditions like liver cirrhosis, HIV infection and cancer significantly increase the odds of death for sepsis patients independently of age, patients over 65 are twice as likely to have a comorbid medical condition in comparison to younger patients. Excluding the effects of comorbidities, advanced age in itself can also increase the risk of mortality among sepsis patients. For example the odds of a patient aged ≥65 years perishing from sepsis are more than double the odds of death from sepsis in younger patients [2]. These independent effects of age on sepsis mortality reflect age-related differences in both innate [3] and adaptive [4,5] immune function. In particular, elderly humans and animal models exhibit a greater amount of immune dysregulation—prolonged, un-resolving inflammation combined with immunosuppression and T cell exhaustion [6-8]. In our epidemiologic survey, we limited the adult data to samples in the age range of 20-60 for two reasons. First, we sought to approximate the age-range of adults showing higher mortality in the 1918 flu. Second, this age range reduces potential confounding by the increased comorbidity and immune dysfunction found in advanced age. However, in our data-mining curation, we sought to increase the power of our meta-analysis by using the maximum number of samples available and decided to use adult samples of all ages >18 (detailed demographics shown in Table 3.1).

Though comorbidity status and advanced age might have affected our comparisons of adults and children, there are a number of reasons why we believe these factors do not fully account for the mortality and transcriptomic differences seen between the two age groups. For example, data by Angus and colleagues (2001) suggest that while comorbidity status does increase the risk of mortality, it does not interfere with the manifestation of increased survival among pre-pubertal children. For example,

while children and adults without comorbidities exhibited mortality rates of 6.3% and 12.8% respectively, those with comorbidities exhibited mortality rates of 16.0% and 27.6% respectively [1]. Additionally, many of the studies that support the theory of pre-pubertal resistance, show an age-associated increase in mortality even among young individuals not typically plagued by comorbidities. For example, in the 1918 pandemic flu, while the specific death rate for pre-pubertal children aged 5-14 was 176.6 deaths per 100,000, the specific death rate for young adults 15-34 was on average, 4.5 times higher [9]. Thus, although the risk of mortality may be increased by comorbidities in older adults, there is still a strong case for the pubertal divide in susceptibility.

Another important factor to consider in our assessment of the human epidemiology and data-mining findings is the well-documented change in leukocyte composition that occurs during development. Though this difference was not replicated in our mouse model, in comparison to older children and adults, pre-pubertal children generally have higher percent blood compositions of B cells (~12.5% vs. ~16.5% of blood lymphocytes) [10-12] and lower percent blood compositions of T cells (~72.5% vs. ~68.5% of blood lymphocytes) [10,12]. Increased percentages of T cells in adults were due specifically to the presence of more $CD4^+$ Helper T cells. In the extreme, there is a formal possibility that the differences seen between pre- and post-pubertal age groups are simply reflecting the differences in starting white blood cell composition. However, it is unlikely that all of the differences seen in the transcriptomic analyses in Chapter 3 as well as in the human children vs. adult epidemiology are due to this slight difference in baseline leukocyte composition.

As evidenced in the endotoxemia data, estrogen appears to play an important role in disrupting the pre-pubertal resistance to mortality. Although our hormonal manipulation studies used only female mice, the epidemiology (Table 1.1) and other experimental data in influenza (Suber et al. manuscript in preparation; [13]) suggest that pre-pubertal resistance to mortality occurs in both sexes. This is logical, considering the essential role that estrogen plays in the pubertal development of both sexes. In males, though the Leydig cells of the testes produce mainly testosterone, they also produce androstenedione, A5-androstenediol, dihydrotestosterone and estrogen. About 25% of the estrogen found in males comes from this testicular secretion, while the other 75% is derived from extra-glandular aromatization of

testosterone and androstenedione. In females, the ovary secretes the majority of estrogen and only a small fraction arises from the extra-glandular conversion of androgens. Plasma estrogen levels are extremely low during pre-puberty in both males and females, but begin to rise at the beginning of puberty. This rising estrogen stimulates the pubertal growth spurt via its effects on Growth Hormone (GH) secretion and subsequent Insulin Growth Factor 1 (IGF-1) production. Though the rise occurs earlier in girls than in boys, the bioactive estrogen levels for growth in both sexes are equivalent when growth velocity peaks. In both sexes, estrogen is critical for promoting skeletal maturation, the eventual cessation of longitudinal growth, and the maintenance of bone mass in adulthood [14].The fact that estrogen has such enormous effects on the pubertal development of both sexes suggests that it may also be able to unilaterally alter the susceptibility to death from sepsis.

In our mouse model of endotoxemia, we found that treatment of pre-pubertal animals with estrogen increased their mortality. In contrast with these findings, there is a sizable body of work in adult rodents that demonstrates the survival benefits of estrogen in sepsis and other forms of critical illness. In various rodent models of critical illness (e.g. sepsis, trauma-hemorrhage, etc.), estrogen (both exogenous and endogenous) has been shown to protect vital organs and improve survival [15,16]. For example, in a CLP model of sepsis, administration of WAY-202196, a selective estrogen receptor β (ERβ) agonist, to both male and female mice significantly improved survival, decreased bacteremia, and protected the gastrointestinal mucosa [17]. In another example, following CLP or a combination of hemorrhage and CLP, female mice maintained splenic immune functionality, exhibited decreased serum pro-inflammatory mediators ,and showed increased survival in comparison to males [18,19]. However, in the clinical setting, there are conflicting reports as to whether female sex is protective during sepsis. These conflicts are most likely related to the lack of information on the hormonal status of the individual at the time of illness (e.g. estrogen in in the peri-ovulatory phase is over 3.5 times that in the mid-follicular phase) as well as differences in study design [20]. An additional complication in understanding the role of estrogen in sepsis is the finding that critically ill individuals often have increased serum levels of estrogen. This is caused by increased rates of peripheral aromatization in response to stress [21] and is associated with increased risk of mortality [22,23].

Understanding the effects of estrogen during sepsis and other critical illnesses, requires us to acknowledge its dynamic, non-monotonic response curve [24]. For example, in the context of immunity, estrogen is anti-inflammatory at high levels and pro-inflammatory at lower levels [25,26]. This might explain why pregnant women, with extremely high estrogen levels, are more susceptible to infectious disease and sepsis while non-pregnant adult women show enhanced immune function, even to the point of predisposing them to auto-immunity [27]. This "bi-potential" effect of estrogen on immune cells has been well-documented in the literature. For example, in human monocytes and macrophages stimulated *ex vivo*, low doses of estrogen enhanced the production of pro-inflammatory cytokines (IL-1, IL-6, TNF) while high concentrations reduced them [28]. Likewise, in T cells, low estradiol concentrations promoted Th1 and Th17 type responses and cell-mediated immunity while high estradiol concentrations augmented Th2 type responses and humoral immunity [26,29,30]

Sex differences in immune function are well established in vertebrates, mammals, and humans and typically relate to the allocation of energy for growth, maintenance (includes immune function), and reproduction [27,31,32]. The interesting question is whether there is an evolutionary reason for the resistance to highly virulent infections seen in pre-pubertal animals. More likely, post-pubertal susceptibility is an accident associated with energetic trade-offs made with reproductive function [32]. If the pre-pubertal resistance is disturbed directly by the first encounter with high levels of estrogens, and occurs equally in males and females, we hypothesize that the effect of interest is most likely an action of estrogen that occurs in both sexes. Unfortunately, it is unknown whether any specific, estrogen-associated changes to immune system function occur during puberty across both males and females. The only study available looked at the effects of puberty on splenic transcriptomes in male and female mice and did not note many similarities in the changing patterns of gene expression. The ultimate finding was that post-pubertal males showed upregulation of genes involved in innate immunity, while females showed upregulation of genes involved in adaptive immunity [33]. Future studies exploring the non-sex-specific effects of puberty on the immune system could help illuminate the mechanisms associated with pre-pubertal resistance.

4.3. Strategies to identify the mechanisms driving pre-pubertal resistance

This thesis has highlighted the effect of pre-pubertal status on murine and human responses to sepsis. Though the precise mechanisms driving these differences are yet to be identified, this work laid the groundwork and generated several hypotheses for future study. In the following section we will describe some of these mechanistic theories and outline strategies to further refine and explore them.

We determined that in comparison to post-pubertal mice, pre-pubertal animals show increased resistance to mortality from endotoxemia. A logical next step is to replicate these findings in a more complex model of sepsis such as bacterial peritonitis, bacterial pneumonia, or cecal ligation and puncture (CLP). Though these models are also imperfect, they do replicate more aspects of the septic disease course seen in humans [34]. Considering that pre-pubertal survival from endotoxemia was associated with robust initial responses to endotoxin, we hypothesize that pre-pubertal animals would also survive better in live bacterial models of sepsis. Throughout this project we attempted to use two live bacterial models of sepsis but were held back in our progress by technical difficulties or inconclusive results.

Our first attempt was an *E.coli*-impregnated fibrin clot model. In this murine model of sepsis, the clot acts as an abscess, promoting a bacterial infection that progresses slowly over time [35]. Normally the fibrin clot is assembled by mixing a known number of bacteria with fibrinogen and thrombin, which forms a small clot that can be surgically inserted into the mouse peritoneal cavity. We attempted to simplify the method, using a slightly different formulation of the fibrinogen/thrombin mixture, so that it could be injected into the mouse peritoneal cavity and upon entry form a solid clot in the animal. Though this method showed promise, the model was abandoned because of a persistent difficulty in standardizing the timing in which animals died. In preliminary trials using adult animals, mice would show few signs of illness for 2 to 3 weeks after injection of the fibrin clot, and then would suddenly succumb to infection. However, given further optimization for the extended timing of the infection and long-term monitoring of animals, there is a possibility that this model could be useful for future sepsis studies. For example, the fibrin-clot model has been found to replicate several important features of sepsis seen in humans such as the hyper-dynamic cardiovascular state, leukocytosis, myocardial depression, cytokine response, and delayed mortality [36]. Another advantage this model holds over others is that the strain and dosage of the

infecting organism can be easily manipulated. Especially if paired with fluid resuscitation and supportive care, this model could be a clinically relevant method to test for the manifestation of pre-pubertal resistance.

We also conducted some preliminary experiments comparing pre- and post-pubertal animals using a CLP model of sepsis. This model is favored in sepsis research because it reproduces the dynamic changes in cardiovascular function seen in humans and the progressive release of pro-inflammatory mediators [36,37]. Four separate trials produced no differences in mortality between pre- and post-pubertal age groups. However, in these initial attempts there were quality control issues with the suture material used for ligation, making mortality highly variable across trials (data not shown). In addition, we were unsure whether we were inflicting similar injury on both age groups, considering that the younger mice on average were at least 25% smaller. Though the ceca appeared to be similarly sized between the two age groups, using the same incision size and needle gauge for both age groups may have inflicted unequal harm on the younger animals, thus interfering with our monitoring of pre-pubertal resistance. Perhaps this could be improved in a larger rodent model, like rats, in which the incision size and needle gauge could be better controlled for animal size.

Another key finding identified by this work was that drug treatments that manipulated sex hormones prior to endotoxemia directly affected survival. Treatment with leuprolide, a GnRH agonist that interferes with the pulsatile release of GnRH required for pubertal development, greatly increased survival. Treatment of pre-pubertal animals with estrogen led to precocious puberty, monitored by early vaginal opening (Supplemental Figure 2.11), and severely decreased the rate of survival. One of the most important next steps is to dissect the role that the estrogen released during puberty plays in priming animals for poor outcomes during endotoxemia. Estrogen acts through the estrogen receptors α and β (ERα and ERβ). To determine what receptor type is required, we can treat pre-pubertal mice with selective agonists for ERα (e.g. PPT) and ERβ (e.g. AC 186) in place of estrogen or compare ERα KO ($Esr1^{tm4.2Ksk}$; Jackson Labs) and ERβ KO ($Esr2^{tm1Unc}$; Jackson Labs) mice with wild-type to determine the effect on survival following endotoxemia. After determining the receptor(s) required for estrogen-induced mortality, we can begin to more easily target the cell types/ organ systems required for these specific

changes. Conditional knockout of ERα, ERβ, or both can be established in many different individual cell types in mice using the Cre-lox system. Interesting cell types to assess for their involvement in estrogen-mediated susceptibility to sepsis would be myeloid cells ($Lyz2^{tm1(cre)Ifo}$; Jackson Labs) and B cells ($Cd19^{tm1(cre)Cgn}$; Jackson Labs). Along with dissecting the molecular biology involved, it is important to identify the ways in which estrogen-primed pre-pubertal animals respond similarly to endotoxin as post-pubertal animals. Useful parameters to analyze in these mice would be cytokine expression, leukocyte dynamics as well as the transcriptomes of whole blood or select peritoneal cells types.

Adoptive transfer of pre-pubertal, but not post-pubertal, peritoneal cells improved the survival of post-pubertal recipient mice, suggesting a particular pre-pubertal cell type might hold the key to this protection from mortality. Important next steps are to identify the cell type(s) responsible for conferring pre-pubertal resistance and to quantify the differences in the immune response occurring in post-pubertal mice following adoptive transfer. Our initial attempts to identify these active cell types were focused on macrophages and lymphocytes. We tried both positive and negative selection strategies using the Miltenyi MACs magnetic bead separation system, but saw no survival benefit from adoptive transfer of any of the separated cells. However, mixing the separated cells back together again also did not rescue the original survival phenotype. This finding suggests that the separation process itself was changing the functionality of the cells. Another way to attempt these separations is using flow cytometric sorting of $CD19^+$ B cells and $F480^+$ macrophages before adoptive transfer. Though this method requires some pre-processing of the cells (e.g. tagging with phenotypic antibodies), it requires fewer incubation steps and involves gentler handling than the Miltenyi MACs system. An additional strategy that should be explored is usage of naïve pre-pubertal peritoneal cells from macrophage [38,39] or B cell deficient mice [40] ($Ighm^{tm1Cgn}$, Jackson Labs). The only major caveat to this method is the uncertainty as to whether more global changes to the immune system milieu have occurred in mice lacking entire cell groups. This may render the pre-pubertal cells for adoptive transfer abnormally responsive or resistant to endotoxin, thus making the model more complex and difficult to interpret.

Eventually identifying the pre-pubertal cell type(s) driving the protection from mortality in the adoptive transfer model will allow us to perform more targeted transcriptomic and *in vitro* studies to

piece apart and understand the associated mechanisms. The absolute need to narrow down the targeted cell type before moving forward was highlighted after multiple attempts at *in vitro* mechanistic studies using the entire cell mix. In particular, we assayed pre- and post-pubertal naïve peritoneal cells for differences in viability, cytokine production, oxidative burst intensity, and metabolic function. In the viability studies, naïve pre- and post-pubertal peritoneal cells were exposed to endotoxin *in vitro* and analyzed by flow cytometry for their changes in viability over time. Overall, post-pubertal cells exhibited greater percent viability than pre-pubertal cells (Supplemental Figure 2.13). The decreased viability among pre-pubertal cells *in vitro* was paired with increased levels of pro-inflammatory cytokine expression. In a single experiment with low sample size (N=4 individuals per age group), pre-pubertal cells exhibited significantly higher expression of IL-6 at 2 hours and higher IL-12 p40, IL-1B, and IL-5 at 6 hours after *in vitro* endotoxin exposure (Supplemental Figure 2.12). Though these findings may be due to the differential compositions of the peritoneal cell mixes in pre- and post-pubertal mice, they do match our *in vivo* cytokine analyses, in which pre-pubertal mice showed equal or increased expression of pro-inflammatory serum cytokines at early time points during endotoxemia. In order to determine whether phagocytic cells from pre-pubertal mice had differences in the intensity of their oxidative burst responses, we used the Fc Oxyburst® Assay. When comparing the accumulation of fluorescence over time (indicative of intra-vacuole production of ROS) there were no significant differences between pre- and post-pubertal animals (Supplemental Figure 2.14). Lastly, we explored the differences in metabolic functionality between peritoneal macrophages from each of the two age groups. To do this we used the Seahorse Flux Analyzer and the Glyco- and Mito- Stress Test Kits to assess changes in O_2 consumption and media acidification following real time treatment with varying concentrations of LPS. Overall, there were no significant differences in the responses of pre- and post-pubertal macrophages (Supplemental Figure 2.15). In sum, these *in vitro* analyses of naïve peritoneal cell function imparted little conclusive information. This may be due to our choice of assays, the noisiness of working with a mixed cell population, or that the differences between pre- and post-pubertal peritoneal cells can only be assessed accurately *in vivo*.

In Chapter 3, we identified putative drug candidates for the treatment of sepsis by data-mining publicly available whole blood transcriptomes from septic adults and children. In light of our success using the Pathprint-PDN pipeline for novel drug discovery, there are several steps that are worth pursuing to strengthen and increase the usefulness of the model. Firstly, it would be useful to explore the utility of these drug candidates in models of murine sepsis that more accurately describe the diverse population of sepsis patients. This should involve testing the drugs in aged mice of both genders, comparing the efficacy of both pre-and post-sepsis treatment protocols, and determining whether the drugs are also relevant in the treatment of a live bacterial infection. For example, the drug topotecan, a topoisomerase often used in the treatment of cancer, was a drug candidate identified via the Pathprint-PDN method that successfully improved survival from endotoxemia. However, topotecan is known to contribute to leukopenia and myelosuppression, which might be protective in a sterile endotoxemia model of sepsis, but could prove deadly in a live bacterial infection. By bringing organ system responses closer to those found in the typical adult with sepsis, we will be better able to assess the translational utility of the drugs identified. The utility of our endotoxemia model could also be improved by exploring the drug testing in other animal models that are more sensitive to LPS like the rabbit [34]. This may help to reduce the artifacts caused by studying endotoxin in a resistant species like mice.

It would also be worth pursuing some post-hoc studies using the Pathprint-PDN method to compare the particular pathways associated with each of the positively and negatively validated drugs. The overlap in pathways among the drugs that produced a positive survival effect could provide clues as to the most important mechanisms involved in the pre- vs. post-pubertal differences. In order to further assess the mechanism by which the positively-validated drugs are acting upon the mice, it would be useful to collect serum for cytokine analysis and monitor changes in blood leukocyte numbers composition.

Another potentially beneficial study would be to compare the pre- and post-pubertal transcriptomes from septic mice to those from septic humans. Though this has been shown to be problematic in other studies [41], a pathway-based analysis may help. It would be most useful to perform these studies using a more complex model of sepsis like fibrin clot peritonitis or CLP. We hypothesize that

if pre-pubertal resistance applies across multiple levels of Mammalia, the pathways differentiating pre- and post-pubertal organisms found in both mice and humans should be of special importance.

4.4. Final Conclusions

This thesis project made substantial progress in: 1) expanding the epidemiologic evidence for a 'honeymoon period' of relative resistance to mortality from severe infections in children; 2) establishing a mouse model of pre-pubertal resistance to endotoxemia, which allowed for experimental analysis of hormonal, cellular and molecular mechanisms; and 3) using meta-analysis of human transcriptome data to identify pathways showing differential expression by age and drug candidates that can reverse the higher susceptibility of adults to sepsis mortality. The results provide many opportunities to further pursue unanswered questions about the fascinating resilience of children in the face of severe infections.

4.5. References

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Supplementary Data

Supplemental Figures

Supplemental Tables

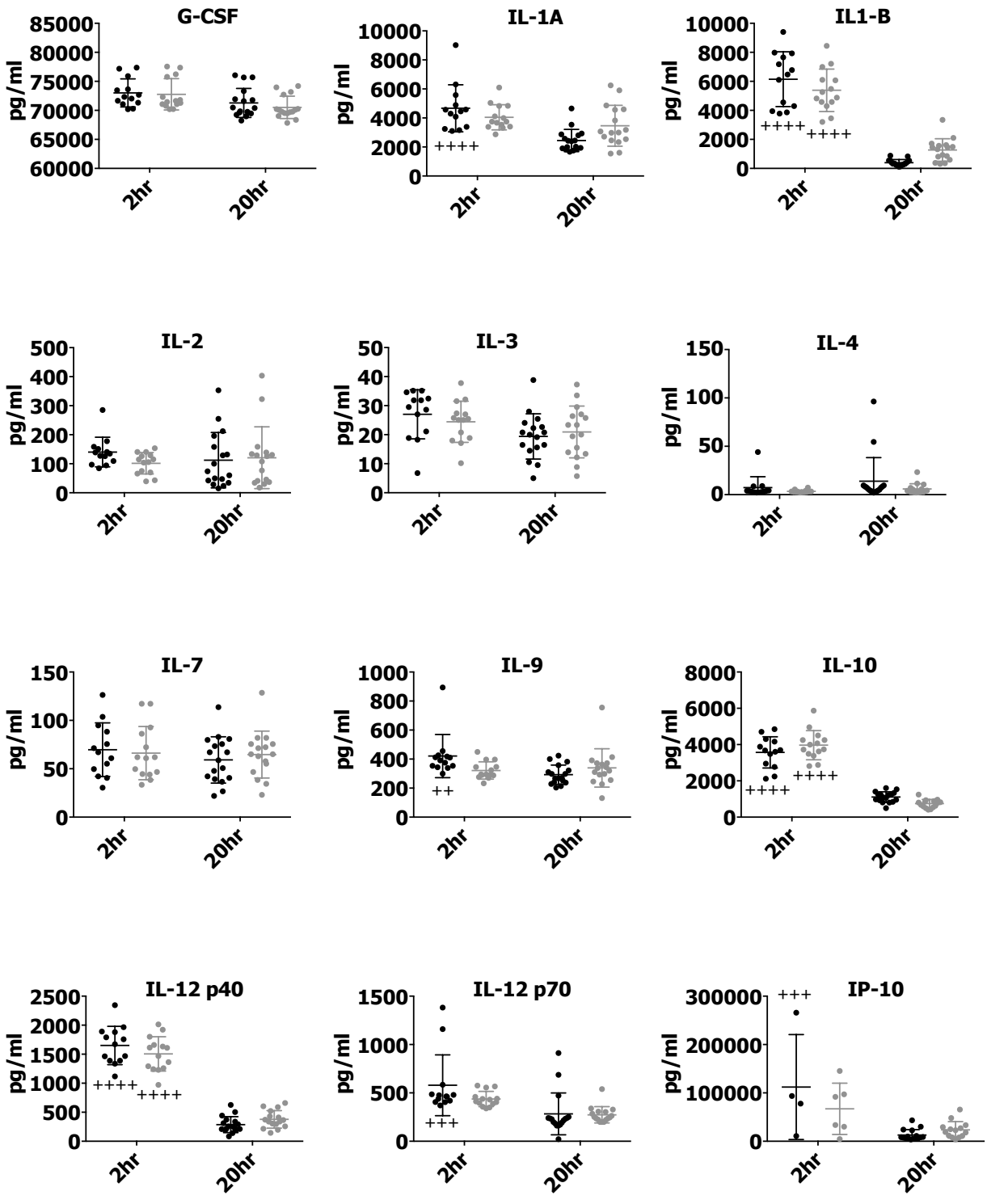
Supplemental Files

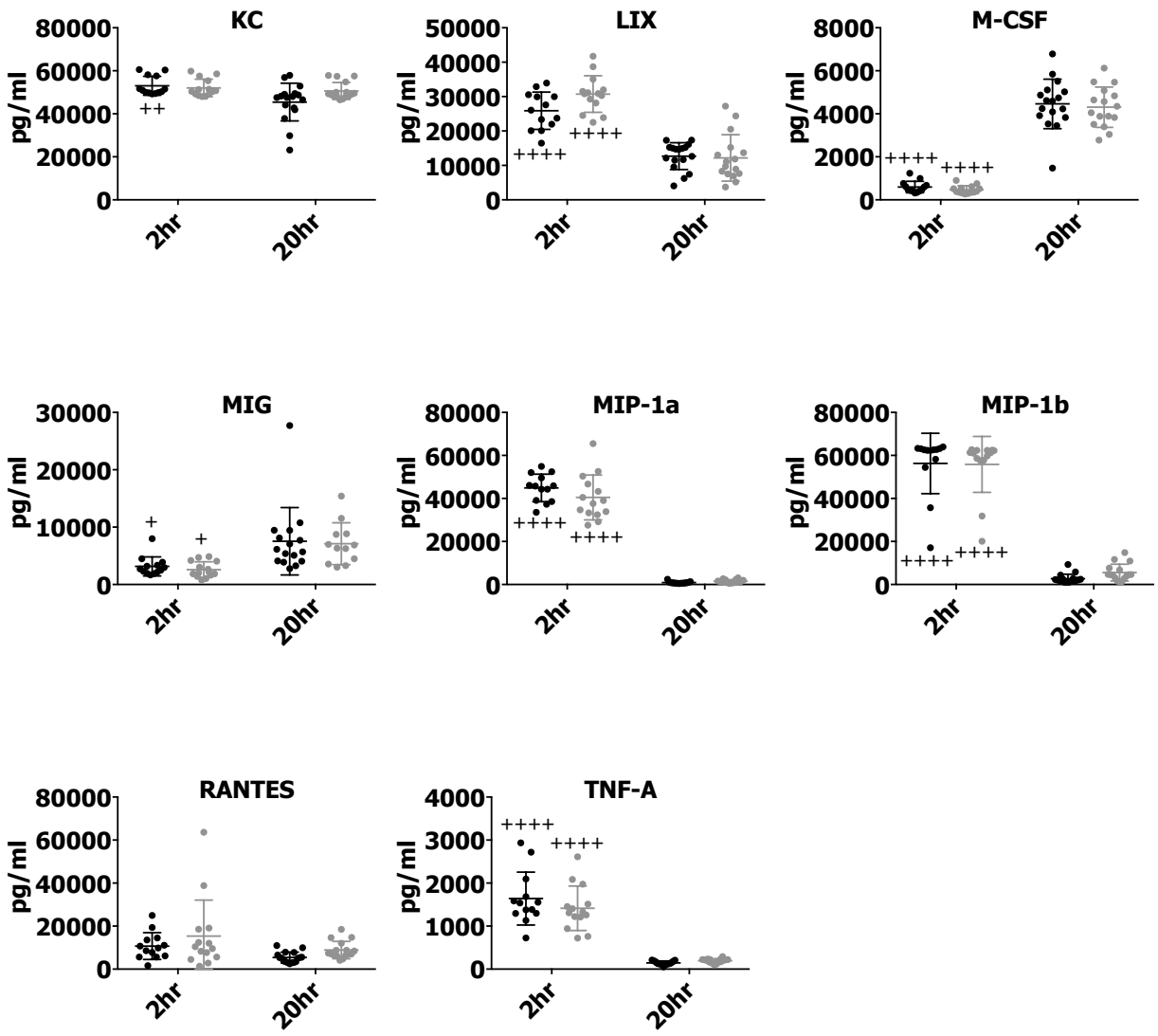
Supplemental File 1. All Pathprint results by counts
Supplemental File 2*. Top 50 pathways: Up in Adults, Down in Children
Supplemental File 3*. Top 50 pathways: Down in Adults, Up in Children
Supplemental File 4. DEGs from BarCode analysis
Supplemental File 5. Drug Curation References (all methods)

*For Supplemental Files 2 and 3, a combined list of all genes from the top 50 pathways in cluster A (Supplemental File 2) or cluster B (Supplemental File 3) was generated. After calculating the frequency of genes in these lists, sub-lists composed of genes appearing 4 or more times were then used to query Enrichr http://amp.pharm.mssm.edu/Enrichr/ or ToppFun https://toppgene.cchmc.org to identify significantly enriched GO Biological Processes. The top 25 GO Biological Processes identified are shown in the Supplemental Files.

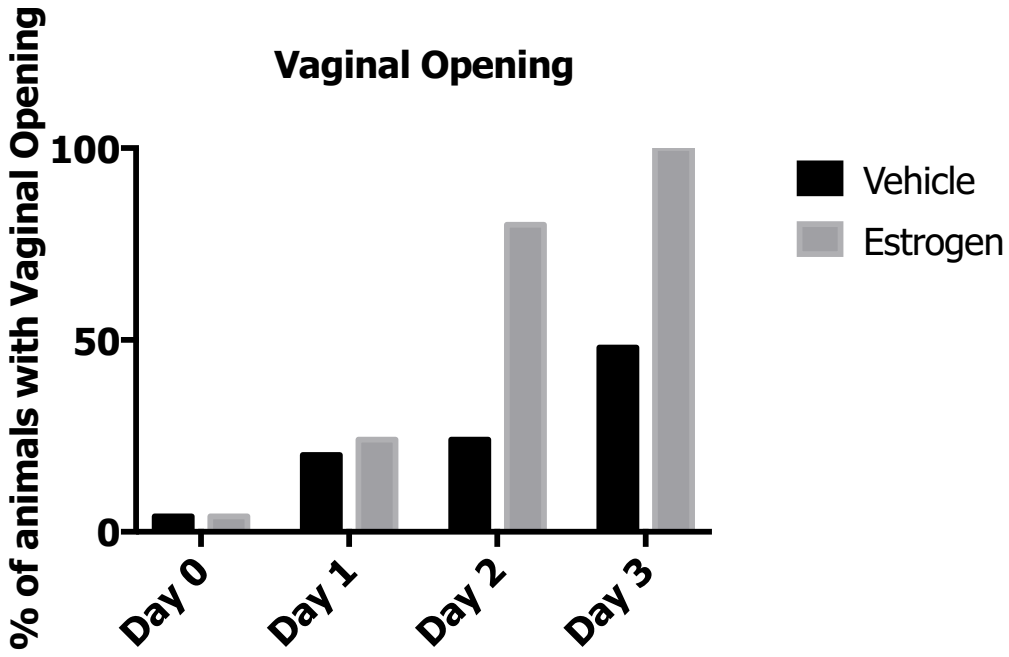
Supplemental Table 2.1. Antibodies used for flow cytometry experiments

Specificity	Fluorophore	Biolegend Product Number
CD11B	Alexafluor 647	101218
CD11B	APC	101212
CD19	APC	115512
CD3	APC	100236
F4/80	FITC	123107
Ly6C	FITC	128005
IgM	FITC	406505
CD8a	FITC	100706
CD117	PE	105807
Ly6G	PE	127607
IgD	PE	405705
CD4	PE	100408

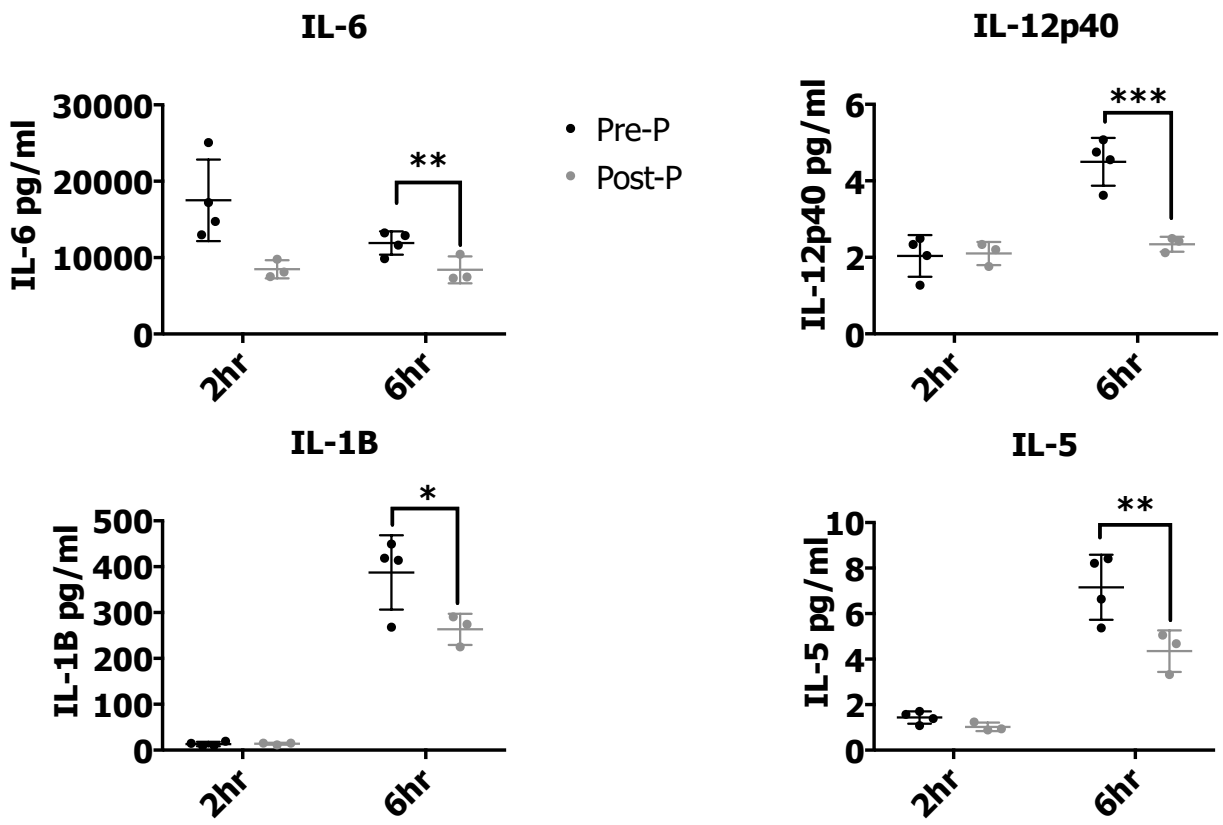

G-CSF
pg/ml
85000
80000
75000
70000
65000
60000
2hr
20hr
IL-1A
pg/ml
10000
8000
6000
4000
2000
0
++++
2hr
20hr
IL1-B
pg/ml
10000
8000
6000
4000
2000
0
++++
++++
2hr
20hr
IL-2
pg/ml
500
400
300
200
100
0
2hr
20hr
IL-3
pg/ml
50
40
30
20
10
0
2hr
20hr
IL-4
pg/ml
150
100
50
0
2hr
20hr
IL-7
pg/ml
150
100
50
0
2hr
20hr
IL-9
pg/ml
1000
800
600
400
200
0
++
2hr
20hr
IL-10
pg/ml
8000
6000
4000
2000
0
++++
++++
2hr
20hr
IL-12 p40
pg/ml
2500
2000
1500
1000
500
0
++++
++++
2hr
20hr
IL-12 p70
pg/ml
1500
1000
500
0
+++
2hr
20hr
IP-10
pg/ml
300000
200000
100000
0
+++
2hr
20hr



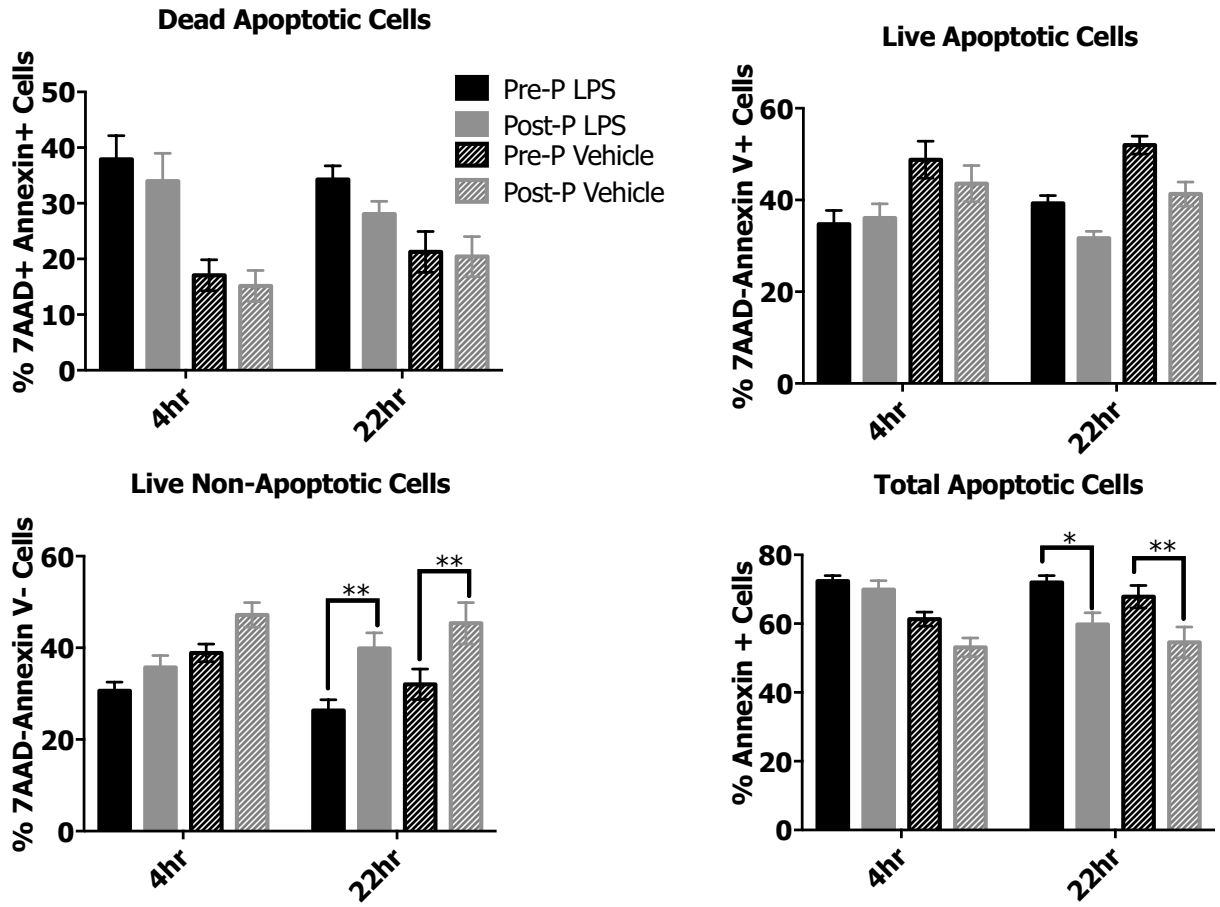
Supplemental Figure 2.10. Serum cytokine expression following endotoxemia
Serum samples from pre- and post-pubertal mice were subjected to a 32-plex cytokine assay (Eve Technologies). Pre-pubertal data are in black and post-pubertal data are in grey. Data points above or below the detectable limit were not included. Significant changes in concentration between 2 and 20 hours for either pre- or post-pubertal mice are labeled with ++++ ($p<0.0001$), +++ ($p<0.001$), ++ ($p<0.01$), or + ($p<0.05$). All comparisons were made using Two-way ANOVA followed by Tukey's test for multiple comparisons.



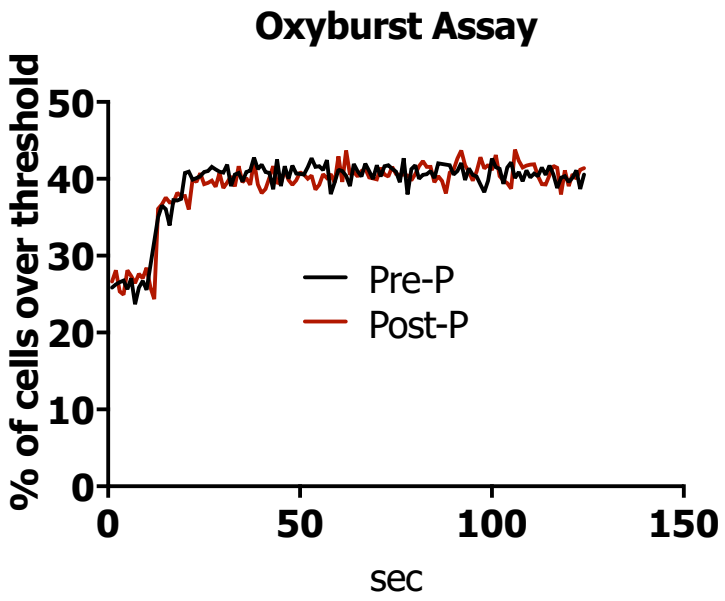
Supplemental Figure 2.11. Vaginal opening following treatment with estrogen or vehicle
Pre-pubertal mice were pre-treated with daily subcutaneous injections of 17β-Estradiol at 100µg/ml (or vehicle (0.4% DMSO) suspended in corn oil for three days prior and once on the day of *E.coli* endotoxin injection. Vaginal opening was examined daily and recorded. Estrogen-treated mice had 100%vaginal opening by day 3 of treatment. N=25 mice per group, over 2 experiments.



Supplemental Figure 2.12. In vitro cytokine production by LPS-stimulated peritoneal cells
Naïve Peritoneal cells were collected from pre- and post-pubertal mice, plated in 24 well tissue culture plates, and treated with *E. coli* LPS for 2, and 6 hours at 37°C. At each of these time points, the supernatants were collected, frozen at -20°C, and later subjected to multiplex cytokine analysis (Eve Technologies). N= 4 individuals per age group. Significant differences in cytokine expression between pre- and post-pubertal mice are labeled *** ($p<0.001$), ** ($p<0.01$), or *($p<0.05$). All comparisons were made using Two-way ANOVA followed by Tukey's test for multiple comparisons.

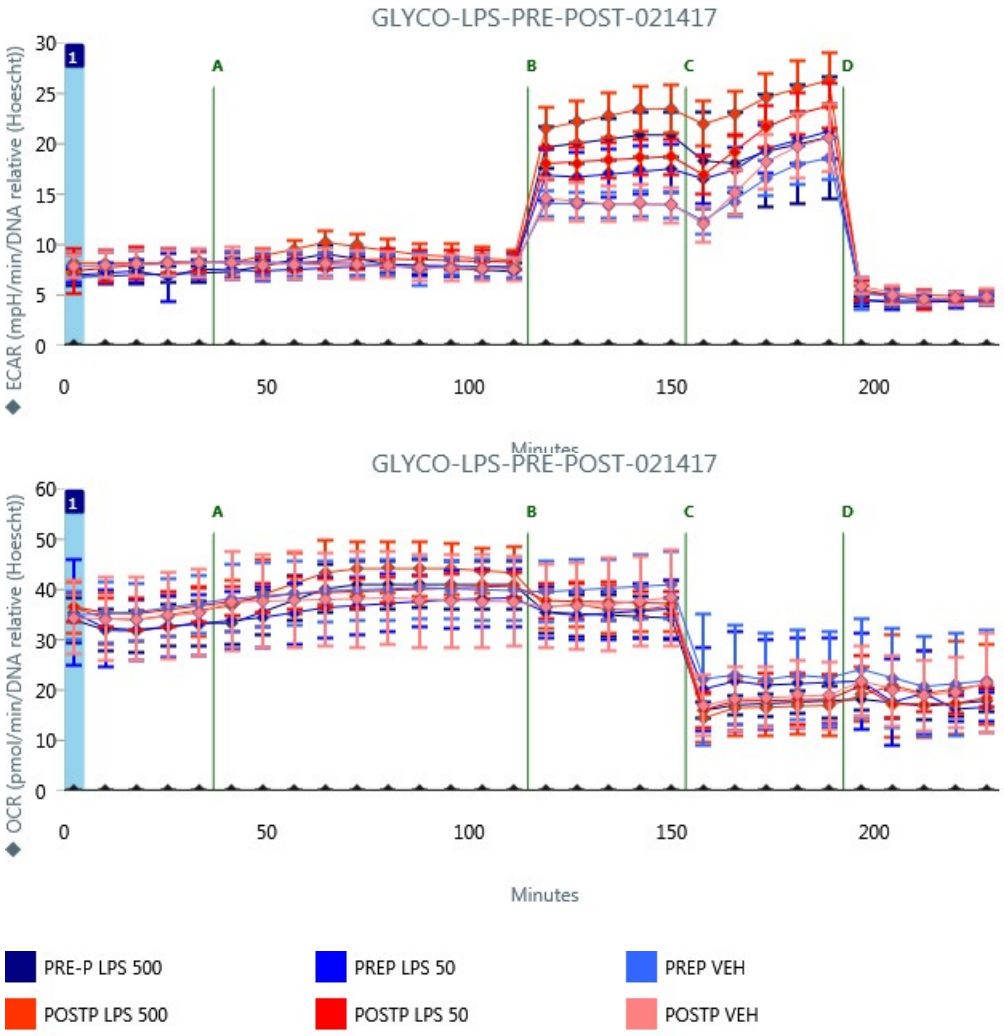


Supplemental Figure 2.13. Viability of peritoneal cells following *in vitro* endotoxin challenge Naïve peritoneal cells were collected from pre- and post-pubertal mice, centrifuged, and resuspended at 1.8 million cells per ml in assay media (phenol red-free RPMI; 10% charcoal-stripped heat inactivated fetal bovine serum). Cells were plated in 96-well plates with ultra-low attachment surface (Corning-3474) and incubated at 37°C 5% CO_2 for 4 and 22 hours with LPS (500ng/ml) or Vehicle (sterile water). At each time point, the plated cells were removed, washed, and resuspended in cell staining buffer. Resupended cells were first incubated for ten minutes with Tru-Stain fcX™ at 4°C, and then stained for cell death (7AAD, Biolegend-420403) and apoptosis (FITC-Annexin V, Biolegend-640945). Cell survival status was assessed by flow cytometry. Significant differences in viability between pre- and post-pubertal mice are labeled ** ($p<0.01$) or *($p<0.05$). All comparisons were made using Two-way ANOVA followed by Tukey's test for multiple comparisons.

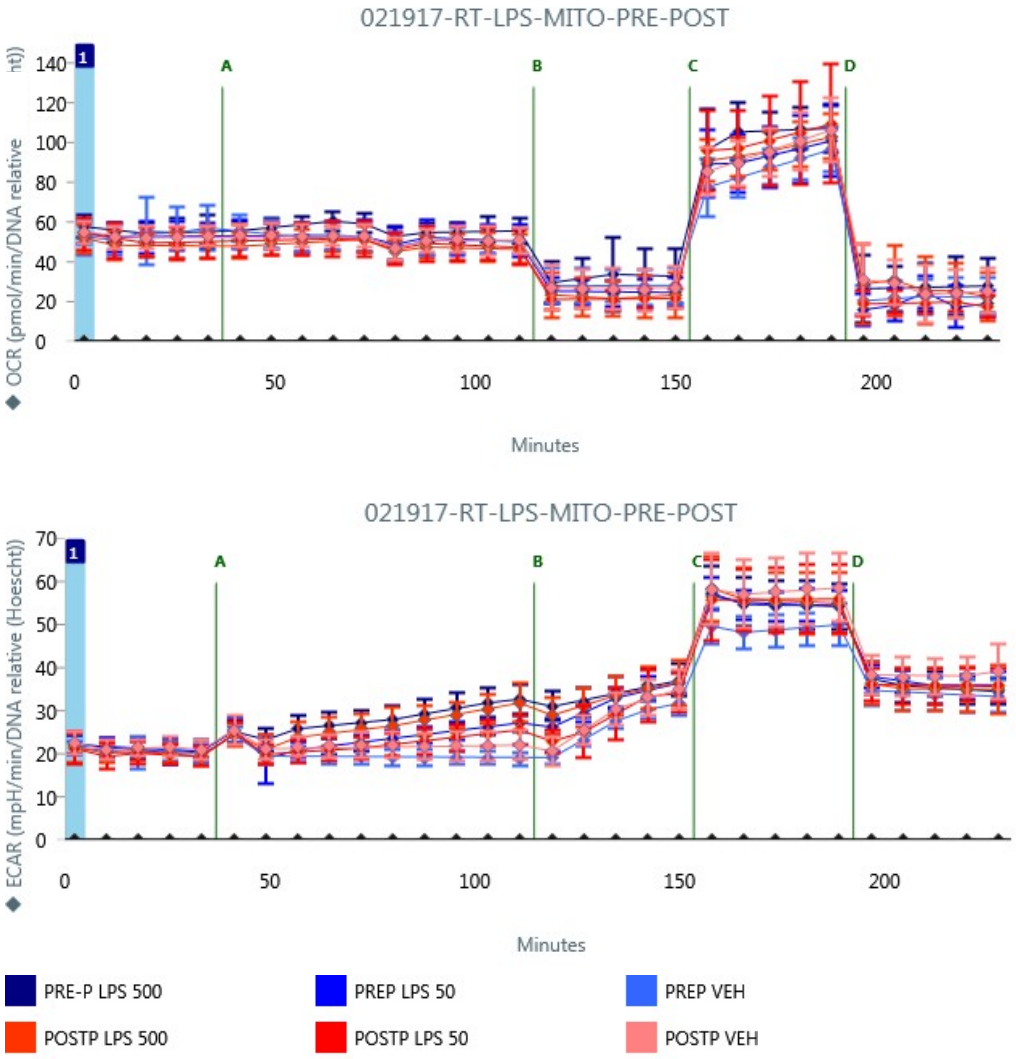


Supplemental Figure 2.14. Oxyburst Assay Results
The Fc Oxyburst® Reagent (Thermofisher, Molecular Probes, F2902) consists of bovine serum albumin (BSA) covalently linked to dichlorodihydrofluorescein (H_2DCF) and complexed with rabbit polyclonal anti-BSA IgG antibodies. When these immune complexes bind to the Fc receptors on phagocytic cells, the non-fluorescent H_2DCF molecules are phagocytized and oxidized to the green fluorescent dichlorofluorescein (DCF). This green fluorescence can be quantified by flow cytometry. Naïve pre- and post-pubertal peritoneal cells were collected by peritoneal lavage, centrifuged at 300G for 10 min at 4°C, resuspended in cell staining buffer and blocked with Tru-stain fcX^{TM}. Cells were then centrifuged and resuspended in sterile assay buffer (PBS pH 7.4, 1mM Ca^{2+}, 1.5mM Mg^{2+}, 5.5mM glucose) and treated with the Oxyburst® immune complex diluted in sterile PBS free of Ca^{2+} and Mg^{2+}. Immediately upon addition of the immune complex, the samples were analyzed for the development of green fluorescence over a period of 2 minutes. The kinetic data was analyzed using FlowJo® software. Cells over threshold were identified as those exhibiting fluorescence above that of the unstained cells.

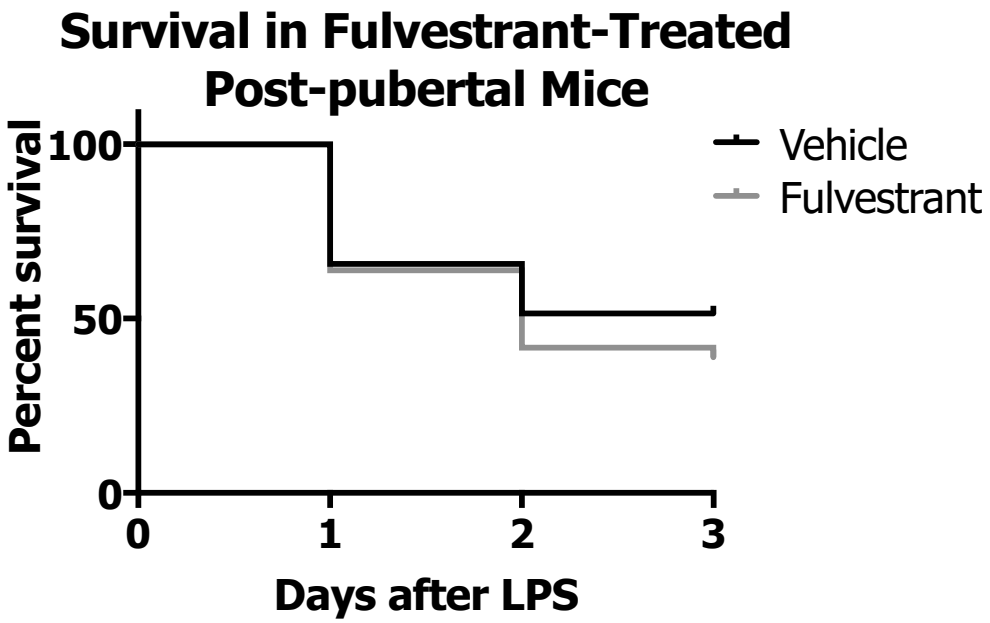
A


GLYCO-LPS-PRE-POST-021417
ECAR (mpH/min/DNA relative (Hoescht))
OCR (pmol/min/DNA relative (Hoescht))
A
B
C
D
Minutes
PRE-P LPS 500
PREP LPS 50
PREP VEH
POSTP LPS 500
POSTP LPS 50
POSTP VEH

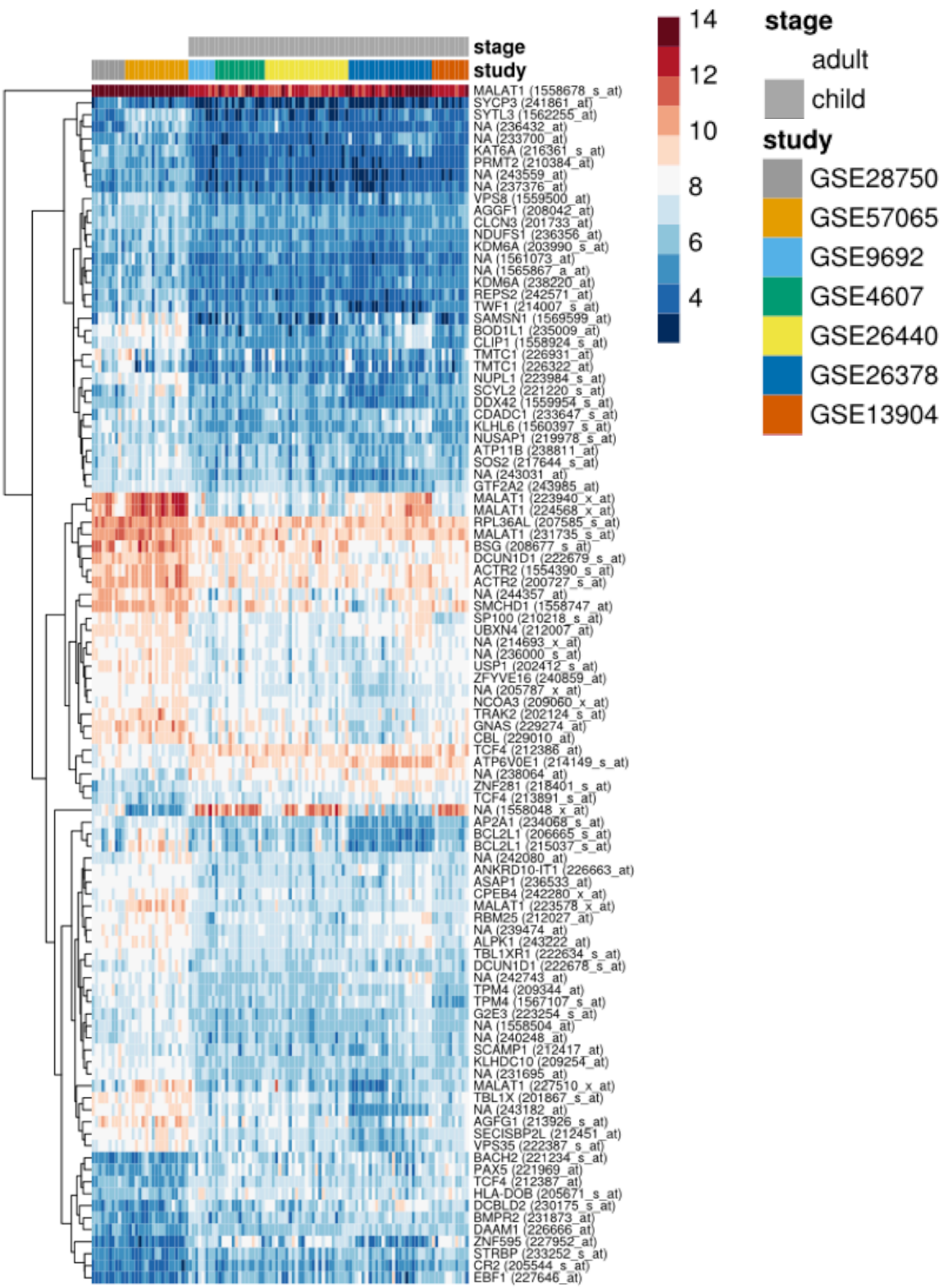
B



Supplemental Figure 2.15. Representative Seahorse Assay Results
Naïve peritoneal cells isolated from pre-pubertal and post-pubertal mice were resuspended at 2 million cells per ml in media (phenol red-free RPMI; 2mM Glutamine; 10% Charcoal-stripped heat inactivated fetal bovine serum), plated in the Seahorse XF^e 96 well plate (Agilent, 102416-100) and incubated for 30 minutes at 37°C in a non-CO_2 incubator. Following this incubation, all but adherent macrophages were removed from the plate by two washes with either Seahorse Glyco Stress Test Assay Media (XF Base Medium (Agilent, 102353-100), 2mM glutamine, pH 7.4) or Seahorse Mito Stress Test Assay Media (XF Base Medium (Agilent, 102353-100), 2mM glutamine, 1mM sodium pyruvate, 10mM glucose, pH 7.4). Following the two washes, cells were plated with 180µl of appropriate Seahorse Assay media and incubated for 30 minutes at 37°C in a non-CO_2 incubator. The pre-hydrated sensor cartridge (Agilent, 102416-100) was loaded with 500ng/ml LPS, 50ng/ml LPS or Vehicle (injection port A) and different combinations of drugs suspended in Seahorse assay media: A) Glyco Stress Test Kit: Glucose (injection port B), Oligomycin (injection port C), 2-DG (injection port D). B) Mito Stress Test Kit: Oligomycin (injection port B), FCCP (injection port C), rotenone/antimycin (injection port D). Data was collected using the Seahorse XF^e 96 analyzer and analyzed using Wave Desktop. Data were normalized for cell number by Hoechst staining for DNA.



Supplemental Figure 2.16. Effects of fulvestrant pre-treatment on endotoxemia survival
Post-pubertal mice were treated with daily subcutaneous 100µl injections of fulvestrant (Sigma; I4409) at 2mg/ml or vehicle (0.8% DMSO) in corn oil for three days prior and once on the day of endotoxin injection. N≥35 per age group. Percent survival was compared using a log rank Mantel Cox test.



Supplemental Figure 3.4. DEGs from standard analysis of U133 arrays
Representative heat map of DEGs from standard limma analysis of all adult and child U133 microarrays.

Supplemental Table 3.4. Pathprint-PDN drug candidate injection location and vehicle composition

* sc=subcutaneous; ip=intraperitoneal

Drug	Injection Location*	Dose	Vehicle Composition
vinpocetine	sc	10 mg/kg	2.5% DMSO, 2.5% cremophor in saline
topotecan	ip	5 mg/kg	saline
topiramate	ip	25-100 mg/kg	1.75-3.5% DMSO
noscapine	ip	25-100 mg/kg	1.25% DMSO, 1.25% cremophor
khellin	ip	10-25 mg/kg	2.5% DMSO, 2.5% cremophor
ganciclovir	ip	10 mg/kg	1% DMSO, 1% cremophor in saline
ethacrynic acid	ip	10-50 mg/kg	2.5% DMSO, 2.5% cremophor
chlorpromazine	ip	50 mg/kg	saline
altretamine	sc	50 mg/kg	10% DMSO
amitriptyline	ip	10 mg/kg	saline

Supplemental Table 3.5. Pathprint clusters A-D: Detailed
*U=unregulated

Cluster	Pathway	Pathprint Scores Summary (1=Up, 0=Unregulated, -1=Down)						P value	Filtering Criterion per Cluster: % of Group Up or Down *	
		-1 child	0 child	1 child	-1 adult	0 adult	1 adult		Children	Adults
A	IL-2 down reg. targets (Netpath)	91	2	2	0	5	162	1.87E-88	95.8	97.0
A	Shigellosis (KEGG)	90	3	2	0	7	160	2.04E-88	94.7	95.8
A	Endocytosis (KEGG)	83	7	5	0	1	166	1.95E-56	87.4	99.4
A	B Cell Receptor down reg. targets (Netpath)	92	1	2	0	26	141	1.69E-109	96.8	84.4
A	Signaling by NGF (Reactome)	82	10	3	0	8	159	9.11E-66	86.3	95.2
A	Pathogenic Escherichia coli infection (KEGG)	93	0	2	0	30	137	1.51E-113	97.9	82.0
A	Pentose Phosphate Pathway (Wikipathways)	83	4	8	0	2	165	1.80E-50	87.4	98.8
A	EGFR1 Signaling Pathway (Wikipathways)	77	15	3	0	1	166	6.46E-57	81.1	99.4
A	p38 MAPK Signaling Pathway (Wikipathways)	79	13	3	0	8	159	2.91E-62	83.2	95.2
A	{HCLS1,17} (Static Module)	93	0	2	30	1	136	1.28E-64	97.9	81.4
B	{MEIS1,22} (Static Module)	2	3	90	138	25	4	1.94E-97	94.7	82.6
B	Steroid Biosynthesis (Wikipathways)	2	8	85	151	14	2	3.90E-84	89.5	90.4
B	Steroid hormone biosynthesis (KEGG)	1	1	93	136	31	0	2.79E-128	97.9	81.4
B	Drug metabolism - cytochrome P450 (KEGG)	3	9	83	161	6	0	7.02E-66	87.4	96.4

Supplemental Table 3.5 (continued)

B	Metabolism of xenobiotics by cytochrome P450 (KEGG)	2	9	84	163	4	0	2.55E-70	88.4	97.6
B	{CTNNB1,130} (Static Module)	2	3	90	158	9	0	2.71E-91	94.7	94.6
C	{EP300,115} (Static Module)	94	1	0	15	140	12	4.30E-75	98.9	U
C	{HDAC1,108} (Static Module)	94	1	0	11	149	7	2.46E-91	98.9	U
C	Keap1-Nrf2 Pathway (Wikipathways)	85	10	0	20	139	8	5.94E-48	89.5	U
C	Kit Receptor up reg. targets (Netpath)	87	8	0	22	143	2	1.92E-52	91.6	U
C	Sulfur relay system (KEGG)	82	12	1	31	135	1	1.00E-29	86.3	U
C	TGF beta receptor up reg. targets (Netpath)	89	6	0	15	152	0	2.87E-67	93.7	U
C	Viral myocarditis (KEGG)	80	15	0	28	136	3	2.03E-32	84.2	U
D	{FLI1,10} (Static Module)	0	27	68	0	128	39	7.52E-15	71.6	U
D	Melanoma (KEGG)	0	22	73	3	141	23	8.98E-26	76.8	U
D	Serotonin Transporter Activity (Wikipathways)	1	25	69	1	128	38	1.73E-14	72.6	U
D	Statin Pathway (Wikipathways)	0	4	91	28	124	15	4.30E-64	95.8	U

Supplemental Table 3.6. Top 10 drugs used to validate Pathprint-PDN method *in vivo*

CMap Drug	Cluster	Cluster Definition	Goal	Category	Substitute Used	Rationale
Topiramate	A	Up in Adults, Down in Children	Reversal	Anticonvulsant		
Ganciclovir	B	Down in Adults Up in Children	Mimic	Antiviral		
Camptothecin	B	Down in Adults Up in Children	Mimic	Topoisomerase inhibitor	Topotecan	Water-soluble analog
Khellin	C	Unchanged in Adults, Down in Children	Reversal	Folk medicine from *Ammi visnaga*		
Piperacetazine	C	Unchanged in Adults, Down in Children	Reversal	Antipsychotic; acidic sphingo-myelinase inhibitor	Chlorpromazine	Same class (phenothia-zine); more commonly used in humans
Noscapine	D	Unchanged in Adults Up in Children	Mimic	Antitussive		
Etacrynic acid	D	Unchanged in Adults, Up in Children	Mimic	Diuretic; NFkB inhibitor		
Altretamine	D	Unchanged in Adults, Up in Children	Mimic	Alkylating agent		
Vincamine	D	Unchanged in Adults, Up in Children	Mimic	Vasodilator	Vinpocetine	Synthetic drug version of natural alkaloid from *Vinca minor*
Desipramine	A&C	see above	Reversal	Antidepressant; acidic sphingo-myelinase inhibitor	Amitriptyline	Same class (tricyclic); evidence for anti-inflamma-tory potential PMID: 25228885