An Investigation of Beta-adrenergic Signaling Pathways among Peripheral Immune Cells in a Murine Model of Chronic Social Stress

by

Catherine P. Walsh

BS in Biomedical Engineering, Johns Hopkins University, 2003

MS in Biotechnology, Johns Hopkins University, 2006

MA in Psychology, New York University, 2014

MS in Psychology, University of Pittsburgh, 2017

Submitted to the Graduate Faculty of the

Dietrich School of Arts and Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2022

UNIVERSITY OF PITTSBURGH

DIETRICH SCHOOL OF ARTS AND SCIENCES

This dissertation was presented

by

Catherine P. Walsh, MS

It was defended on

November 2, 2022

and approved by

Stephen B. Manuck, PhD, Distinguished Professor, Department of Psychology

Peter J. Gianaros, PhD, Professor, Department of Psychology

Dana H. Bovbjerg, PhD, Professor, Department of Psychiatry

Alan F. Sved, PhD, Professor, Department of Neuroscience

Committee Co-Chair: Anna L. Marsland, PhD, RN, Professor, Department of Psychology

Committee Co-Chair: John Sheridan, PhD, Professor, Division of Biosciences,
The Ohio State University

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Chronic social stress associates with an increase in peripheral pro-inflammatory markers and primed myeloid cells that can contribute to inflammatory health risk. Physiological pathways linking social stress to peripheral proinflammatory phenotypes remain unclear. Converging evidence shows chronic activation of the hypothalamic-pituitary(HPA)-axis in response to stress results in cellular resistance to anti-inflammatory effects of glucocorticoids. However, this does not fully account for stress-related proinflammatory phenotypes. Thus, it is possible that stress-induced activation of the sympathetic-adrenal-medullary(SAM)-axis may contribute. In this study, a well-established murine model of chronic social stress, repeated social defeat(RSD), was employed to examine whether stress induces changes in β-adrenergic receptor (β-AR) expression and related intracellular signaling within CD11b+ peripheral monocytes and granulocytes, which may contribute to proinflammatory phenotypes. Specifically, the possibility that stress induces a downregulation in β-AR surface expression and a shift from canonical β-AR-regulated intracellular signaling via CREB, to non-canonical signaling via MAPK was examined. As hypothesized, RSD associated with downregulation in β-AR surface expression and upregulation in pro-inflammatory gene transcription in CD11b+ cells isolated from the spleen and peripheral blood. There was also evidence for a "switch" in intracellular signaling, including a downregulation in β-AR canonical signaling and an upregulation in non-canonical β-AR signaling. The results were less clear when examining the cellular response to *ex vivo* isoproterenol

stimulation, although initial evidence suggested stimulation with supraphysiologic levels of a β-adrenergic agonist may attenuate pro-inflammatory gene transcription via promotion of β-AR canonical signaling and attenuation in β-AR non-canonical signaling. Together, these results suggest a desensitization of peripheral monocytes and granulocytes from RSD mice to physiologic levels of adrenergic signaling, which may be adaptive in response to prolonged or repeated SAM-activation during chronic social stress. A molecular "switch" in β-adrenergic intracellular signaling may also preserve energy resources within the cell. However, the resultant promotion of pro-inflammatory gene transcription may pre-dispose the organism to inflammatory diseases of aging. Greater knowledge of cellular responses to chronic stress promotes understanding of proinflammatory phenotypes that associate with health risk and aids identification of novel targets for pre-emptive interventions that decrease risk for diseases with inflammatory pathophysiology known to accompany chronic social stress.

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Acknowledgments

I'm not even sure where to begin in thanking the numerous people who have supported me and believed in me throughout my graduate school journey, not only for this project alone. Thank you to Anna Marsland, for teaching me how to be a more independent researcher and to continually challenge myself to grow as a person, a researcher, and a communicator. Thank you to John Sheridan, for believing in my ideas and giving me the opportunity to come learn and work in your lab, testing out the ideas for this project. Thank you to Steve Manuck, for teaching me how to be a better writer, communicator, and researcher. It has been a true pleasure to have you as my secondary mentor. Thank you to Dana Bovbjerg for encouraging me to pursue this project in the first place and for always pushing the envelope. And to Pete Gianaros for being an introspective thinker, listener, and supporter; for always providing an encouraging word or a new way of viewing a problem. Thank you also to Alan Sved for your ideas and teaching. My sincere thanks also go to Denise Bellinger and Dianne Lorton, who met with John Sheridan and I over Zoom in a pandemic to discuss their ideas, which are the basis for this project; and to Dan McKim for similarly discussing the project and sharing his ideas.

This project would not have been possible without the help of those in the Sheridan lab – especially Natalie Gallagher and Ren Yin. Natalie was the laboratory mastermind behind the execution of all of the animal work and keeping everything (and everyone) together. On experiment days, she was stressing animals early in the morning and we were isolating RNA until late at night. She constantly challenges all who encounter her and she raises others up with her while doing so. She is an amazing scientist and I hope she sticks around to keep everyone in the Sheridan lab together; both personally and professionally! Ren Yin was the mastermind behind

the genomics analysis. While the sequencing alignment was performed by the OSU genomics core, Ren sat with me on many occasions over coffee, drinks, or while multitasking in the lab to teach me how to analyze the genomics data. I am grateful that she constantly challenged me to think in new ways and to grow as a scientist and thinker. Thank you also to Becca Biltz for sitting with me through R programming problems. I think I would still be trying to make volcano plots not gray. To all the friends I have made in the Sheridan and Godbout labs, I am truly grateful. Thank you to Ethan, Sam, Linde, Shane, Chelsea, and Damon for your ideas, connections, reagents, and support. Also Amara 2, because she came second ;P.

To my friends at Pitt who have supported me in my graduate school journey – I would not have made it without you. Christie Musket – I literally do not know how you always manage to be there for me when I need you most, but you always are. I am so grateful for your friendship. To my comps work buddies and friends Marissa Bowman and Will Woods, you guys are the best and you make me smile and laugh. To Kimberly Lockwood, who basically showed me the ropes of graduate school and OEH for the first five years of my graduate student journey. Thank you for being my mentor, my buddy, and my friend. To Brianna Natale, I'm sad we did not overlap long, but I'm so glad we have had the opportunity. Thank you for your endless encouragement. You've got this. And to the Meta-monkeys – you guys renew my spirit every time we talk. Also thank you to Emily Lindsay – boy we've been through a lot together. You are always an inspiration; thank you for being a mentor, a collaborator, and a friend.

Finally, thank you to my family – my husband Scott and daughter Amara. Thank you for walking (running, chasing), and supporting me in this journey, and for cooking delectable food and pastries along the way. I would never have embarked on this journey without encouragement and support from my partner for life, and I might not have made it to the finish line without him.

Thank you also to his parents, who are always there for us, and to my dad and stepmom, who hosted me in Ohio to do the work on this project, who were part of our pandemic bubble, and who continue to support me along my personal and professional journey.

List of Abbreviations

α	alpha
AC	adenylate cyclase
ACTH	adrenocorticotropic hormone
Antigen	any foreign substance that can induce an immune response in the body
AP-1	activator protein-1
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
β	beta
β-AR	beta-adrenergic receptor
β1-AR	beta1-adrenergic receptor
β2-AR	beta2-adrenergic receptor
β-arrestin	beta-arrestin, an intracellular scaffolding molecule for the GPCR
CBP	CREB-binding protein
CD14	cell surface marker for monocytes in humans
CD16	cell surface marker of early myeloid lineage in humans (low levels)
CD11b	cell surface marker for monocytes in mice
CREB	cAMP response-element binding protein
CRH	corticotropin hormone
ERK1/2	extracellular signal-regulated kinases ½
HPA	hypothalamic pituitary axis
GC	glucocorticoid
GPCR	G-protein coupled receptor
GR	glucocorticoid receptor
GRK	G-protein coupled receptor kinase
Ly6c	cell surface marker of early myeloid lineage in mice

MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
NFκB	nuclear factor kappa-light-chain-enhancer
Pathogen	a micro-organism that can cause disease
PKA	protein kinase A
PVN	paraventricular nucleus
RSD	repeated social defeat
SAM	sympathetic-adrenal-medullary
SNS	sympathetic nervous system

1.0 Background and Significance

1.1 Chronic stress engenders risk for diseases with inflammatory pathophysiology

Chronic social stress associates with risks for a number of diseases with inflammatory pathophysiology (Cohen et al., 2007; Tosevski & Milovancevic, 2006), including cardiovascular disease, cancer, asthma, and rheumatoid arthritis (Cutolo & Straub, 2006; Kozyrskyj et al., 2008; Leserman et al., 2002; Steptoe & Kivimaki, 2013). Indeed, chronic stress increases risk of CVD by 40-60% (Ford & Capewel1, 2011; Steptoe & Kivimaki, 2012, 2103), a magnitude similar to the impact of lipids and smoking (Yusuf et al., 2004). Psychological stressors have been defined by Lazarus & Folkman (1984) as environmental demands that pose a threat to self that exceed the perceived ability to cope (Lazarus, 1966; Lazarus & Folkman, 1984). Drawing on this definition, stressors that are considered chronic tend to be environmental demands and/or stressful life events that remain in the environment for a prolonged period with uncertainty about when or if they will end. Demands that present a threat to social roles and/or the integrity of social relationships, such as caregiving, social isolation/loneliness, economic strain, and bereavement are particularly provocative chronic stressors (Cohen, Gianaros, & Manuck, 2016; Cohen et al., 2007; Baumeister & Leary, 1995; Kennedy, Kiecolt-Glaser, & Glaser, 1988; Kiecolt-Glaser and Glaser, 1989). Individuals experiencing these chronic social stressors are at increased risk for inflammatory diseases and related morbidity (Eng, Rimm, Fitzmaurice, & Kawachi, 2002; Nausheen, Gidron, Peveler, & Moss-Morris, 2009; Buckley, McKinley, Tofler, & Bartrop, 2010; Kaprio, Koskenvou, & Rita, 1987; Mortensen et al., 2017; Shaffer, Kim, Carver, Cannady, & Author, 2017; de Mestral 2017 & Stringhini, 2017; Singh, & Jemal, 2017; Vilen, Baldassari, & Callahan, 2017). Although

the pathways that link chronic social stress to inflammatory disease risk remain unclear and are likely complex, growing evidence suggests immune processes may play a role.

1.2 Systemic inflammation as a plausible pathway

The immune system is a complex network of cells and soluble mediators that protect the body from foreign matter (antigens). For descriptive purposes, immune processes are classified as innate or adaptive. Innate immunity includes nonspecific responses to any compound not recognized as part of one's own body. Innate immune responses are functional at birth and are activated within minutes or hours in response to antigen. Adaptive immunity is slower (days) and provides a more targeted response against specific antigens.

The innate immune system includes physical barriers (e.g., skin, mucus, tears and saliva) and cells that recognize common foreign pathogens (e.g., natural killer ([NK] cells and granulocytes, monocytes, dendritic cells, and macrophages; Janeway et al., 2017). NK cells recognize and destroy altered self-cells (e.g., tumor or virally-infected cells), and granulocytes are phagocytic cells that engulf and destroy foreign material present in extracellular fluids. Macrophages are an important subset of monocytic cells that have migrated into tissues. When activated in response to antigen, monocytes help coordinate inflammatory responses by producing soluble factors called cytokines in peripheral circulation. Pro-inflammatory cytokines attract immune cells to infection sites and systemically activate an acute phase response to contain pathogens (Janeway et al., 2017). The acute phase response includes the production and release of proteins by the liver to prevent the spread of infection. For example, C-reactive protein (CRP) is a liver-derived acute phase protein that coats and marks bacteria for destruction (Janeway et al.,

2017). The cytokines released by activated monocytes that initiate the acute phase response include tumor necrosis factor alpha (TNF-α), interleukin (IL)-1, and IL-6. Of these cytokines, IL-6 is most readily detected in serum and is primarily responsible for the initiation of the acute phase response (Gruys, Toussaint, Niewold, & Koopmans, 2005; Naugler & Karin, 2008). In sum, activation and signaling by the innate immune system, especially monocytes and signaling by pro-inflammatory cytokines, is an important response that protects the individual from infectious pathogens.

1.2.1 Pro-inflammatory cytokines

Increasing evidence suggests that some individuals may have chronically elevated levels of inflammatory cytokines in peripheral circulation, which are independent of antigen exposure or injury (Rohleder, 2014). These pro-inflammatory cytokines (e.g., IL-6) and acute phase proteins (e.g., CRP), measured in peripheral blood among otherwise healthy individuals, predict risk for incident inflammatory disease. For example, circulating levels of IL-6 and CRP in healthy individuals prospectively predict development of cardiovascular disease and cancer (Dibaba et al., 2019; Guo 2013; Emerging Risk Factors Collaboration, 2010; Kaptoge et al., 2014), and anti-inflammatory treatment can reduce risk (Nguyen et al, 2019; Wong et al, 2020).

Accumulating evidence supports a positive association between chronic social stress and circulating levels of inflammatory mediators, possibly contributing to stress-related risk for chronic inflammatory disease. For example, meta-analytic evidence shows a small but significant association between chronic social isolation and higher levels of inflammatory markers (e.g., IL-6, CRP; Uchino et al., 2018), and a recent systematic review found that bereaved individuals generally demonstrate higher levels of systemic inflammation compared to non-bereaved controls (Knowles, Ruiz, & O'Connor, 2019). Significant research also exists on the long-term stress of

caregiving, with caregivers demonstrating elevated markers of inflammation both cross-sectionally and longitudinally compared to non-caregiving controls (Lovell & Wetherell, 2011; Potier, Degryse, & Saint-Hubert, 2017; Roth et al., 2019). Thus, across a number of chronic social stressors, studies demonstrate higher levels of peripheral inflammation among those exposed to the stress than among their non-stressed counterparts.

The origin of these circulating markers of inflammatory mediators is likely multi-determined, with many cell subtypes contributing to peripheral levels. For example, sources of IL-6 may include circulating immune cells, contracting muscle cells (myocytes), adipocytes, and epithelial cells that line blood vessels (Fantuzzi, 2005; Hansson, 2005; Steensberg et al., 2002). Many factors also influence cytokine production and release by these cells. For example, activation of neuroendocrine pathways that result in the peripheral release of glucocorticoids and catecholamines can modulate cytokine production by all these cellular sources (Blalock, 1989; Elenkov, Wilder, Chrousos, & Vizi, 2000). Evidence from animal models suggests that activation of neuroendocrine pathways in response to stress can also modify the activation state of immune cells in peripheral circulation (Bailey et al., 2007; Hanke et al., 2012). The number of different subtypes and activation state of immune cells present in peripheral circulation can also influence levels of circulating markers of inflammatory mediators (Fantuzzi, 2005; Khaodiar, Ling, Blackburn, & Bistrian, 2004). Thus, an understanding of the mechanisms that contribute to peripheral pro-inflammatory states is key to identifying modifiable pathways of risk for chronic inflammatory disease.

1.2.2 Number and subtype of circulating immune cells

Evidence suggests that immune cells migrate between lymphoid organs, the margins of blood vessels, and lymphatic and peripheral circulation (Dhabhar et al., 1995, 1997). In the context of social stress, endocrine signaling may play a role in coordinating this movement, specifically by recruiting innate immune cells to peripheral circulation (Blalock, 1989; Dhabhar, Miller, McEwen, & Spencer, 1995; Dhabhar & McEwen, 1997; Elenkov et al., 2000). In addition to their contribution to levels of peripheral pro-inflammatory mediators, epidemiological and clinical studies demonstrate that elevated circulating numbers of leukocytes can independently predict risk for diseases with inflammatory pathophysiology, such as cardiovascular disease (Majidid et al., 2004; Nahrendorf, 2018). Here, growing evidence suggests that the accumulation of a particularly pro-inflammatory monocyte is associated with increased risk of CVD, hematologic cancers, and all-cause mortality (Majidid et al., 2004; Nahrendorf, 2018). These pro-inflammatory monocytes express the human monocyte indicator CD14 [CD14+], and low levels of the cell surface marker CD16 [$CD16^{lo}$], a unique indicator of early myeloid lineage (Yang, Zhang, Yu, Yang, & Wang, 2014). Thus, while there are normal variations of immune cell subtypes in circulation, accumulating evidence suggests that stably elevated numbers of peripheral leukocytes associate with risk for diseases with inflammatory pathophysiology, with a particular focus on the accumulation of $CD14+CD16^{lo}$ monocytes.

An early meta-analytic review suggests that the relationship between chronic social stress and alterations in peripheral cell subtype composition is inconsistent (Segerstrom & Miller, 2004). While a number of more recent studies similarly present mixed findings (e.g., Bauer et al., 2000; Miller et al., 2008, 2014; Mills et al., 1997; Cole et al., 2007, 2011; Gerra et al., 2003; O'Connor et al., 2014), some studies have focused specifically on increases in phagocytic cells, such as

monocytes and granulocytes. For example, at least one study reports that circulating neutrophil percentages were elevated among socially isolated individuals compared to those not socially isolated (Cole et al., 2008). Additionally, at least one study reports evidence that the chronic stress of caregiving may promote an increase in the number of monocytes in peripheral circulation (Walsh et al., 2018). Other studies use gene expression data to approximate relative prevalence of leukocyte subset mRNA (i.e., mRNA for specific cell surface markers) within the total mRNA pool from peripheral leukocytes. Using this measurement method, an additional study among lonely individuals reports an elevation in percent of peripheral monocytes compared to non-lonely individuals (i.e., CD14+ cells; Cole et al., 2015). Among studies of economic strain, at least one study demonstrates an increase in number of peripheral CD14+CD16- monocytes, as assessed by both bioinformatic analysis and peripheral blood count (Powell et al., 2013). Thus, while not all studies are consistent, there is at least initial support for an association between chronic social stress and an upregulation in monocyte number, with one study demonstrating elevations in the CD14+CD16^{lo} subtype specifically.

1.2.3 Pro-inflammatory gene transcription

Variability in cellular activation of pro-inflammatory gene transcription associates with variation in adult lifespan (Christensen, Johnson, & Vaupel, 2006; Dato et al., 2017), presumably through increased susceptibility to diseases of aging. Signaling at the immune cell surface (e.g., by endocrine factors) initiates intracellular secondary signaling cascades that also result in individual variation in pro-inflammatory gene transcription (Elenkov, 2000; Bellinger & Lorton, 2018; Schoneveld & Cidlowki, 2007). In the context of chronic stress, genetic variation in inflammatory gene transcription may interact with stress exposure to further contribute to

individual variation in pro-inflammatory gene transcription (Dato et al., 2017). These alterations in transcription patterns may then promote the release of inflammatory proteins from immune cells (Janeway et al., 2017). Thus, while exact pathways are still being revealed, individual variation in pro-inflammatory gene transcription may also contribute to inflammatory disease risk and overall mortality.

Consistent evidence exists regarding the association between exposure to chronic social stress and upregulation in pro-inflammatory gene transcription among peripheral immune cells. To investigate these associations, researchers have examined molecular and genetic mechanisms that may contribute to alterations in inflammatory gene transcription by (a) quantifying amounts of messenger ribonucleic acid (mRNA, the genetic transcript that codes for protein) and (b) quantifying the activation or inhibition of promotors that stimulate transcription of mRNA (Gibson, 2003). These elements can be assessed individually or as common transcriptional control pathways for gene expression (e.g., using Transcription Element Listening System [TELiS]; Cole et al., 2005). Using TELiS, at least two studies report an upregulation in pro-inflammatory gene transcription that is specific to peripheral monocytes among caregivers compared to non-caregiving controls (Miller et al., 2008; 2014). Evidence also exists for elevated expression of pro-inflammatory genes among peripheral leukocytes from those high vs. low economic strain (Powell et al., 2013), as well as from bereaved individuals compared to non-bereaved individuals (O'Connor et al., 2014). A number of studies also find an upregulation in pro-inflammatory gene transcription within peripheral leukocytes among individuals who are socially isolated compared to individuals who are not socially isolated (Cole et al., 2007, 2011, 2015), with one study providing support for upregulation in pro-inflammatory programming that is specific to peripheral monocytes (Cole et al., 2015). Findings from these studies suggest that there is growing evidence

for a per-cell upregulation in pro-inflammatory gene transcription, in particular among monocytes, that may contribute to elevated markers of inflammation observed among these chronically stressed populations.

In sum, a number of immune mechanisms likely contribute to the concentration of pro-inflammatory cytokines in peripheral circulation, including the absolute number or concentration of immune cells in the periphery, the subtype and activation state of these cells, and activation of gene transcription processes within cells that regulate cytokine production. Individually, each of these markers may have some predictive validity for diseases with inflammatory pathophysiology; together, they may provide a more robust understanding of pro-inflammatory processes.

1.2.4 Animal Models of Chronic Stress

In parallel with human literature, there is also a large body of animal literature on the association between chronic stress and physiological markers of stress, including markers of inflammation. This literature draws on a number of different animal models, including chronic variable stress, repeated immobilization stress, and chronic social defeat stress. While both chronic variable stress and repeated-restraint stress paradigms associate with upregulation in pro-inflammatory states (e.g., Voorhees et al., 2013; Lu, Xu, et al., 2019; Lu, Ho, et al., 2017), the ecological validity of these paradigms is questionable. One difference between these paradigms and chronic social defeat is that the natural biological rhythms of the animals tend to be disrupted (i.e., natural circadian rhythm, especially with regard to stress hormones), whereas in chronic social defeat these rhythms are preserved. Another difference is that it is more difficult to translate the paradigms of chronic variable stress and repeated immobilization stress to human models of

chronic social stress. In contrast, animal models of chronic social defeat involve a key social component that has garnered attention as a more naturalistic model for chronic human social stress.

1.3 Repeated Social Defeat murine model of Chronic Stress

Animal models of social conflict show ethological relevance and capture some of the key complexities of stress in humans (Huhman, 2006; Golden et al., 2011). In particular, the model of repeated social defeat (RSD) developed by John Sheridan and colleagues involves repeated disruption of an established and stable social hierarchy of three to five mice via introduction of an unfamiliar aggressor mouse. The resident mice exhibit physiological and behavioral stress responses to the aggressor mouse, whose presence disrupts previously established social hierarchy within the cage (Avitsur, Stark, & Sheridan, 2001). More specifically, after undergoing social disruption for 6 days, mice demonstrate behavioral alterations that are similar to chronic social stress responses in humans, such as increased anxiety-like behaviors, self-imposed social isolation, and decreased grooming behavior (i.e., self-care in humans; Wohleb et al. 2011, Wohleb, Powell, Godbout, & Sheridan, 2013). Anxiety-like behaviors can be observed for at least a week after the end of the formal exposure period of RSD, and these behaviors can be re-elicited after single exposure to an aggressor mouse up to 1 month after the initial stress period (Wohleb et al., 2014). Mice subject to RSD also demonstrate physiological consequences that parallel those observed in humans, including the promotion of pro-inflammatory phenotypes. They also display normal circadian variation in peripheral levels of stress hormones such as glucocorticoids and catecholamines (Avitsur et al., 2001; Engler et al., 2005, 2008; Hanke et al., 2012; Merlot et al., 2004; Stark et al., 2001). Thus, as an animal model of chronic social stress, RSD captures some of

the crucial behavioral components of stress in humans, and offers an opportunity to investigate physiological mechanisms.

1.3.1 RSD associates with a pro-inflammatory phenotype

Consistent evidence demonstrates that mice exposed to RSD show elevated levels of pro-inflammatory markers when compared to controls. For example, levels of IL-6, TNF-α and IL-1β are consistently elevated in peripheral blood (Engler et al., 2008; Hanke, Powell, Stiner, Bailey, & Sheridan, 2012; Niraula, Wang, et al., 2018; Niraula, Witcher, et al., 2018; Stark, Avitsur, Hunzeker, Padgett, & Sheridan, 2002; Engler et al., 2008; Hanke et al., 2012). Interestingly, one study that measured levels of IL-6 in other tissue compartments found that IL-6 was significantly elevated in the livers of RSD mice compared to controls (Stark et al., 2002); this finding was consistent with activation of systemic pro-inflammatory signaling pathways such as the acute phase response. However, elevation in an acute phase protein among RSD mice has never been examined. In this regard, serum amyloid P-component (SAP) has been identified as a major acute phase reactant across multiple strains of mice (Mortensen, Beisel et al., 1983; Pepys et al., 1979)). Importantly, SAP is a well-documented homologue of CRP in humans, and the proteins share 60-70% sequence homology (Mortensen, Beisel et al., 1983). Functionally, SAP has been shown to respond to both acute and chronic inflammatory stimuli in vivo, from parasitic and bacterial infections to tumor growth, although there is some inter-strain variation in the magnitude of response (Mortensen, Beisel et al., 1983; Pepys et al., 1979). The SAP response has also been shown to correlate with the activity of macrophage derived IL-1 containing supernatants in a strain specific manner that parallels the pathogen response (Mortensen, Beisel et al., 1983). Thus, SAP demonstrates a number of parallels in protein sequence and function to human CRP. Therefore, an

Exploratory Aim of this investigation included the assessment of peripheral levels of SAP in the plasma of RSD mice as an acute phase protein and a marker of systemic inflammation. It was predicted that SAP would be elevated in the peripheral blood of RSD mice compared to controls given consistent evidence for elevations in IL-6, TNF-α and IL-1β.

The chronic social stress of RSD also associates with an increase in circulating numbers of monocytes that are of a pro-inflammatory phenotype. These monocytes express CD11b and/or Ly6c on their cell surfaces, which parallel expression markers in humans that are used to indicate monocyte subtype (CD14) and early myeloid lineage (low CD16 expression), respectively. Murine monocytes expressing high levels of Ly6c have been shown to mature in the bone marrow, and expression of this marker on the cell surface is thought to indicate recent release into peripheral circulation (Yang et al., 2014). Experimental evidence in the RSD model demonstrates that production of Ly6c+ cells in the bone marrow is increased in response to social stress (Wohleb et al., 2013; McKim et al., 2018), and that sympathetic mechanisms are responsible for their release into peripheral circulation (McKim et al., 2018; Niraula, Wang, et al., 2018). In this regard, reliable evidence suggests that RSD results in a four- to five-fold increase in CD11b+ peripheral monocytes in the peripheral blood of RSD mice compared to controls (e.g., Engler et al., 2008; Powell et al., 2013), with a significant proportion of these CD11b+ monocytes expressing Ly6c (CD11b+Lyc6[high]; Powell et al., 2013; Wohleb et al., 2013; McKim et al., 2016).

On a cellular level, there is also evidence of upregulation in pro-inflammatory gene transcription among peripheral monocytes from RSD mice compared to controls (Niruala, Witcher, et al., 2018; Powell et al., 2013). Evidence suggests that this upregulation in gene transcription may be specific to peripheral CD11b+ monocytes (Powell et al., 2013), particularly the CD11b+Lyc6(high) subtype (Niraula, Witcher, et al., 2018). Altogether, the chronic social

stress of RSD promotes elevations in pro-inflammatory cytokines as well as the accumulation of peripheral pro-inflammatory monocytes.

1.4 Pathways by which Chronic Stress may promote pro-inflammatory states

1.4.1 The Hypothalamic-Pituitary-Adrenal (HPA)-axis

There are a number of biological pathways by which chronic social stress may modulate inflammatory processes. One established pathway is the hypothalamic-pituitary-adrenal (HPA)-axis, which releases glucocorticoids (GCs) when activated (cortisol in humans, corticosterone in mice). In response to social threat, activation of central neural systems results in the production of corticotropin-releasing hormone (CRH) by the paraventricular nucleus (PVN) of the hypothalamus (Castro, Elias, Elias, & Moreira, 2011; Schoneveld & Cidlowski, 2007). CRH travels to the anterior lobe of the pituitary gland, where it stimulates the release of adrenocorticotropic hormone (ACTH) into peripheral circulation. ACTH stimulates receptors in the adrenal cortex, resulting in the production and release of glucocorticoids into the bloodstream (Castro et al., 2011).

Although it is generally thought that chronic stress drives persistent release of glucocorticoids into peripheral circulation, human findings have been inconsistent (Miller et al., 2007). These inconsistencies may be due to heterogeneity of measurement techniques, variability in timing of assessment during the course of the chronic stress, and poor reliability of measurement across studies (Miller et al., 2007). In contrast to human studies, the murine model of RSD has consistently demonstrated elevated levels of peripheral corticosterone compared with controls

(Engler et al., 2005, 2008; Hanke et al., 2012; Nirula, Wang, et al., 2018; Merlot, Moze, Dantzer, & Neveu, 2004; Stark et al., 2001).

Mechanistically, GCs bind to GC receptors (GRs) within immune cells, then translocate to the nucleus and downregulate inflammatory processes. Because GCs are traditionally considered anti-inflammatory, synthetic corticosteroid therapies are often used to treat inflammatory diseases (Boumpas, Chrousos, Wilder, Cupps, & Balow, 1993). In the absence of GC binding, GRs are sequestered in the cytoplasm by a complex of proteins that dissociate from the receptor upon GC binding. In the nucleus, the GR-GC complex can inhibit pro-inflammatory transcription factors, such as NFκB and AP-1. These pro-inflammatory transcription factors are important in initiating the transcription of genes involved in promoting activation and trafficking of lymphocytes into the periphery (Garvy et al., 1993a; Garvy et al., 1993b; Zhang et al., 2007), as well as initiating transcription of pro-inflammatory cytokines (e.g., IL-6, IL-1, TNF-α; Medzhitov & Horng, 2009). The GR-GC complex also promotes transcription of anti-inflammatory proteins (e.g., Iκβ) via the GC response element (GRE) transcription factor. As an anti-inflammatory protein, Iκβ is important in sequestering the pro-inflammatory transcription factor NFκB in the cytoplasm (Hermoso & Cidlowski, 2003). Thus, the GR-GC complex is instrumental in downregulating inflammation at the molecular level via inhibition of NFkB and AP-1, as well as the initiation of important local anti-inflammatory feedback loops via the GRE and Ikβ. Together, systemic activation of the HPA axis and binding of GC receptors results in immune cell activation and trafficking, as well as suppression of the release of pro-inflammatory cytokines.

1.4.1.1 Development of Glucocorticoid Resistance

Chronic exposure to cortisol from chronic social stress may change the responsivity of the glucocorticoid receptor, such that it no longer downregulates pro-inflammatory gene transcription

and the subsequent release of pro-inflammatory proteins (i.e., development of GC resistance; Miller, 2008; Raison & Miller, 2003). Receptor alterations that may reduce sensitivity to cortisol include changes in the number, binding affinity, or functional signaling capacity of glucocorticoid receptors (Raison & Miller, 2003). In humans, evidence supports GC resistance among leukocytes from chronically stressed populations (Bauer et al., 2000; Cole et al., 2007; Miller et al., 2002; Rohleder, Marin, Ma, & Miller, 2009; Walsh et al., 2018). In parallel with human models of chronic stress, RSD mice demonstrate development of GC resistance among peripheral leukocytes (Niraula, Wang et al., 2018; Powell et al., 2013; Quan et al., 2003). Moreover, GC resistance has been shown to be specific to peripheral monocytes, particularly the pro-inflammatory monocyte subtype, CD11b+Lyc6^{high} (Niraula, Witcher, et al., 2018; Powell et al., 2013).

Across both murine and human studies, evidence suggests that GC resistance accounts for some, but not all, variation in upregulation of inflammatory processes engendered by chronic social stress (e.g., Powell et al., 2013; Niruala, Wang et al., 2018; Miller et al., 2014; Rohleder et al., 2009; Walsh et al., 2018). Moreover, in the context of RSD, initial evidence suggests that both HPA-axis and SNS signaling may play a role in modulating the development and trafficking of GC resistant cells. Specifically, adrenalectomy and pre-stressor treatment with metyrapone (MTP), a drug that prevents corticosterone synthesis, abrogates RSD-related increase in peripheral pro-inflammatory markers (e.g., IL-6) and the number of circulating GC resistant CD11b+Lyc6^{high} monocytes in the periphery (Niruala, Wang, et al. 2018). While development of GC resistance and increase in pro-inflammatory processes are thought to be driven by activation of the HPA-axis and subsequent release of corticosterone, exclusivity for this pathway has not been established. In this regard, pre-treatment with propranolol, a non-specific beta-adrenergic antagonist, abrogates GR resistance and reduces the RSD-related up-regulation in pro-inflammatory gene expression among

circulating CD11b+Lyc6^{high} monocytes (Hanke et al., 2012; Powell et al., 2013). Thus, experimental evidence suggests that RSD-related activation of both HPA-axis and SNS signaling may contribute to elevated numbers of GC resistant CD11b+ and/or CD11b+Lyc6^{high} monocytes in peripheral circulation, as well as upregulation of pro-inflammatory gene transcription among these cells. In sum, findings from available research using RSD and human models suggest the SNS functions in the promotion and redistribution of GC resistant monocytes, as well as the upregulation in pro-inflammatory gene transcription.

1.4.2 Autonomic Nervous System

1.4.2.1 Parasympathetic Nervous System

The parasympathetic branch of the ANS is also involved in bidirectional communication between the brain and peripheral immune processes (Pavlov & Tracey, 2015). Specifically, the vagal nerve may play a role in the systemic regulation of peripheral inflammatory processes, largely functioning to downregulate the peripheral inflammatory response (Bellinger & Lorton, 2014; Martelli, McKinley, & McAllen, 2014). Although this pathway is important in the regulation of pro-inflammatory signaling, it is not a relevant pathway of interest here. In particular, CD11b+Lyc6^{high} GC resistant cells have been shown to originate in the bone marrow (Engler et al., 2005; McKim et al., 2018; Wohleb et al., 2013), and to date there is no definitive neuroanatomical evidence of parasympathetic innervation within the bone marrow (Nance and Sanders, 2007; Maryanovich, Takeishi, and Frenette, 2018). Thus, it is unlikely that the development of a pro-inflammatory phenotype among these cells would be driven by parasympathetic mechanisms.

1.4.2.2 Sympathetic Nervous System

Chronic stress activates the sympathetic branch of the ANS through central neural systems that stimulate norepinephrine (NE) secreting neurons in the brainstem (i.e., the medulla & pons; Elenkov et al., 2000; Janig, 2014). These neurons innervate multiple organs, including the adrenal gland, where they stimulate the release of epinephrine (Epi) into peripheral circulation (Bellinger & Lorton, 2014, 2017; Elenkov et al., 2002). The sympathetic nervous system (SNS) also innervates all primary and secondary lymphoid organs, including the bone marrow, where it can activate immune cells and stimulate the selective release of neutrophils and monocytes into peripheral circulation (Elenkov et al., 2000; Dhabhar et al., 2012; McKim et al., 2018; Wohleb et al., 2013). Together, the activation of the SNS and subsequent release of NE into organs and Epi into peripheral circulation from the adrenal medulla is synonymous with the activation of the sympathetic-adrenal-medullary (SAM)-axis. Throughout this document, we use the term SNS to indicate activation of the SAM-axis, including both the release of NE at nerve terminals and the stimulation of Epi into peripheral circulation by the adrenal medulla.

Chronic sympathetic activation may also shift circulating monocytes toward a pro-inflammatory phenotype, a process blocked by β-adrenergic receptor (AR) antagonism (e.g., Geissmann et al., 2010; Hanke et al., 2012; Powell et al., 2013). Sympathetic modulation of immune cells in the periphery depends on adrenergic receptor type (i.e., α or β) and density, with monocytes and granulocytes expressing a greater number of β-ARs than other lymphocytes (Marino & Cosentino, 2013). Under homeostatic conditions, evidence suggests that α-adrenergic receptors are largely absent from peripheral monocytes and granulocytes (Casale & Kaliner, 1984; Elenkov et al., 2000; Marino & Cosentino, 2013) and β-adrenergic signaling pathways predominate (Bellinger & Lorton, 2014); thus, the focus of this project is on β-adrenergic signaling

pathways. In sum, central activation of SNS signaling results in the peripheral release of catecholamines and the selective activation and peripheral release of monocytes and granulocytes, a process of that is likely mediated by β-AR signaling.

1.4.2.3 Physiology and expression of β-adrenergic receptors (β-ARs) on immune cell surface

β-ARs are located on the immune cell surface and are G-protein coupled receptors (GPCRs; Bellinger & Lorton, 2018). When Epi or NE binds to the GPCR, an intracellular complex (the G-protein complex) dissociates from the receptor, initiating a downstream signaling cascade. A few studies have reported that among chronically-stressed human populations, there is a downregulation in the expression of β-ARs on the surface of immune cells (Dimsdale et al., 1994; Mills et al., 2004; Mills et al., 1997). In contrast, among murine models of chronic stress, one study using 5 days of chronic restraint stress revealed no difference in β-AR density on lymphocytes from the blood compared to controls (Kubera et al., 1992). More recently, Li et al. (2018) found that 7 days of chronic restraint stress resulted in no changes to β-AR receptor expression among splenocytes; however, an upregulation in β-AR receptor expression occurred after 21 days of restraint stress. Although, as previously discussed, models of restraint stress lack ethological relevance to human models of chronic stress, and to date, there is no data regarding β-AR surface expression among peripheral blood cells or splenocytes in the RSD model of chronic stress. Thus, the expression of β-AR on peripheral $Ly6c^{hi}$ monocytes from RSD mice vs. controls remains unknown.

1.4.2.4 β-ARs activate intracellular pathways that promote release of pro-inflammatory cytokines

Intracellular signaling through β-ARs is complex, with reports of the promotion of anti-inflammatory and pro-inflammatory phenotypes, depending on context. Ample evidence suggests the most typical, or canonical, signaling pathway for β-AR activates adenylate cyclase (AC), which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), a second messenger protein. In turn, cAMP activates protein kinase A (PKA), an enzyme that mediates downstream gene transcription via the cAMP response element binding protein (CREB). In the nucleus, CREB can promote the transcription of pro-inflammatory cytokines such as IL-6 (Elenkov et al., 2000; Luo and Zheng, 2016;). However, signaling through the canonical pathway has most commonly been studied in context of immune stimulation, in which its effects can be anti-inflammatory (Elenkov et al., 2000; Newell et al., 1994; Sag et al., 2008; Scanzano and Cosentino, 2015). For example, β-AR mediated inhibition of TNF-α has been observed in the context of immune-system activation by lipopolysaccharide (LPS), a component of gram-negative bacteria that induces NFkB-mediated pro-inflammatory transcription (Avitsur et al., 2005; Elenkov et al., 2000; Newell et al., 1994; Severn et al., 1992).

Mechanistically, inhibition of TNF-α in this context could be a result of cAMP inhibition of NFkB-mediated transcription and/or competition between CREB and NFkB for limited amounts of the transcriptional co-activator protein CREB-binding protein (CBP; Elenkov et al., 2000; Newell et al., 1994). Thus, in the context of both β-AR and LPS stimulation, inhibition of TNF-α at the level of the immune cell may be primarily mediated by attenuation of LPS-induced NFkB transcription, while the pro-inflammatory β-AR-CREB signaling pathway (e.g., transcription of IL-6) may still be activated (Elenkov et al., 2000; Tan et al., 2007). This is one example of how

signaling through β-ARs can be context dependent, with multiple factors influencing whether a pro-inflammatory or anti-inflammatory phenotype is observed.

Signaling through the canonical pathway can be detected by measuring cAMP production and/or by quantifying the differential regulation of genes with CREB promotor regions. For example, one measure of β-AR sensitivity in immune cells involves quantification of cAMP accumulation after stimulation with isoproterenol, a non-specific β-AR-agonist (Lorton et al., 2013; Mausbach et al., 2007, 2008; Mills et al., 1997). Additional quantification of the differential regulation of genes with a common CREB transcriptional control element after β-AR stimulation could indicate greater activation of the canonical β-AR intracellular signaling pathway. In sum, catecholamine signaling via β-ARs at the immune cell surface initiates an intracellular signaling cascade that can be detected via cAMP accumulation, together with monitoring of CREB-mediated gene transcription.

1.4.2.5 Alternate β-AR signaling pathways may lead to differences in pro-inflammatory gene expression

Alterations in β-AR intracellular signaling pathways within immune cells may contribute to the relationship between chronic social stress and the development of systemic pro-inflammatory conditions. Three mechanisms may moderate the magnitude of the cell's inflammatory response. The first two occur within the context of the canonical signaling pathway, in which GPCR desensitization (via uncoupling or internalization without degradation) and downregulation of GPCR expression (via degradation pathway) can suppress β-adrenergic signaling (Figure 1; Bellinger & Lorton, 2014, 2018). At a molecular level, receptor desensitization, or "uncoupling," occurs upon conformational change in the β-AR by PKA phosphorylation. The recruitment of G-protein coupled receptor kinase (GRK)-1/2 and β-arrestin-

1 to the β-AR can induce receptor internalization. Internalized receptors are either recycled back to the cell surface, ending a relatively brief desensitization period, or transported to lysosomes for degradation, resulting in a more prolonged downregulation of β-AR signaling. Receptor downregulation has been quantified by evaluation of cAMP accumulation upon β-AR stimulation, and/or by radioligand assay measuring the density of β-ARs on the cell surface (e.g., Lorton et al., 2013; Mills et al., 1997, 2004). Practically, downregulation in signaling through the β-AR canonical pathway may be evidenced by (1) a decrease in intracellular cAMP protein levels on stimulation of the β-AR, (2) a decrease in the density of β-ARs on the cell surface, and/or (3) a downregulation in CREB-mediated gene transcription compared to controls. Thus, **Aim 1a and 1c** of this study were to characterize alterations in β-AR regulated intracellular signaling pathways within peripheral monocytes from RSD mice vs. control mice via quantification of cAMP after β-adrenergic stimulation and characterization of differential gene transcription patterns. More specifically, we evaluated whether β-adrenergic stimulation resulted in reduced accumulation of cAMP among RSD mice and whether differential gene expression between RSD mice and controls revealed an alteration in CREB-promoted gene transcription.

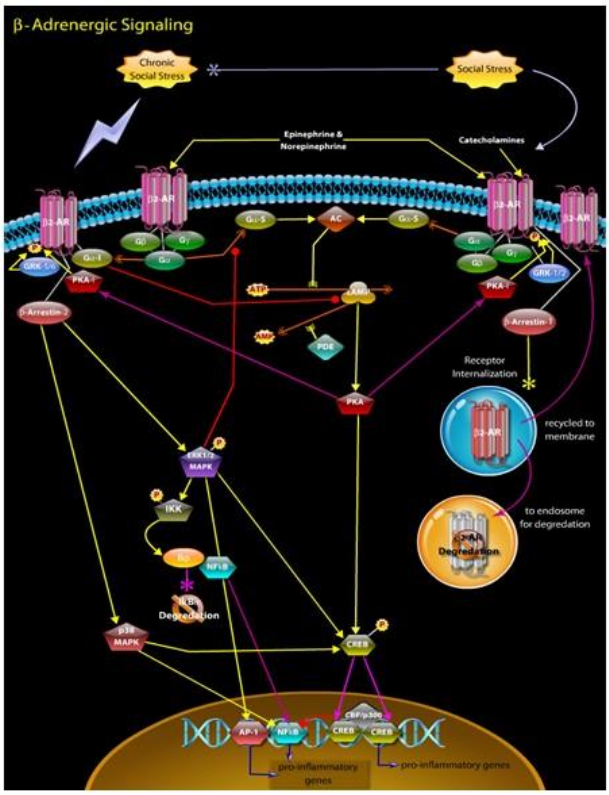


Figure 1. Intracellular β-adrenergic signaling

Diagram of canonical and non-canonical intracellular β-adrenergic signaling pathways thought to be engaged in conditions of social stress and chronic social stress, respectively.

Notes: **GPCR** = G-protein coupled receptor; **β2-AR** = β-adrenergic receptor; **Gα, Gγ, Gβ** = G-proteins that dissociate from the β-AR on catecholamine binding, mediating downstream signaling; **$G\alpha_s$** = G-protein-alpha-S, dissociation from GPCR activates AC, initiating canonical signaling pathway**;** **$G\alpha_I$** = G-protein-alpha-I, recruitment inhibits signaling through canonical pathway; **ATP** = adenosine triphosphate; **cAMP** = cyclic adenosine monophosphate; **AMP** = adenosine monophosphate; **AC** = adenylate cyclase, enzyme that catalyzes the conversion of ATP to cyclic AMP; **PKA** = protein kinase A; **PDE** = phosphodiesterase, an enzyme that converts cAMP to AMP; **CREB** = cAMP response element binding protein; **CBP/p300** = CREB-binding protein, a transcription co-activator; **GRK** = G-protein coupled receptor kinase; **β-arrestin** = a scaffolding protein that changes the conformation of the GPCR, can mediate intracellular signaling independent of the β-AR; **MAPK** = mitogen activated protein kinase, a family of intracellular signaling proteins; **ERK1/2** = extracellular signal-regulated kinase, a conventional MAPK signaling pathway (typically: Ras-Raf-MEK-ERK chain of proteins); **p38** = a conventional MAPK signaling pathway; **AP-1/ NFκB** = Activator protein-1/nuclear factor kappa-light-chain-enhancer of activated B cells, pro-inflammatory transcription factors; **IκB** = inhibitory proten that sequesters NFκB in the cytoplasm; **IKK** = IκB kinase, enzyme important in dissociation of IκB from NFκB, allowing NFκB to move into the cytoplasm. *Refs*: Bellinger & Lorton, 2014; 2018; Cargnello & Roux, 2011; Elenkov, 2000; Scanzano & Cosentino, 2015.

Given that signaling through the canonical pathway may produce an anti-inflammatory or pro-inflammatory phenotype depending on context, downregulation of this signaling pathway is similarly complex. If we consider signaling through the β-AR to be primarily pro-inflammatory (i.e., transcription of IL-6), diminished signaling through the β-AR would result in attenuation of signaling through this pro-inflammatory pathway, likely as a local negative feedback mechanism. This β-AR downregulation mechanism is widely recognized in homeostatic processes (Bellinger & Lorton, 2018). Alternatively, if we consider signaling through the β-AR to be primarily anti-inflammatory (i.e., attenuation of TNF-α transcription), diminished signaling through the canonical pathway would result in a more pro-inflammatory phenotype, consistent with that observed in the context of chronic stress. However, this anti-inflammatory phenotype has been primarily observed in the context of LPS or other immune stimulants (e.g., Avitsur et al., 2005; Newell et al., 1994; for reviews, see Elenkov et al., 2000; Scanzano and Cosentino, 2015).

One alternate β-AR intracellular signaling pathway that has been described in mice may help explain associations between chronic stress and heightened levels of circulating inflammatory markers (Bellinger and Lorton, 2018). Specifically, initial evidence suggests that β-ARs may switch to a non-canonical GPCR-independent signaling pathway in conditions of chronically-elevated sympathetic tone (Bellinger & Lorton, 2015, 2018), such as those thought to exist in the context of chronic stress. Under conditions of high Epi concentration, phosphorylation of the β-AR by PKA can recruit GRK-5/6 to the receptor along with β-arrestin-2, initiating a switch away from the canonical cAMP-CREB pathway and toward a non-canonical signaling cascade by way of MAPK signaling cascades (Figure 1; Bellinger & Lorton, 2015, 2018; DeFea, 2008; Lefkowitz, Pierce, & Luttrell, 2002). Similar to the canonical signaling pathway, phosphorylation of the β-AR by PKA and GRK5/6 induces a β-arrestin-2 mediated receptor internalization and

desensitization of the β-AR. In contrast to the canonical pathway, MAPK signaling results in largely pro-inflammatory gene transcription (Cargnello & Roux, 2011). Thus, a switch from canonical β-AR signaling pathways to non-canonical MAPK signaling pathways could help explain positive associations between chronic stress and peripheral pro-inflammatory states. To assess this possibility, **Aim 1b** of this study was to characterize alterations in β-AR regulated intracellular signaling pathways within peripheral monocytes from RSD mice vs. control mice via quantification of the relative expression of β-arrestin-1 vs β-arrestin-2 protein on stimulation with catecholamines. Specifically, we aimed to evaluate whether protein expression of β-arrestin-2 vs 1 differed between RSD mice vs. controls; here, greater expression of β-arrestin-2 vs 1 among RSD mice vs controls was indicative of engagement of the non-canonical signaling pathway.

Initial evidence from a comprehensive literature review investigating differential gene transcription as a consequence of RSD stress in mice and chronic social stress among humans found that chronic social stress may associate with a downregulation in CREB-mediated gene transcription (Walsh, Bovbjerg, Marsland, 2021), although not all findings were consistent. At least one study reviewed in each species also found that chronic social stress associated with an upregulation in MAPK-mediated gene transcription (Walsh, Bovbjerg, Marsland, 2021). Importantly, some of these studies provided evidence that these alterations in gene transcription were specific to peripheral pro-inflammatory monocytes (Walsh, Bovbjerg, Marsland, 2021). Thus, it is plausible that a shift in β-AR signaling pathways among monocytes toward a β-arrestin-2/MAPK mediated pro-inflammatory signaling pathway may help explain observed positive associations between chronic stress and pro-inflammatory states. However, these pathways have never before been experimentally examined in the RSD model of chronic social stress.

Given current evidence, a primary goal of this study was to characterize β-AR regulated intracellular signaling pathways within peripheral monocytes from RSD mice vs. controls (**Aim 1a, 1b**). Another goal was to examine whether alterations in β-AR regulated intracellular signaling pathways would contribute to the upregulation of pro-inflammatory gene expression observed among peripheral monocytes from RSD mice vs. controls (**Aim 1c**). More specifically, we aimed to examine whether alterations in β-AR intracellular signaling pathways among RSD mice vs. control mice would lead to a relative upregulation in MAPK-promoted gene transcription via the described non-canonical signaling pathway (**Aim 1c**). Finally, an **Exploratory Aim** was to test whether SAP, a potential murine acute phase marker, was elevated among RSD mice vs. control mice.

2.0 Aims and Hypotheses

2.1 Aim 1a

Aim 1a characterized whether RSD induces β-AR "signal switching" by measuring the production of the second messenger cAMP on stimulation with isoproterenol, among peripheral monocytes from RSD mice compared to controls. Cells for these assessments were harvested immediately after the end of the last cycle of RSD. *Hypothesis: CD11b+Ly6c+ cells from RSD mice vs. controls would show diminished cAMP accumulation on stimulation with isoproterenol consistent with β-AR "signal switching."*

2.2 Aim 1b

Aim 1b characterized whether RSD induced β-AR "signal switching" by measuring the presence of key signaling proteins β-arrestin-1 and 2 among peripheral monocytes from RSD mice compared to controls. Cells for these assessments were harvested immediately after the end of the last cycle of RSD. *Hypothesis: CD11b+Ly6c+ cells from RSD mice vs. controls would show greater β-arrestin-2 utilization vs β-arrestin-1 consistent with β-AR "signal switching."*

2.3 Aim 1c

Aim 1c characterized whether RSD induced β-AR "signal switching" by measuring differential gene expression on stimulation with isoproterenol among peripheral monocytes from RSD mice compared to controls. Cells for these assessments were be harvested immediately after the end of the last cycle of RSD. Gene transcription was amplified using mRNA Illumina NextGen Sequencing, with subsequent grouping of differential gene expression by key transcription factors (e.g., NFkB, CREB, MAPK). *Hypothesis: CD11b+Ly6c+ cells from RSD mice vs. controls would show an upregulation of proinflammatory genes consistent with β-AR "signal switching" (i.e., ↑MAPK and ↓CREB mediated gene transcription).*

2.4 Exploratory Aim

An **Exploratory Aim** examined whether SAP, an acute phase marker of systemic inflammation in mice, was elevated in the peripheral blood from RSD mice compared to controls. Plasma for this assay was obtained immediately after the end of the last cycle of RSD. *Hypothesis: SAP would bel be elevated in the peripheral blood from RSD mice vs. controls, consistent with activation of the acute phase response and the presence of long-term systemic inflammatory processes in response to chronic social stress.*

3.0 Research Design and Methods

3.1 Repeated Social Defeat (RSD)

Male C57BL/6 (6-8 weeks old) and CD-1 retired breeders (12 months) were purchased from Charles River Breeding Laboratories and housed at the Postle Hall animal facilities at The Ohio State University. The C57BL/6 mice were housed in cohorts of three, whereas the CD-1 mice were individually housed. All experimental procedures were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals with approval from The Ohio State University Institutional Laboratory Animal Care and Use Committee (IACUC).

Mice were subjected to RSD as previously described (McKim et al., 2015; Wohleb et al., 2011, 2013, 2014). In brief, resident mice were exposed to an aggressive intruder CD-1 mouse for 2 hours (17:00-19:00) daily, for six consecutive nights. To ensure a successful stress exposure, the resident mice were observed during each stress cycle for submissive behavior including fleeing, upright posture, crouching and huddling. The intruder was removed intermittently during the exposure to manage the arousal and wounding of the C57BL/6 mice; the CD-1 mice were removed completely at the end of each 2-hour cycle. The resident mice were carefully observed for injuries. Control mice were left undisturbed in their cages in the control room until sacrifice. For this study, mice were exposed to one additional stress exposure on the morning of sacrifice, such that cells were harvested within 3 hours of the last cycle of RSD.

Animals were sacrificed by CO_2 asphyxiation in groups of 3-5 according to the IACUC protocol. This method induces rapid loss of consciousness in the animal with minimal safety concerns to humans (Bovin et al., 2017); however, of relevance here, CO_2 asphyxiation can act as

an acute stressor resulting in systemic activation of both the HPA-axis and SAM systems (Bovin et al., 2017). Indeed, peripheral norepinephrine levels have been shown to be elevated within 5 seconds of CO_2 exposure. Despite this, norepinephrine levels have been shown to be elevated in RSD animals for three hours after the last cycle of RSD above and beyond levels in control mice (Hanke et al., 2012).

3.2 Anxiety-like behavior

There are reliable alterations in anxiety-like behavior and social avoidance as a consequence of RSD. Specifically, mice experiencing RSD demonstrate greater anxiety-like behaviors compared to controls when measured using the open field test, the light-dark preference test, and the elevated plus maze (Wohleb et al. 2011, Wohleb, Powell, Godbout, & Sheridan, 2013). They also demonstrate reduced grooming behavior and greater social avoidance as measured by the social avoidance test compared to controls (Wohleb et al. 2011, Wohleb, Powell, Godbout, & Sheridan, 2013). Given that these alterations in behavior are well-established, they were not repeated as a part of these experiments to reduce the number of manipulations of the mice and isolate the effect of RSD on peripheral cells.

3.3 Preparation of Cells

3.3.1 Splenocytes.

Spleens were removed from mice and stored in HBSS (experiments with splenocytes only: Cat # 14065-056; 10x diluted to 1x in deionized water prior to use) or PBS pH7.2 (experiments with CD11b+ cell isolation: 1.37M NaCl Fisher Chemical#S640, 81.70mM $Na_2HPO_4*7H_20$ Sigma#71642, 17.64mM KH_2PO_4 Sigma#P5655, 26.8mM KCL Sigma#P3911] on ice until processing. For processing, spleens were homogenized in HBSS (experiments with splenocytes only) or PBS (no Ca/Mg; for CD11b+ isolation) and passed through a 70um nylon mesh. Red blood cells (RBCs) were lysed in 5mL RBC Lysis Buffer (Biolegend Cat# 420301; 10x diluted to 1x in deionized water prior to use) for 4-5min on ice with occasional shaking. Cells were washed one time by adding 20-30mL HBSS (experiments with splenocytes only) or PBS (no Ca/Mg; for CD11b+ isolation) to stop the reaction. Cells were then pelleted (350g for 6 min at 10°C). For experiments with splenocytes only, cells were resuspended cold supplemented RPMI (sRPMI: RPMI Gibco Cat#11875-093 + 1% Pen/Strep Sigma Cat# P7539 + 5% FBS Sigma Cat#F4135) for counting (~10mL/spleen). For experiments involving CD11b+ isolation, cells were resuspended in cold MACs® buffer (1xPBS pH7.2, 1%BSA, 1mM EDTA) for counting (~1-2mL/animal).

3.3.2 Peripheral Blood.

Blood was removed via cardiac puncture (~500ul-900uL) using 26G needles coated with 50mM EDTA (EDTA, Invitrogen #15575-020, diluted in deionized water, stored at RT) and stored

in 1.5mL Eppendorf tubes on ice until processing. For processing, blood was added to 15mL 1x RBC lysis buffer (Biolegend Cat# 420301), gently vortexed, and incubated at RT in the dark for 10min. Cells were then washed one time by addition of ~35mL PBS (no Ca/Mg; for CD11b+ isolation) and pelleted (350g for 6 min at 4°C). The supernatant was pipetted off and cells were gently vortexed to break up the pellet. Cells were then resuspended in cold MACs® buffer (1xPBS pH7.2, 1%BSA, 1mM EDTA) for counting (~1-2mL/animal).

3.3.3 Counting Cells.

Cells were counted using a 10x dilution into Acridin Orange/Propidium Iodide Stain (Logos Biosystems Cat #F23001) using Photon Slide Ultra Low Fluorescence Counting Slides (Cat # L12005) on a Logos Luna-FL machine (Model #L20001).

3.3.4 CD11b+ isolation.

Based on prior experiments, we estimated that 10-20% of total splenocytes (~4-50x10^6 cells) and 6-10% of the total peripheral blood cells (~1.8-3.1x10^6 cells for an average blood draw) would be CD11b+ (Powell et al., 2013; Niraula, Witcher, et al., 2018). Thus, to have enough cells for subsequent assays (2.4-3.0x10^6 total CD11b+ cells) we determined that each isolation would need to have a starting number of at least 2.5-5x10^7 cells. If one mouse sample had fewer than this range of cells, then we made the determination to either 1) reduce the number of technical replicates for the controls (RPMI or Forskolin – see section 3.4), or 2) combine samples (based on mice with the most similar spleen weight or if two samples had particularly low yield). MACs® buffer (1xPBS pH7.2 [PBS: 1.37M NaCl Fisher Chemical#S640, 81.70mM Na_2HPO_4*$7H_20$

Sigma#71642, 17.64mM KH_2PO_4 Sigma#P5655, 26.8mM KCL Sigma#P3911], 1%BSA Sigma Cat# A9647, 1mM EDTA, Invitrogen #15575-020) was prepared and degassed and stored at 4°C until use. On the day of use, MACs® buffer was kept on ice. After counting/combining of samples, cells were pelleted (300×g for 10 minutes at 4°C) and the supernatant was pipetted off. Each sample was resuspended in 90uL MACs® buffer and 10uL CD11b+ Microbeads (Miltenyi Biotec Cat #130-049-601) per $1x10^7$ total cells. The solution was mixed well and incubated for 15 min at 4-8°C (in fridge). After incubation, cells were washed with 1-2mL MACs® buffer/$1x10^7$ cells and pelleted (300×g for 10 minutes at 4°C); supernatant was pipetted off and each sample was resuspended in 500uL MACs® buffer/$1x10^8$ cells. Samples were pre-filtered through a 30μm nylon mesh (Miltenyi Pre-Separation Filters # 130-041-407) before loading onto MS positive selection columns (Miltenyi #130-042-201), following the manufacturer directions. Positively selected cells were eluted in 1mL MACs® buffer and kept on ice before counting.

3.3.5 Ly6c+ cells.

Based on prior experiments, we estimated that 10-40% of CD11b+ splenocytes (~.4-$20x10^6$ cells) and 5-20% of the CD11b+ peripheral blood cells (~.9-$6.2x10^5$ cells for an average blood draw) would be Ly6c+ (Niraula, Witcher, et al., 2018; Powell et al., 2013). Thus, based on calculations described in section 3.3.4 (2.4-$3.0x10^6$ total CD11b+Ly6c+ cells needed for stimulation assay), it would be difficult to isolate enough Ly6c+ cells for our assays. In addition, Lyc6+ cells would need to be isolated using Fluorescence-Activated Cell Sorting (FACS), which can reduce the viability of the cells due to the time and rigor of the isolation procedure. Thus, we used CD11b+ cells in all experiments.

3.4 β-adrenergic receptor (β-AR) stimulation

3.4.1 Preparation of reagents.

3.4.1.1 Isobutylmethylxanthine (IBMX)

Isobutylmethylxanthine (IBMX; Sigma Cat #I5879) was reconstituted at .9M in DMSO, divided into 150uL aliquots, and stored at -20ºC. In preparation for the assay, aliquots were thawed at RT (and/or warmed briefly in a 37ºC water bath if unable to dissolve into solution) and diluted to 1mM in warmed supplemented RPMI (sRPMI: RPMI Gibco Cat#11875-093 + 1% Pen/Strep Sigma Cat# P7539 + 5% FBS Sigma Cat#F4135). For volumes less than ~10ml, the IBMX often precipitated out of solution when added to the sRMPI. In this case, the 1mM IBMX sRPMI solution was rewarmed briefly at 37ºC and vortexed.

3.4.1.2 Isoproterenol

Isoproterenol (ISO; Sigma Cat #I5627) was reconstituted at 200mM in H_20, divided into 20uL and 50uL aliquots, and stored at -20ºC. In preparation for the assay, aliquots were thawed on ice and diluted to 10mM in H_20 before dilution to 1mM in warmed sRPMI.

3.4.1.3 Forskolin

Forskolin (Cayman Chemical Item #11018) was reconstituted at 5mM in 60%DMSO/H_20, divided into 110uL aliquots, and stored at -20ºC. In preparation for the assay, aliquots were thawed on ice and diluted to 10uM in warmed sRPMI.

3.4.1.4 Sodium Acetate

Sodium acetate (Sigma Cat#S2889) was prepared at 50mM in Millipore H_2O within 24 hours of use and stored at RT.

3.4.2 Stimulation for determination of cAMP accumulation by ELISA

Splenocytes or CD11b+ peripheral cells (see section 3.3) were resuspended at a concentration of $2x10^6$ cells/mL in cold sRPMI and kept on ice before addition into assay. For splenocytes, we diluted 1mL of cell suspension in sRPMI to a concentration of $2x10^6$ cells/mL. CD11b+ peripheral cells were pelleted at 300g for 10min at 4°C, the supernatant was pipetted off, and cells were re-suspended in the required volume of sRPMI for $2x10^6$ cells/mL.

The following solutions were prepared and .9mL was aliquoted to 5mL Eppendorf tubes (Eppendorf Cat # 0030119401; 900uL/tube) in triplicate for each condition:

1. sRMPI alone [stimulation and IBMX negative control]
2. sRPMI containing 1mM IBMX, a cAMP phosphodiesterase inhibitor [stimulation (ISO and forskolin) negative control]
3. sRPMI containing 1mM IBMX + 10uM isoproterenol (ISO) [β-AR cAMP stimulation condition]
4. sRPMI containing 1mM IBMX + 10uM forskolin [non-specific cAMP stimulation positive control]

The aliquoted solutions were warmed for 10min in a 37°C water bath prior to the addition of 100uL of $2x10^6$ cells/mL cell suspension ($2x10^5$ total cells in assay). Cells and solutions were then incubated for 10 minutes at 37°C (i.e., tubes were submerged in 37°C water bath). The

reaction was stopped via dilution by the addition of 3 mL ice cold sRPMI to the tubes and placing them on ice. The samples were then centrifuged at 300g for 6 min at 4°C and supernatants were decanted with extra wiping of the liquid closest to the top of the tube to eliminate extra volume ("dump and dab"). The cell pellet was then resuspended in 0.5 mL 50 mM sodium acetate buffer, followed by lysis using two cycles of freezing (dry ice) and boiling (100°C water bath) for 5 minutes each. Tubes were then centrifuged for 10 minutes at 3,000 rpm (1700g) to remove cellular debris and supernatants were collected and stored at −70°C until cAMP analysis (section 3.5).

3.4.3 Stimulation for determination βarrestin-1/2 utilization and Gene Expression

The stimulation protocol described in Section 3.3.2 was used with modification that IMBX was not added to solutions. This ensured that intracellular signaling cascades would proceed as normal. Thus, there were only three stimulation conditions for these protocols:

1. sRMPI alone (stimulation negative control)
2. sRPMI containing 10uM isoproterenol (ISO) [β-AR specific stimulation condition]
3. sRPMI containing 10uM forskolin [non-specific cAMP stimulation control condition]

3.4.3.1 βarrestin-1/2 western blot

To prepare for ***βarrestin-1/2 western blot***, after the 10-minute incubation of cells as described above, the reaction was stopped via dilution by the addition of 3 mL ice cold HBSS (Gibco Cat # 14065-056, diluted to 1x in deionized water) to the tubes and placing them on ice. The samples were then centrifuged at 300g for 6 min at 4°C and supernatants decanted with extra wiping of the liquid closest to the top of the tube to eliminate extra volume ("dump and dab").

Each cell pellet was then resuspended in 50uL RIPA buffer solution[1] (RIPA buffer, Thermo # 89901; 1% Protease Inhibitor, Thermo #87786; 1% Phosphatase Inhibitor, Thermo # 78420), transferred to a new 1.5mL Eppendorf tube, and incubated for 15min with vigorous shaking at 4-8°C (in the fridge). The cells were then lysed by two cycles of freezing (dry ice) and thawing (37°C water bath) for 10 min each[2], centrifuged for 15 min at 14,000g in microcentrifuge at 4°C to remove cellular debris, and supernatants were transferred to 0.2mL tubes (~35uL/tube) to be stored at −70°C until analysis (Section 3.6).

3.4.3.2 Gene Expression

To prepare for ***gene expression***, the three reactions described in the beginning of this section were stopped after 10 min via dilution by the addition of 3 mL ice cold HBSS (Gibco Cat # 14065-056, diluted to 1x in deionized water) to the tubes and placing them on ice. The samples were then centrifuged for 10min at 3000g at 4°C and the supernatants were aspirated under a laminar flow hood. To lyse cells, prevent unwanted changes in the gene expression profile, and prevent RNA degradation (via inactivation RNAses and other proteins), 350uL Qiagen Buffer RLT+β-mercaptoethanol (Buffer RLT, from Qiagen Kit#74004; 2-mercaptoethanol (β-ME), BioRad Cat#1610710) was added to the cell pellet and pipetted to mix, followed by transferring the mixture to a 1.5mL RNAse-free tube. The reaction tube was then rinsed with 350uL RLT+β-ME and the rinse solution added to the lysate in the RNAse-free tube. The entire 700uL sample

[1] Total protein extracted from splenocytes using RIPA buffer was significantly greater compared to using TPER buffer (Thermo #78510) for cell lysis ($t(8) = 2.31$, $p = .008$; RIPA = 93.69μg ± SE 5.25, TPER = 72.41 ± SE 3.10).

[2] Because β-arrestin-1 and 2 are membrane associated proteins, we included the use of sonication in the development of this protocol. We found that there was no significant difference in total protein extracted from splenocytes using sonication vs freeze-thaw in two separate experiments ($t(8) = 2.31$, $p = .770$; $t(7) = .370$, $p = .370$)

volume was then vortexed for 1min[3]. One replicate of each sample was isolated using the Qiagen Micro Kit (Cat # 74004) on the same day of the experiment; the remaining samples were frozen at -70°C. For the Qiagen Micro Kit, DNAse I was used to remove contaminating genomic DNA via incubation on the column membrane for 15min. RNA was eluted into RNAse free water. For *splenocytes*, RNA was eluted using two separate aliquots of 15uL RNase-free water, with incubation on the column membrane 1 min each time (total volume = 23uL, due to 2uL column bed volume; goal: greater RNA yield). For *peripheral cells*, RNA was first eluted using 15uL RNase-free water with incubation on the column membrane for 1 min; this eluate was then used for a second elution from the same column (incubation time 1min, final volume 13uL; goal: greater concentration). Eluted RNA was kept on ice and then at -20°C overnight until it was sent for QC analysis prior to sequencing (Section 3.7).

3.5 Measurement of cyclic adenosine monophosphate (cAMP)

cAMP levels were determined from cell lysates stored at -70°C from stimulation protocol (Section 4.4.2). Samples were assayed using a competitive enzyme linked immunoassay kit (ELISA; Cayman Chemical, Cat # 501040), according to the manufacturer's directions with acetylation of the samples and standards. The range of detection for this assay is 0.09 – 200pmol/mL, with sensitivity of 0.6 pmol/mL.

[3] Disruption/homogenization using DNA/RNA free syringe resulted in overall lower yields due to volume loss with the syringe methods; manufacturer recommendations suggested that for ≤ 1x105 cells, the lysate can be homogenized by vortexing.

3.6 Measurement of β-arrestin-1/2 protein

Recruitment and/or utilization of β-arrestin-1/2 after stimulation may be determined using Western Blot protein analysis on cell lysates from the stimulation protocol described in Section 3.3.3. To do this, total protein amounts were measured using a BCA Assay (Thermo Cat#23225) with the lysate buffer as a standard diluent[4]. The maximum amount of protein based on concentration was loaded onto pre-cast mini-PROTEAN gels (10% gradient, 50uL wells, BioRad Cat # 4561034) under reducing conditions (4x Lammeli Sample Buffer BioRad Cat #1610747, 2-mercaptoethanol (BioRad Cat#1610710 [10% of volume, final conc: 1.42M]), denatured at 70°C for 20min). Cerebellar lysates were loaded as protein controls and the Precision Plus All Blue Protein Standard (BioRad Cat#161-0373) was used as a standard protein marker. Tris-Glycine-SDS Running Buffer (BioRad Cat# 1610772) was used to run the gel at 50V for 28min (or until dye was pulled from the wells into the gel), then 80V for 1h 28min on a Biorad Mini-PROTEAN Tetra cell gel electrophoresis system to separate the proteins. Protein was transferred onto nitrocellulose membranes (Trans-Blot® Turbo™ Mini Nitrocellulose Transfer Packs #1704158) using the Turbo Mini TGX setting on the Bio-Rad Trans-blot Turbo Transfer System. Membranes were blocked in 1xTBS Blocking Buffer (Intercept TBS Blocking Buffer, Li-Cor Cat #927-60001) for 1h at RT on an orbital shaker. The membrane was then probed for presence of β-arrestin-1 and 2 (~47kDa, Rabbit anti-ARR1, Abcam#ab31868; ~49kDa, Rabbit anti-ARR2, ThermoFisher #PA1-732) in 1xTBS blocking buffer with .2% Tween-20 (1xTBS-T.2) overnight at 4-8°C (in the fridge). For protein loading standardization, we either probed for Cofilin as a loading control

[4] Lysate buffer (RIPA and TPER) were compared to the recommended diluent of deionized water and we found no significant difference in measurement of protein concentration across two separate experiments (Two-way ANOVA, $F(2,14)=.52$, $p = .60$; $F(2,12)=1.06$, $p = .38$)

protein (~20kDa; Rabbit anti-Cofilin, Abcam# ab42824), or used the Revert Total Protein Stain Kit (Li-Cor Cat#926-11010) according to manufacturer protocols after initial imaging of the membrane. After primary antibody incubation, the membrane was washed using 1xTBS with .1% Tween-20 (1xTBS-T.1) prior to probing with secondary detection antibody in 1xTBS.2 (IRDye® 800CW Donkey anti-Rabbit Cat # 926-32213) for 1 hour at RT. Membranes were washed with 1xTBS-T.1 before imaging using the Odyssey LICOR system.

3.7 Measurement of Gene Expression

3.7.1 RNA preparation, mRNA library construction, and Illumina Sequencing

Purified RNA was sent for quality control (QC) analysis using the Agilent RNA 6000 Pico Assay run on an Agilent 2100 Bioanalyzer through the OSU Genomics Core. The range for this assay is 50-5000pg/uL. Samples were selected for library generation and sequencing based concentration (> 90pg/uL) and high quality RIN number (RIN > 8.0; Schroeder et al., 2006). If no replicates for a sample type met these criteria, we tried drying and concentrating high-quality samples using a Savant speed vac concentrator system. Of note, all the following steps were performed by OSU Genomics Core with sequencing at Nationwide Children's Hospital. Specifically, global pre-processing (i.e., mRNA amplification using oligo(dT) primers and generation of cDNA) for low input RNA (10pg-1ng) was performed using the Clontech SMART-Seq HT kit (Takara Bio Inc., Mountain View, CA, Cat #634456). cDNA was checked for concentration and quality prior to library generation using the Qiagen Qubit and High Sensitivity DNA1000 Bioanalyzer. DNA fragmentation and library generation was performed using the

Nextera XT DNA Library Prep Kit (Illumina, Inc., San Diego, CA, Cat #FC-131-1096). Samples were then sequenced at a depth of 15-21million 2x100bp paired-end clusters on an Illumina NovaSeq6000 platform (Illumina, Inc., San Diego).

3.7.2 Sequence alignment and generation of gene count matrices

Individual FASTQ files were trimmed for adapter sequences and filtered for a minimum quality score of Q20 using Adapter Removal v2.2.0 (Schubert et al., 2016). Preliminary alignment using HISAT2 v2.0.6 (Kim et al., 2015) was performed to a composite reference of rRNA, mtDNA, and PhiX bacteriophage sequences obtained from NCBI RefSeq (O'Leary et al., 2016); reads aligning to these sequences were removed from subsequent analyses. Primary alignment was performed against mouse genome reference GRCm38p4 using HISAT2 (Kim et al., 2015). Gene expression values for genes described by the GENCODE (Harrow, Denoeud et al., 2006; Harrow, Frankish et al., 2012;) Gene Transfer Format (GTF) release M14 (mouse) were quantified using the featureCounts tool of the Sub-read package v1.5.1 (Liao, Smyth, & Shi, 2013, 2014) in unstranded mode. Sequencing alignment quality control was performed by verifying aligned read quality using RNA-SeQC (DeLuca et al., 2012) and RSeQC (Wang, Wang, & Li, 2012). Parameters evaluated included the alignment rate (the percentage of reads aligning to the reference genome), and the duplication rate (the percentage of reads that were identified as PCR duplicates). The uniquely mapped read ratio was then calculated as 100%-(the duplication rate).

3.7.2.1 CLEAR processing

CLEAR is a sequence processing workflow that can be used to select robust/low-noise transcripts when using samples with low RNA input amounts in deep sequencing technologies

(Walker et al., 2020). Using the CLEAR workflow has been shown to reliably identify quantifiable transcripts across replicate samples from single cell and limiting cell inputs (i.e., 10-1000pg), as well as result in superior separation of cell types based on principle components analysis of gene expression data (Walker et al., 2020). Files that are used as input into the CLEAR workflow include those generated using the 'genomecov' utility of BedTools v2.27.0 (ref 43) in BedGraph format (using the 'split output mode' to reduce the size of the output files). These files contain information about the coverage depths across the aligned reference sequence at a per base resolution. Using the NCBI RefSeq GRCm38 as reference, CLEAR calculates the positional mean of the read distribution of the aligned sequence between the 5' and 3' ends for each transcript annotated in the reference sequence (μ_i: [-1, 1]). When the positional mean is closer to 0, it means that the read distribution of the aligned sequence is symmetrical along the length of a transcript and will likely be included in further downstream analyses (i.e., deemed an acceptable transcript by CLEAR). In the case that a gene contains multiple isoforms, the longest transcript in the reference sequence is used for the calculation. Transcript positional means [-1, 1] for all transcripts quantified by the featureCounts tool (Section 3.7.1) are then ranked and binned by the transcript read coverage (i.e., the expression of that gene in the aligned sequence) and fit to a bimodal (double beta) distribution model using the 'optimize' module of the Python 'scipy' package (for exact equation, see Walker et al., 2020). When the distribution of μ_i's start to deviate from a unimodal distribution around 0 (i.e., an even distribution around the center of the transcripts), a bimodal distribution emerges (indicating uneven sequencing coverage of the transcript). This is evidenced in the model by a change in the fitting parameters a and b. When either a or b exceeds a value of 2, these transcripts are excluded by CLEAR for downstream analyses. For confirmation that these

transcripts are in fact bimodal, violin plots can be examined using the matplotlib package in Python for visualization.

3.7.3 Estimation of Differential Gene Expression

After CLEAR selection for robust/low-noise transcripts, we used the DESeq2 package in R (Love et al., 2014) to quantify differential gene expression. When there are a small number of samples per replicate condition (here, N = 3), analyzing each gene individually can be underpowered and may result in uncertain estimation due to high within-sample variation. This limitation can be overcome by relying on assumptions about the similarity of variance across genes within the same experiment. DESeq2 assumes that genes with similar average expression strength across all samples have similar dispersion. In practice, DESeq2 first estimates the dispersion of each gene, plots the distribution of these estimates, and fits a smooth curve to estimate the average dispersion. The algorithm then uses a Bayes approach to estimate how close the true dispersion values tend to be to the fit line; this determines the "shrinkage" estimate, or how much the gene-wise dispersion estimates would need to change to fall on the predicted curve. Thus, as sample size increases, the shrinkage estimates decrease in strength and can become negligible. In the case of small sample sizes, the curve can aid in providing information for distribution estimation. One downside of this practice is that inference based on shrunken dispersion estimates can lead to false positives in differential gene expression. Thus, if an individual sample contributes a dispersion estimate more than 2 standard deviations above the curve, DESeq2 uses the individual gene-wise dispersion estimate instead (Love et al., 2014).

The goal of the differential gene expression analysis is to estimate the log2 fold change between groups and identify genes that are significantly different after multiple test adjustment.

However, log fold change can be skewed (artificially inflated) when gene counts are low; in addition, the genes that are determined to be significantly different can depend on sample size as well as other aspects of the experimental design (Love et al., 2014). To partially address these limitations, we manually filtered out all genes with an average median count of ≤ 2 (across all samples) after CLEAR pre-processing and prior to input into DESeq2. In addition, DESeq2 relies on a statistical framework to facilitate gene ranking based on stable estimates of log fold change. Specifically, read counts are modeled as a negative binomial distribution (gamma-Poisson distribution), with the mean read count proportional to the concentration of reads for a gene in the sample; these means are then scaled by normalization factor that can account for differences in sequencing depth between samples. Within sample variance is modeled from the dispersion data as previously described. When information about a gene's mean and dispersion is low, DESeq2 artificially shrinks the log fold change estimates toward zero. Practically, this is done using a Bayes approach: an ordinary GLM is fit to the data to obtain maximum likelihood estimates for the log fold change; these estimates are then fit to a zero-centered normal distribution. This distribution is then used as a prior for a second round of GLM fits and these estimates are kept as the final estimates for the mean fold change. The standard error for each estimate is derived from the posterior's curvature at its maximum. This procedure ensures that the log fold change estimates are more evenly spread around zero and artificially "shrinks" the log fold change estimates for genes with less information available to estimate parameters of the GLM. This method for estimation of log fold change has been shown to be more reproducible in quantification of transcriptional differences than other approaches (Love et al., 2014), and can help correct for artificially inflated log fold change estimates when gene counts are low. These shrunken log fold change estimates and standard errors are then used in a Wald test (the estimate divided by its

standard error and compared to a standard normal distribution) to determine significance for the differential expression. This also allows for testing of individual coefficients, or contrasts of coefficients, without the need to fit a new model to the reduced data. Adjustment for multiple comparisons is performed using the procedure of Benjamini and Hochberg (Love et al., 2014).

3.8 Measurement of peripheral inflammation

Plasma samples were collected immediately after the end of the last cycle of RSD in ~5mM EDTA final concentration (blood is drawn into 50mM EDTA solution prepared in ddH20). Samples were then frozen at -80°C until analysis.

3.8.1 Measurement of IL-6

Samples were assayed in duplicate using a quantitative sandwich enzyme immunoassay kit (BD OptEIA Mouse IL-6 ELISA Cat#555220; BD Biosciences, San Jose, CA), according to the manufacturer's directions. The range of this assay as run in the lab is 15.6pg/mL to 1000pg/mL.

3.8.2 Measurement of Serum Amyloid Protein (SAP)

Samples were assayed in duplicate using the Mouse Pentraxin 2/SAP Quantikine ELISA (R&D Systems, Cat# MPTX20), according to the manufacturer's directions. The range of this assay is 1.56 – 100 ng/mL, with sensitivity of 0.159ng/mL.

4.0 Analytic Plan

Analyses were performed using Graphpad Version 9.4 software. Observations above or below three standard deviations from the mean were considered to be outliers and were removed from analyses. We checked the data when appropriate for a normal distribution using a skewness and kurtosis within +/- 2.0. Correlations were performed using Pearson's product moment coefficient. Main effects were determined using mixed ANOVA, with follow-up pairwise comparisons adjusted for multiple comparisons.

4.1 Gene Expression Analysis

Analyses were performed using RStudio Open Source Software. To analyze our data using DESeq2, we performed pairwise comparisons for within-group and between-group contrasts, and used a group X treatment interaction term for between group contrasts across sample types (e.g., RSD_I vs CON_R comparison). For IPA, core analysis were performed with the default settings in IPA, selected for specific species (mus musculus) and cell types (splenocytes or peripheral mononuclear cells & leukocytes). The biological relationships between genes in a canonical pathway are available from My Pathways in IPA. Panther over-representation analyses were performed using default settings, with mus musculus as the organism and Panther Pathways as the annotation dataset. The reference list for genes was the mus musculus default whole-genome set.

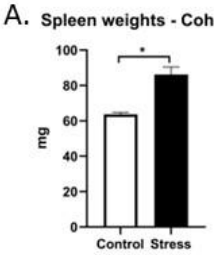
5.0 Results

5.1 Confirmation of stress effect

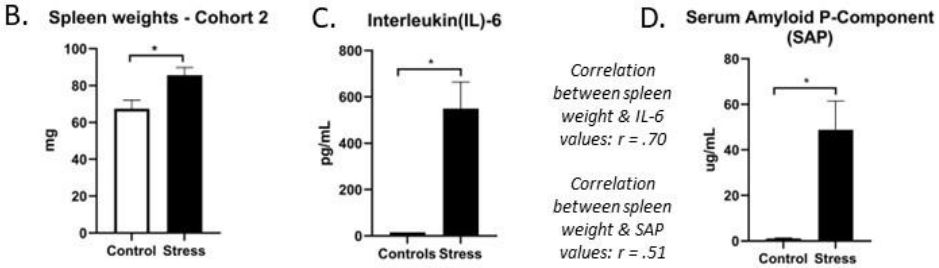
5.1.1 Spleen weight

Spleen weight is used as a quantitative measure of the stress effect after sacrifice of the C56/BL6 mice. Splenomegaly represents the known increase in monocytes, granulocytes, and erythrocytes in the spleen as a consequence of RSD (McKim et al., 2018). Spleen weights for all experiments can be seen in Figure 2 (A, B, E, F, and I). Spleens weights from RSD mice were significantly greater than spleens from Control mice for all experiments except Cohort 4 (Figure 2E). For Cohort 4, there was wide variation in spleen weight in both the RSD and Control groups, likely resulting in the non-significant difference between groups (p = .06). There were no significant outliers (> 3SD above or below the mean) and body weight did not account for these differences (p = .629). For Cohort 5 and 6, samples from only 3 out of 6 of the mice in the cohort were sent for sequencing. Thus, a separate analysis containing only the subset of mice sent for sequencing can be seen in Figure 2G and Figure 2J; the spleen weight for these subsets of mice were significantly different between RSD and Control groups (RSD > Control).

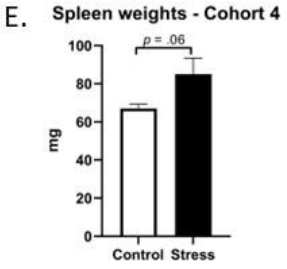
Cohort 1 – cAMP assay on splenocytes



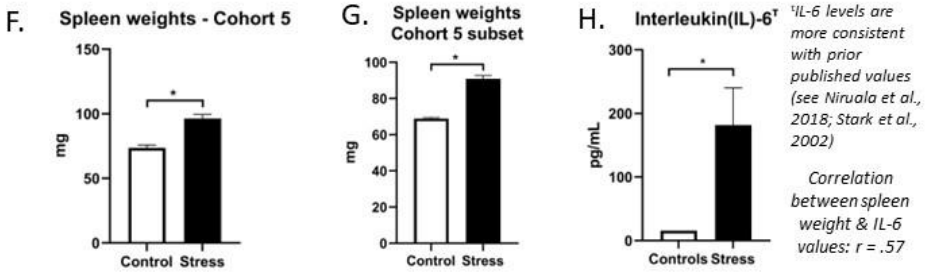
Cohort 2 – splenocyte lysates for Western Blot, plasma for IL-6 & SAP



Cohort 4 – cAMP assay on CD11b+ Peripheral Cells



Cohort 5 – CD11b+ Splenocytes for mRNA isolation; plasma for IL-6



Cohort 6 – CD11b+ Peripheral Cells for RNA isolation

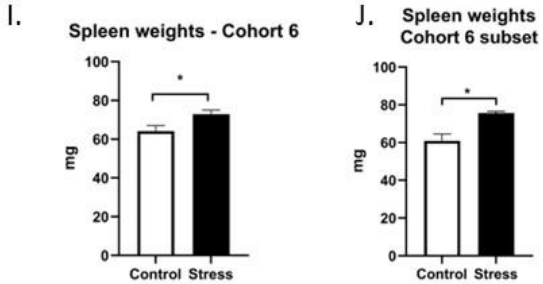


Figure 2. Confirmation of Stress Effects

Quantitative measures of stress in animals experiencing repeated social defeat (RSD/"Stress") include splenomegaly and elevated levels of peripheral pro-inflammatory proteins. This figure displays spleen weights (in mg; A, B, E, F, I) and concentration of peripheral pro-inflammatory proteins (IL-6 (C, H) and SAP (D), when measured) in peripheral plasma for control and RSD mice in all cohorts in the study.

Notes: *$p < .05$; cAMP = cyclic adenosine monophosphate; CD11b+ = cell surface protein marker indicating mouse monocytes and granulocytes; mRNA = messenger ribonucleic acid, IL= interleukin.

Cohort 1 (A): Splenocytes from these mice were used to measure levels of cAMP after stimulation with isoproterenol, a non-specific β-adrenergic agonist and forskolin, a non-specific activator of adenylate cyclase (AC), which in turn increases levels of intracellular cAMP. These mice were 7.5weeks old at sacrifice; there was no significant difference in body weight between groups (Control/Stress, $p > .05$).

A. RSD ("Stress") mice had significantly greater spleen weights, on average (mean = 86.22mg ± SE 4.25, N = 6), compared to Control mice (mean = 63.67mg ± SE 1.02, N = 6), indicating a significant stress effect.

Cohort 2 (B-D): Plasma from these mice was used to quantify levels of interleukin(IL)-6, an early response pro-inflammatory protein, and Serum Amyloid P-Component (SAP), a possible marker of the acute phase response in mice. Splenocytes from these mice were stimulated with isoproterenol, a non-specific β-adrenergic agonist and forskolin, a non-specific activator of adenylate cyclase (AC), and frozen for quantification of levels of β-arrestin-1 and 2, key signaling proteins in canonical and non-canonical β-adrenergic receptor signaling, respectively. These mice were 11.3 weeks at sacrifice, there was no significant difference in body weight between groups (Control/Stress, $p > .05$).

B. RSD ("Stress") mice had significantly greater spleen weights, on average (mean = 85.63mg ± SE 4.12, N = 6), compared to Control mice (mean = 67.44mg ± SE 4.67, N = 5), indicating a significant stress effect.

C. RSD ("Stress") mice had significantly greater levels of IL-6, on average (mean = 550.0pg/mL ± SE 114.2, N = 6), compared to Control mice (mean = 15.63pg/mL ± SE 0.00, N = 5), indicating a significant stress effect.

D. RSD ("Stress") mice had significantly greater levels of SAP, on average (mean = 48.84ug/mL ± SE 12.63, N = 6), compared to Control mice (mean = 1.18ug/mL ± SE 0.17, N = 5), suggesting longer-term inflammatory process as a consequence of stress than an elevation in IL-6.

Cohort 4 (E): CD11b+ peripheral cell from these mice were used to measure levels of cAMP after stimulation with isoproterenol, a non-specific β-adrenergic agonist and forskolin, a non-specific activator of adenylate cyclase (AC), which in turn increases levels of intracellular cAMP. Mice were 8.9 weeks at sacrifice, there was no significant difference in body weight between groups (Control/Stress, $p > .05$).

E. RSD ("Stress") mice had quantitatively greater spleen weights, on average (mean = 85.00mg ± SE 8.46, N = 6), compared to Control mice (mean = 67.03mg ± SE 2.28, N = 6); however, this difference was not statistically significant ($p = .06$).

Cohort 5 (F-H): RNA was isolated from CD11b+ splenocytes from these mice after stimulation with isoproterenol, a non-specific β-adrenergic agonist and forskolin, a non-specific

activator of adenylate cyclase (AC). These mice were 9.1 weeks at sacrifice, there was no significant difference in body weight between groups (Control/Stress, $p > .05$).

F. RSD ("Stress") mice had significantly greater spleen weights, on average (mean = 96.43mg ± SE 2.97, N = 6), compared to Control mice (mean = 73.70mg ± SE 2.19, N = 6), indicating a significant stress effect.

G. RSD ("Stress") mice in this subset had significantly greater spleen weights, on average (mean = 90.83mg ± SE 1.98, N = 3), compared to Control mice (mean = 68.93mg ± SE 0.59, N = 3), indicating a significant stress effect. The samples from the subset of mice in this graph were sent for poly-A library preparation and lc-RNA-seq (polyA = poly-A tail was used to amplify mRNA using oligo(dT) primers for mRNA library generation (the poly-A tail is a long chain of adenosine nucleotides added to 3' end RNA to prevent degradation and mark it for transport to ribosomes to be translated into protein); lc-RNASeq = mRNA library generation for limiting cell (lc = low input RNA amounts: 10pg-1ng) was performed using the Clontech SMART-Seq HT kit (Takara Bio Inc., Mountain View, CA, Cat #634456) and the the Nextera XT DNA Library Prep Kit (Illumina, Inc., San Diego, CA, Cat #FC-131-1096). RNA-Seq = Samples were then sequenced at a depth of 15-21million 2x100bp paired-end clusters on an Illumina NovaSeq6000 platform (Illumina, Inc., San Diego)).

H. RSD ("Stress") mice had significantly greater levels of IL-6, on average (mean = 181.9pg/mL ± SE 58.48, N = 6), compared to Control mice (mean = 15.63pg/mL ± SE 0.00, N = 6), indicating a significant stress effect.

Cohort 6 (I-J): RNA was isolated from CD11b+ peripheral cells from these mice after stimulation with isoproterenol, a non-specific β-adrenergic agonist and forskolin, a non-specific activator of adenylate cyclase (AC). These mice were 9 weeks at sacrifice, there was no significant difference in body weight between groups (Control/Stress, $p > .05$).

I. RSD ("Stress") mice had significantly greater spleen weights, on average (mean = 72.97mg ± SE 1.99, N = 6), compared to Control mice (mean = 64.18mg ± SE 2.82, N = 6), indicating a significant stress effect.

J. The samples from the subset of mice in this graph were sent for poly-A library preparation and lc-RNA-seq. polyA = poly-A tail was used to amplify mRNA using oligo(dT) primers for mRNA library generation (the poly-A tail is a long chain of adenosine nucleotides added to 3' end RNA to prevent degradation and mark it for transport to ribosomes to be translated into protein); lc-RNASeq = mRNA library generation for limiting cell (lc = low input RNA amounts: 10pg-1ng) was performed using the Clontech SMART-Seq HT kit (Takara Bio Inc., Mountain View, CA, Cat #634456) and the the Nextera XT DNA Library Prep Kit (Illumina, Inc., San Diego, CA, Cat #FC-131-1096). RNA-Seq = Samples were then sequenced at a depth of 15-21million 2x100bp paired-end clusters on an Illumina NovaSeq6000 platform (Illumina, Inc., San Diego). RSD ("Stress") mice in this subset had significantly greater spleen weights, on average (mean = 75.67mg ± SE 0.84, N = 3), compared to Control mice (mean = 60.93mg ± SE 3.54, N = 3), indicating a significant stress effect.

5.1.2 Measurement of peripheral inflammation

5.1.2.1 Measurement of IL-6.

The average intra-assay CV for samples in Cohort 2 was 2.65%; the average intra-assay CV for samples in Cohort 5 was 1.73%. There was only one plate of samples for each cohort so no inter-assay CV was calculated. Levels of IL-6 are an additional quantitative indicator of a significant stress effect in RSD animals. Levels of peripheral IL-6 were significantly greater in RSD mice vs Control mice in both Cohort 2 and Cohort 6 (Figure 2C, Figure 2H, *p*'s < .05). The average correlation between spleen weight and peripheral levels of IL-6 across both cohorts was r = .64.

5.1.2.2 Measurement of Serum Amyloid Protein (SAP).

The average intra-assay CV for samples in Cohort 2 was 6.34%. There was only one plate of samples so no inter-assay CV was calculated. Levels of peripheral SAP were significantly greater in RSD mice vs Control mice in Cohort 2 (Figure 2D, $p < .05$). The correlation between spleen weight and peripheral levels of SAP was r = .51. The correlation between peripheral levels of IL-6 and peripheral levels of SAP was r = .92.

5.2 Aim 1a.

Aim 1a assessed whether RSD induced β-AR "signal switching" by measuring the production of the second messenger cAMP on stimulation with isoproterenol among peripheral monocytes from RSD mice compared to controls. (*Note*: We only addressed CD11b+ monocytes

in the study due to technical issues obtaining enough Ly6c^{hi} monocytes for the stimulation assay; See Section 3.3.5). Cells for these assessments were harvested immediately after the end of the last cycle of RSD. *Hypothesis: CD11b+ cells from RSD mice vs. controls would show diminished cAMP accumulation on stimulation with isoproterenol, consistent with β-AR "signal switching."*

5.2.1 Splenocytes from RSD mice had lower cAMP accumulation on stimulation with isoproterenol compared to splenocytes from control mice

5.2.1.1 cAMP Assay Quality Control.

Samples from Control mice were paired with samples from RSD mice by spleen weight and run on the same plate. Samples from each mouse were run on the same plate in complete sets (i.e., RPMI, isoproterenol, and forskolin treated samples). The plates were developed with Ellman's reagent (in the dark) for approximately 60 minutes, or until the corrected maximum binding activity of the tracer (B_0 wells) was between .03 and 1.0 AU (blank subtracted). We found that this occurred around 60 minutes, which is less than the manufacturer's recommendation of 90-120 minutes. On average, the assay blank across plates at OD405 was .16 AU ± SE .002. After blank subtraction, the average non-specific binding activity of the assay tracer (NSB) was NSB = .005 AU ± SE .007, the average specific activity of the tracer (TA) was TA = 1.18 AU ± SE .001, and the average maximum binding of the tracer in the absence of free analyte (B_0) was B_0 = .56 AU ± SE .04. The average measure of the fit for the standard curve (R^2) was R^2 =.994 ± SE .001.

5.2.1.2 cAMP Assay Results: Control Mice

A one-way analysis of variance was performed on cAMP levels (pmol/mL) among samples from control mice as a function of treatment. There were 4 treatment conditions (RPMI, 1mM

IBMX, 10mM forskolin + 1mM IBMX, and 10mM isoproterenol + 1mM IBMX; see section 3.4.2). There was a significant difference in cAMP levels across treatment conditions ($F(3, 19) = 38.88$, $p < .0001$). Post-hoc pair-wise comparisons were performed using Holm-Sidak's multiple comparison test (Guo & Romano, 2007). There was no significant difference in cAMP levels between RPMI and IBMX treatment groups (RPMI = .032 pmol/mL ± SE .012, 1mM IBMX = .037 pmol/mL ± SE .009; $p = .942$; Figure 3B), indicating that IBMX had no independent effect on the assay. Compared to the IBMX treatment group, both forskolin and isoproterenol resulted in significantly greater accumulation of cAMP (forskolin vs IBMX, $p = .047$; isoproterenol vs IBMX, $p < .0001$; Figure 3B), indicating that both treatment conditions stimulated the production of cAMP. There was also a significant difference between isoproterenol and forskolin stimulation, with isoproterenol resulting in significantly greater cAMP levels compared to forskolin (10mM forskolin + 1mM IBMX = .196 pmol/mL ± SE .055, 10mM isoproterenol + 1mM IBMX = .645 pmol/mL ± SE .069; $p < .0001$; Figure 3B).

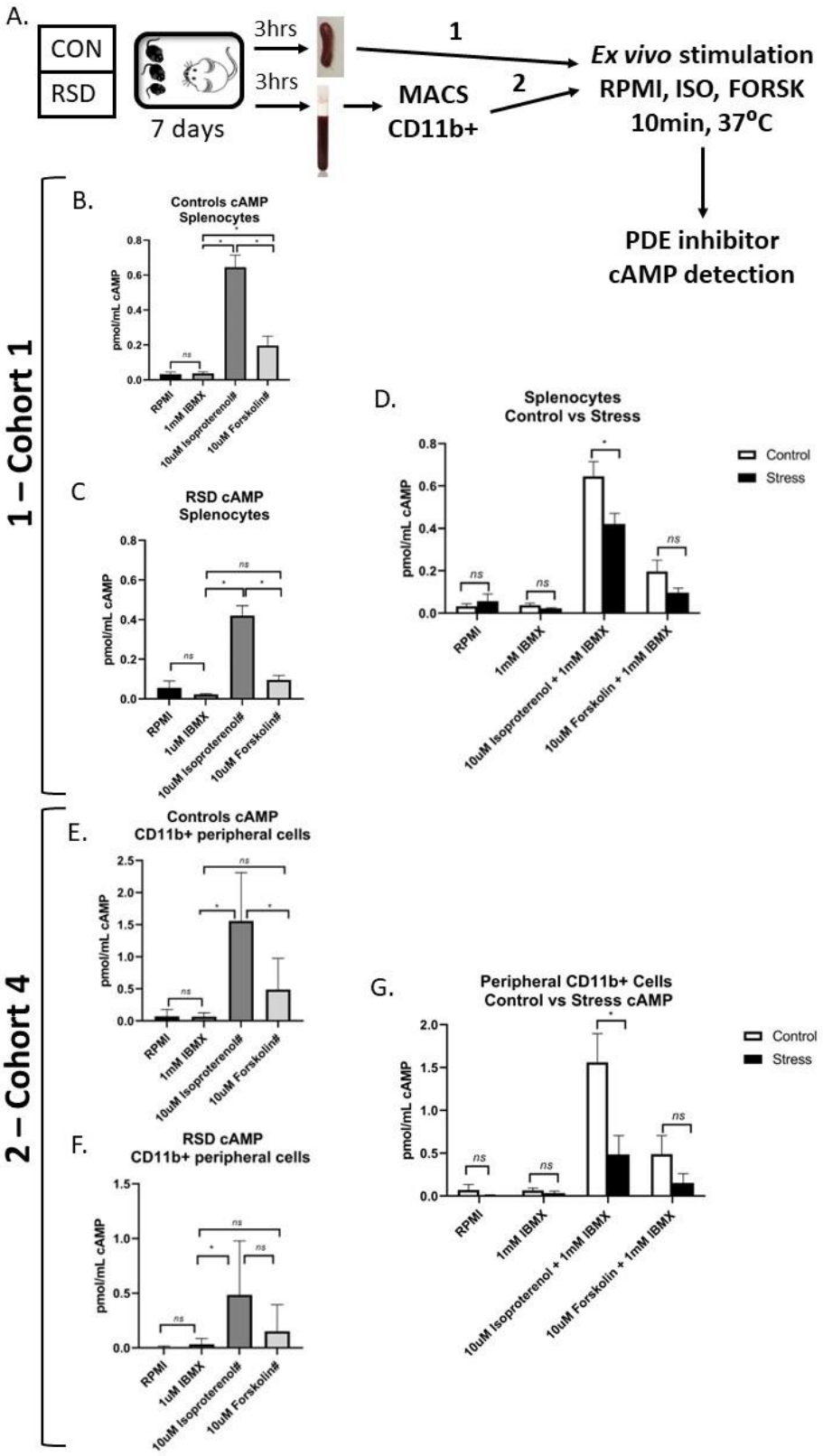


Figure 3. cAMP Assay

Levels of cyclic adenosine monophosphate (cAMP; pmol/mL) detected in splenocytes (Cohort 1) and CD11b+ peripheral cells (Cohort 4) that were unstimulated (RPMI 1640 cell culture media,* isobutylmethylxanthine *(IBMX) treatment conditions) or stimulated (isoproterenol (ISO) or forskolin (FORSK) treatment conditions) among animals in the control (CON; B&E) or Repeated Social Defeat (RSD/"Stress"; C&F) groups, and treatment conditions compared by animal group (Control/Stress; D&G).

Notes: $*p < .05$, ns = not significant ($p > .05$); CON = Control group; RSD = Repeated Social Defeat group, used interchangeably with "Stress" group; hrs = hours; MACS = Miltenyi Biotec buffer for cell separation; CD11b+ = cell surface protein marker indicating mouse monocytes and granulocytes*;* RPMI = Roswell Park Memorial Institute 1640, a cell culture media used in all experiments; ISO = isoproterenol, a non-specific β-adrenergic agonist; FORSK = forskolin, a non-specific activator of adenylate cyclase (AC); PDE = phosphodiesterase, an enzyme that breaks down cAMP to AMP; cAMP = cyclic adenosine monophosphate; IBMX = isobutylmethylxanthine, a PDE inhibitor; # = indicates the additional inclusion of 1mM IBMX in the culture media. All values are based on the average of 3 replicates per mouse, N = 6 mice in each CON vs RSD/Stress group.

A. Diagram of experiment. Either splenocytes (1) or CD11b+ cells isolated from peripheral blood (2) were either left unstimulated (RPMI 1640 cell culture media alone) or were subject to stimulation by isoproterenol (ISO), a non-specific β-adrenergic agonist, or forskolin (FORSK), a non-specific activator of adenylate cyclase (AC) for 10 minutes at 37ºC, in the presence of a phosphodiesterase (PDE) inhibitor (IBMX) to prevent the breakdown of cAMP after stimulation. cAMP was detected using a competitive enzyme linked immunoassay kit (ELISA; Cayman Chemical, Cat # 501040), according to the manufacturer's directions with acetylation of the samples and standards.

Cohort1: Splenocytes from Control (B) and RSD mice (C) were unstimulated (RPMI, IBMX treatment conditions) or stimulated with isoproterenol (ISO) or forskolin (FORSK) in the presence of a PDE inhibitor (IBMX) to prevent the breakdown of cAMP.

B. There was a significant difference in cAMP levels (pmol/mL) across treatment conditions among Control mice. Both forskolin and isoproterenol resulted in significantly greater accumulation of cAMP, compared to the IBMX condition. There was no significant difference in cAMP levels between RPMI and IBMX treatment groups, suggesting that IBMX had no independent effect on the assay. There was a significant difference between isoproterenol and forskolin stimulation, with isoproterenol resulting in significantly greater cAMP levels compared to forskolin.

C. There was a significant difference in cAMP levels (pmol/mL) across treatment conditions among RSD mice. Only the isoproterenol stimulation condition resulted in significantly greater accumulation of cAMP, compared to the IBMX condition. There was no significant difference in cAMP levels between RPMI and IBMX treatment groups, suggesting that IBMX had no independent effect on the assay. There was a significant difference between isoproterenol and forskolin stimulation, with isoproterenol resulting in significantly greater cAMP levels compared to forskolin.

D. There was a significant effect of stress on cAMP levels (pmol/mL). Specifically, cAMP levels were significantly lower in RSD samples treated with 10mM isoproterenol + 1mM IBMX compared to Control samples. No other comparisons were significant, suggesting specificity for

decreased cAMP on stimulation with isoproterenol in splenocytes from stressed mice compared to controls.

Cohort 4: CD11b+ cells from peripheral blood of Control (E) and RSD mice (F) were unstimulated (RPMI, IBMX treatment conditions) or stimulated with isoproterenol (ISO) or forskolin (FORSK) in the presence of a PDE inhibitor (IBMX) to prevent the breakdown of cAMP.

E. There was a significant difference in cAMP levels (pmol/mL) across treatment conditions among control mice. Only the isoproterenol stimulation condition resulted in significantly greater accumulation of cAMP, compared to the IBMX condition. There was no significant difference in cAMP levels between RPMI and IBMX treatment groups, suggesting that IBMX had no independent effect on the assay. There was a significant difference between isoproterenol and forskolin stimulation, with isoproterenol resulting in significantly greater cAMP levels compared to forskolin.

F. There was a significant difference in cAMP levels (pmol/mL) across treatment conditions among RSD mice ($H(4) = 11.63$, $p = .008$). Only the isoproterenol stimulation condition resulted in significantly greater accumulation of cAMP, compared to the IBMX condition. There was no significant difference in cAMP levels between RPMI and IBMX treatment groups, suggesting that IBMX had no independent effect on the assay. There was no significant difference between isoproterenol and forskolin stimulation conditions.

G. There was a significant effect of stress on cAMP levels (pmol/mL). Specifically, cAMP levels were significantly lower in RSD samples treated with 10mM isoproterenol + 1mM IBMX compared to Control samples. No other comparisons were significant, suggesting specificity for decreased cAMP on stimulation with isoproterenol in CD11b+ peripheral cells from stressed mice compared to controls.

5.2.1.3 cAMP Assay Results: RSD Mice

A one-way analysis of variance was also performed on cAMP levels (pmol/mL) among samples from RSD mice as a function of treatment. Among samples from RSD mice, there was a significant difference in cAMP levels across treatment conditions ($F(3, 20) = 33.14$, $p < .0001$). Post-hoc pair-wise comparisons using Holm-Sidak's multiple comparison test (Guo & Romano, 2007) revealed no significant difference between RPMI and IBMX treatment groups (RPMI = .056 pmol/mL ± SE .035, 1mM IBMX = .023 pmol/mL ± SE .004; $p = .758$; Figure 3C), indicating that IBMX had no independent effect on the assay among RSD samples. While forskolin samples had numerically greater cAMP levels compared to IBMX samples (unadjusted $p = .017$), this difference was not significant after correction for multiple comparisons ($p = .222$; Figure 3C). In contrast, isoproterenol samples resulted in significantly greater cAMP accumulation compared to

IBMX samples ($p < .0001$; Figure 3C). There was also a significant difference in cAMP accumulation between isoproterenol and forskolin stimulated samples ($p < .0001$), with isoproterenol resulting in significantly greater cAMP levels compared to forskolin (10mM forskolin + 1mM IBMX = .097 pmol/mL ± SE .021; 10mM isoproterenol + 1mM IBMX = .421 pmol/mL ± SE .049; Figure 3C).

5.2.1.4 cAMP Assay Results: RSD Mice vs Control Mice

To compare treated samples from Control mice to treated samples from RSD mice, we used independent samples t-tests with a single pooled variance across samples. To correct for multiple comparisons, we used the two-stage step-up method of Benjamin, Krieger, and Yekutieli (Benjamini, Krieger, & Yekutieli, 2006). Using this correction for multiple comparisons, there was no significant difference in cAMP levels between Control and RSD samples treated with RMPI ($p = .602$; Figure 3D) or 1mM IBMX ($p = .602$; Figure 3D). There was also no significant difference in cAMP accumulation between Control and RSD samples treated with 10mM forskolin + 1mM IBMX ($p = .122$; Figure 3D). In contrast, there was a significant difference between Control and RSD samples treated with 10mM isoproterenol + 1mM IBMX ($p < .001$), with cAMP levels significantly lower in samples from RSD mice (mean = .421 pmol/mL ± SE .049) compared to Control mice (mean = .645 pmol/mL ± SE .069; Figure 3D).

5.2.2 Peripheral CD11b+ cells from RSD mice had lower cAMP accumulation on stimulation with isoproterenol compared to peripheral CD11b+ cells from control mice

Note: Although we report results for the cAMP assay in peripheral CD11b+ cells, these findings should be interpreted with caution given that the difference in spleen weight between RSD mice and Control mice for this cohort was not statistically significant (p = .06; Figure 2E).

5.2.2.1 cAMP Assay Quality Control

Samples were assayed for cAMP levels as described in Section 5.2.1.1. One modification to the treatment protocol from Section 3.4.2 was made; specifically, the cell pellet after stimulation was resuspended in .160mL 50 mM sodium acetate buffer, rather than 0.5 mL. This modification was made for ease of acetylation prior to measurement of cAMP and to reduce the number of freeze-thaw cycles for samples. Of note, the values from the cAMP assay reported in peripheral cells have been transformed mathematically (multiplied by .32) to match the cAMP concentrations measured in splenocytes. For the cAMP assays in peripheral CD11b+ cells, the average assay blank across plates was .06 AU ± SE .055 (OD405). After blank subtraction, the average non-specific binding activity of the assay tracer (NSB) was NSB = -.009 AU ± SE .005, the average specific activity of the tracer (TA) was TA = AU 1.44 ± SE .019, and the average maximum binding of the tracer in the absence of free analyte (B_0) was B_0 = .99 AU ± SE .005. The average measure of the fit for the standard curve (R^2) was R^2 = .990 ± SE .005.

5.2.2.2 cAMP Assay Results: Control Mice

A one-way analysis of variance was performed on transformed cAMP levels (pmol/mL) among samples from Control mice as a function of treatment. There were 4 treatment conditions (RPMI, 1mM IBMX, 10mM forskolin + 1mM IBMX, and 10mM isoproterenol + 1mM IBMX; see section 3.4.2). Of note, there were not enough cells for technical replicates in the RPMI treatment condition for CD11b+ peripheral cells from two Control mice (see Section 3.3.4 for planned reduction in samples).

Among cells from Control mice, there was a significant difference in cAMP levels across treatment conditions ($F(3, 14) = 9.99$, $p = .0009$). Post-hoc pair-wise comparisons using Holm-Sidak's multiple comparison test (Guo & Romano, 2007) found no significant difference in cAMP levels between RPMI and IBMX treatment groups (RPMI = .084 pmol/mL ± SE .074; 1mM IBMX = .077 pmol/mL ± SE .032; $p = .987$; Figure 3E), indicating that IBMX had no independent effect on the assay. While forskolin samples had numerically greater cAMP levels compared to IBMX samples, this difference was not significant ($p = .338$; Figure 3E). In contrast, isoproterenol samples resulted in significantly greater accumulation of cAMP compared to IBMX samples ($p = .0009$; Figure 3E). There was also a significant difference in cAMP accumulation between isoproterenol and forskolin stimulated samples ($p = .01$), with isoproterenol resulting in significantly greater cAMP levels compared to forskolin (10mM forskolin + 1mM IBMX = .573 pmol/mL ± SE .255; 10mM isoproterenol + 1mM IBMX = 1.83 pmol/mL ± SE .394; Figure 3E).

5.2.2.3 cAMP Assay Results: RSD Mice

A one-way analysis of variance was also performed on cAMP levels (pmol/mL) among samples from RSD mice as a function of treatment. Of note, there were not enough cells for technical replicates in the RPMI treatment condition for one set of samples from RSD mice (see

Section 3.3.4 for planned reduction in samples due to low cell number). In addition, these samples did not meet the assumption of normality of residuals (Shapiro-Wilk's test, $p = .002$). Thus, we used a non-parametric Kruskal-Wallis test with Dunn's multiple comparison test for post-hoc comparison between samples (Dunn, 1964).

Among samples from RSD mice, there was a significant difference in cAMP levels across treatment conditions ($H(4) = 11.63$, $p = .008$). Post-hoc pair-wise comparisons revealed no significant difference between RPMI and IBMX treatment groups (RPMI = .010 pmol/mL ± SE .006, 1mM IBMX = .039 pmol/mL ± SE .028; $p > .999$; Figure 3F), indicating that IBMX had no independent effect on the assay among RSD samples. While forskolin samples had numerically greater cAMP accumulation compared to IBMX samples, this difference was not significant ($p = .222$; Figure 3F). In contrast, isoproterenol samples resulted in significantly greater accumulation of cAMP compared to IBMX samples ($p = .039$; Figure 3F). Interestingly, there was no significant difference in cAMP accumulation between isoproterenol and forskolin stimulated samples (10mM forskolin + 1mM IBMX = .178 pmol/mL ± SE .127, 10mM isoproterenol + 1mM IBMX = .569 pmol/mL ± SE .259; $p = .462$; Figure 3F).

5.2.2.4 cAMP Assay Results: RSD Mice vs Control Mice

To compare treated CD11b+ peripheral cells from Control mice to treated CD11b+ peripheral cells from RSD mice, we used independent samples t-tests with a single pooled variance across samples. To correct for multiple comparisons, we used the two-stage step-up method of Benjamin, Krieger, and Yekutieli (Benjamini, Krieger, & Yekutieli, 2006). Using this correction for multiple comparisons, there was no significant difference between Control and RSD samples treated with RMPI ($p = .680$; Figure 3G) or 1mM IBMX ($p = .680$; Figure 3G). There was also no significant difference in cAMP accumulation between Control and RSD samples treated with

10mM forskolin + 1mM IBMX (p = .281; Figure 3G). In contrast, there was a significant difference between Control and RSD samples treated with 10mM isoproterenol + 1mM IBMX (p = .0005), with cAMP levels significantly lower in samples from RSD mice (mean = .569 pmol/mL ± SE .259) compared to Control mice (mean = 1.83 pmol/mL ± SE .394; Figure 3G).

5.3 Aim 1b

Aim 1b intended to assess whether RSD induced β-AR "signal switching" by measuring the presence of key signaling proteins β-arrestin-1 and 2 among peripheral monocytes from RSD mice compared to controls. (*Note*: We only addressed CD11b+ monocytes in the document due to technical issues obtaining enough Ly6c^{hi} monocytes for the stimulation assays; See Section 3.3.5). Cells for these assessments were harvested immediately after the end of the last cycle of RSD. *Hypothesis: CD11b+ cells from RSD mice vs. controls would show greater β-arrestin-2 utilization vs β-arrestin-1 consistent with β-AR "signal switching."*

Brain cerebellum samples were used as a positive control for β-arrestin-1 and β-arrestin-2 antibodies (Figures 4A, 4C, and 5A) as these proteins are expressed in high abundance in brain tissue (Lefkowitz, 1998). Confirmation of secondary antibody binding (Donkey-α-Rabbit) was visualized using Rabbit IgG protein as a positive control (Figure 4A). Loading control proteins included Cofilin (20kDa) and GAPDH (35kDa; although this protein was not visualized in Figure 4C due to technical error).

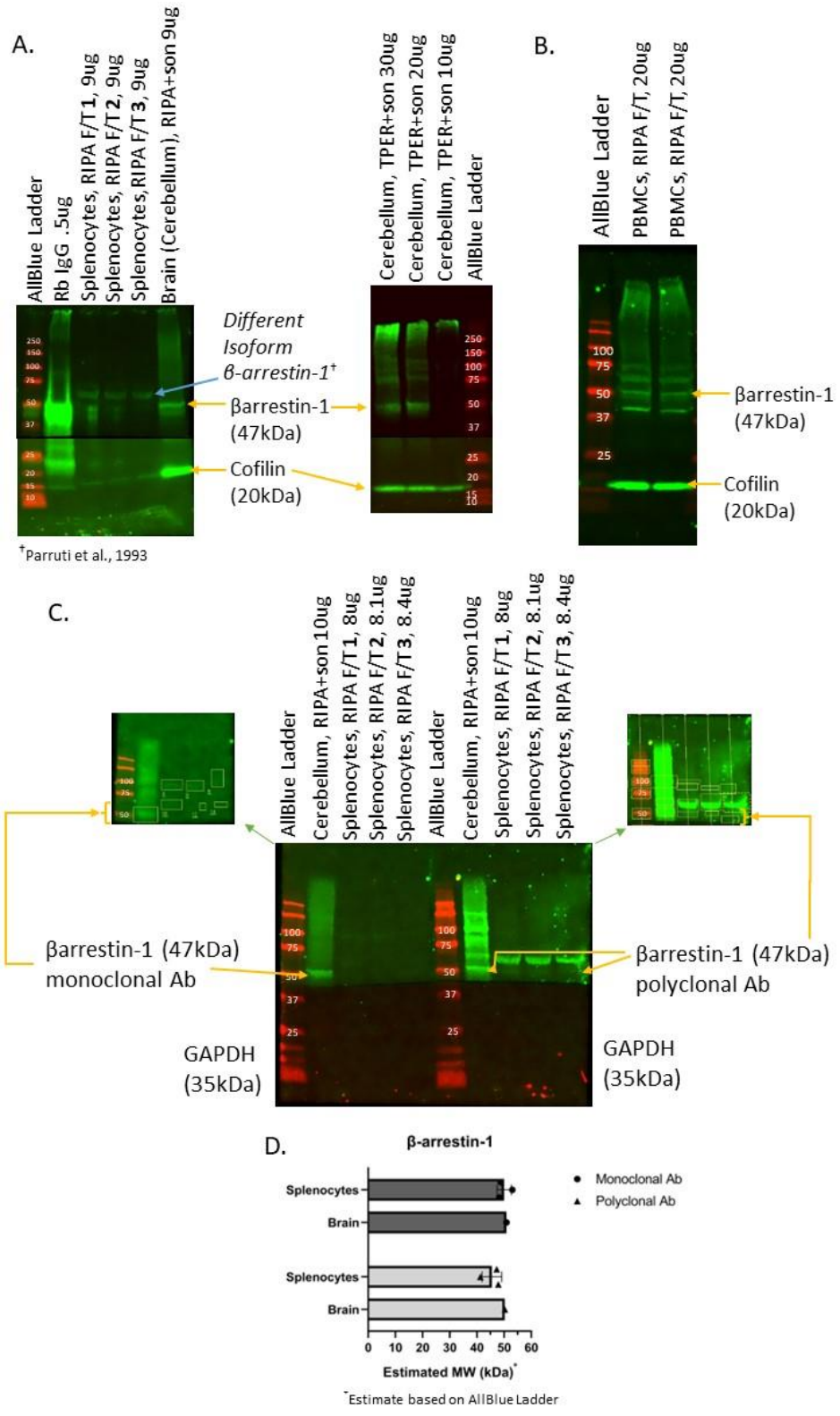


Figure 4. β-arrestin-1 protein

Visualization of the β-arrestin-1 protein in splenocytes (A, C) and peripheral mononuclear cells (B) using Western Blot technique. β-arrestin-1 is a key signaling protein involved in canonical β-adrenergic receptor signaling.

Notes: AllBlue Ladder = Protein Standard Marker used to estimate the molecular weight of unknown proteins (Precision Plus All Blue Protein Standard (BioRad Cat#161-0373)); Rb = Rabbit, IgG = Immunoglobin G, Rb IgG = secondary antibody loading control protein, used to confirm binding of the secondary antibody; RIPA = Cell lysis buffer (RIPA buffer, Thermo # 89901; 1% Protease Inhibitor, Thermo #87786; 1% Phosphatase Inhibitor, Thermo # 78420); F/T = cells were lysed by two cycles of freezing (dry ice) and thawing (37°C water bath) for 10 min each; son = cells were lysed by sonication; TPER = Tissue lysis buffer (TPER buffer, Thermo #78510; 1% Protease Inhibitor, Thermo #87786; 1% Phosphatase Inhibitor, Thermo # 78420); PMBCs = peripheral blood mononuclear cells; kDa = kilo-Daltons, a measure of molecular weight (MW); Ab = antibody; Cofilin = protein loading control, used to confirm that protein loading is the same across the gel; GAPDH = protein loading control, used to confirm that protein loading is the same across the gel. Protein concentration for splenocyte, PBMC, and brain cerebellar samples were measured using BCA assay ((Thermo Cat#23225) with the lysate buffer as a standard diluent). All proteins were loaded onto pre-cast mini-PROTEAN gels (10% gradient, 50uL wells, BioRad Cat # 4561034) under reducing conditions (4x Lammeli Sample Buffer BioRad Cat #1610747, 2-mercaptoethanol (BioRad Cat#1610710 [10% of volume, final conc: 1.42M]), denatured at 70°C for 20min). Gels were run at 50-80V using Tris-Glycine-SDS Running Buffer (BioRad Cat# 1610772). Protein was transferred onto nitrocellulose membranes and blocked using Tris-Buffered-Saline (TBS). Imaging was performed using the Odyssey LICOR system.

- **A.** Western blots demonstrate presence of β-arrestin-1 protein in bulk splenocytes, and brain cerebellar samples using a polyclonal antibody. Confirmation of secondary antibody binding is demonstrated using Rb IgG. Cofilin (20kDa) was used as a loading control protein. The amount of total protein loaded onto the gel is indicated in the figure (in ug). Primary antibodies used: Rb-α-B-arr1 Abcam#31868 (47kDa) 1:1000, Rb-α-Cofilin Abcam#42824 (20kDa) 1:1000; secondary antibody used: Dk-α-Rabbit 800CW 1:5000 LI-COR#926-32213.
- **B.** Western Blot demonstrates the presence of β-arrestin-1 protein in PBMCs using a polyclonal antibody. The amount of protein loaded onto the gel is indicated in the figure (in ug). Primary and secondary antibodies used were the same as in A.
- **C.** Western Blot using a monoclonal antibody confirms presence of β-arrestin-1 protein in bulk splenocytes and brain cerebellar samples. The amount of protein loaded onto the gel is indicated in the figure (in ug). Primary antibodies used: Monoclonal Rb-α-B-arr1 Abcam#32099 [clone E274] 1:1000 vs. Polyclonal α-B-arr1 Abcam#31868 (47kDa) 1:1000, Ms-α-GAPDH; secondary antibodies used: Dk-α-Rabbit 800CW 1:5000 LI-COR#926-32213, Dk-α-Mouse 680RD LI-COR#925-32210
- **D.** Based on the Western Blot in C, LICOR software was used to detected the bands of interest. The AllBlue protein standard ladder was used to estimate their molecular weight. In splenocytes, the monoclonal antibody detected a band for β-arrestin-1 at an average molecular weight of 50kDa ± SD 2.62 across three replicate samples, and the polyclonal antibody detected a band for β-arrestin-1 at an average molecular weight of 45.5kDa ± SD

3.65 across three replicate samples. These findings suggest that the proteins identified as β-arrestin-1 are similar in MW and are likely identifying the same isoform of β-arrestin-1.

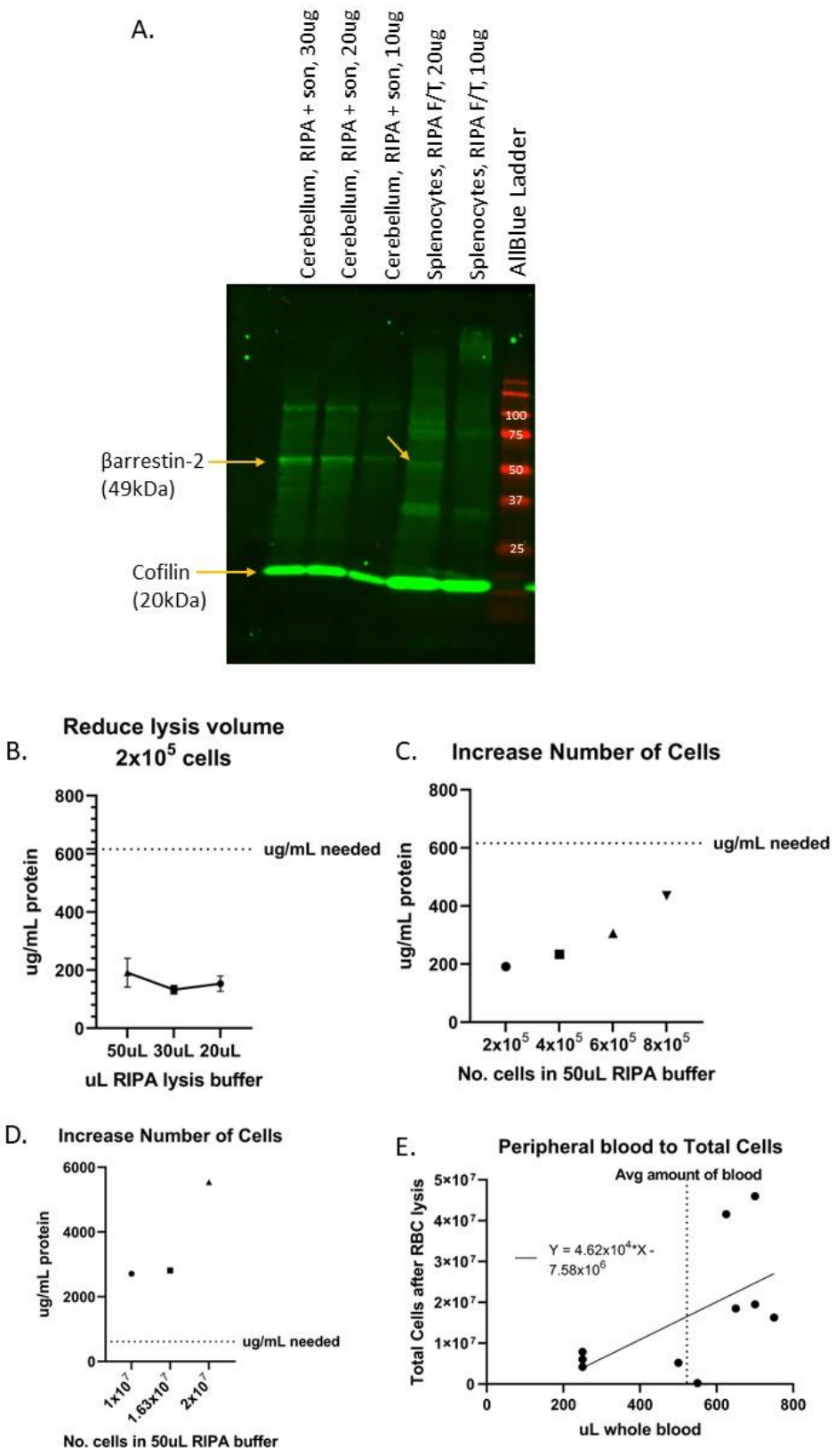


Figure 5. β-arrestin-2 protein

Visualization of the β-arrestin-2 protein in splenocytes (A) using Western Blot technique. β-arrestin-2 is a key signaling protein involved in non-canonical β-adrenergic receptor signaling. Pilot studies were designed to increase the concentration of protein from cell lysates to 616ug/mL (B-E).

Notes: AllBlue Ladder = Protein Standard Marker used to estimate the molecular weight of unknown proteins (Precision Plus All Blue Protein Standard (BioRad Cat#161-0373)); RIPA = Cell lysis buffer (RIPA buffer, Thermo # 89901; 1% Protease Inhibitor, Thermo #87786; 1% Phosphatase Inhibitor, Thermo # 78420); F/T = cells were lysed by two cycles of freezing (dry ice) and thawing (37°C water bath) for 10 min each; son = cells were lysed by sonication; kDa = kilo-Daltons, a measure of molecular weight (MW); Cofilin = protein loading control, used to confirm that protein loading is the same across the gel. Protein concentration for splenocyte and brain cerebellar samples were measured using BCA assay ((Thermo Cat#23225) with the lysate buffer as a standard diluent. All proteins were loaded onto pre-cast mini-PROTEAN gels (10% gradient, 50uL wells, BioRad Cat # 4561034) under reducing conditions (4x Lammeli Sample Buffer BioRad Cat #1610747, 2-mercaptoethanol (BioRad Cat#1610710 [10% of volume, final conc: 1.42M]), denatured at 70°C for 20min). Gels were run at 50-80V using Tris-Glycine-SDS Running Buffer (BioRad Cat# 1610772). Protein was transferred onto a nitrocellulose membrane and blocked using Tris-Buffered-Saline (TBS). Imaging was performed using the Odyssey LICOR system

A. Western Blot demonstrates the presence of β-arrestin-2 protein in bulk splenocytes and brain cerebellar samples using a polyclonal antibody. The amount of protein loaded onto the gel is indicated in the figure. Primary antibodies used: Rb-α-B-arr2 Thermo#PA1-732 (49kDa) 1:1000, Rb-α-Cofilin Abcam#42824 (20kDa) 1:1000; secondary antibody used: Dk-α-Rabbit 800CW 1:5000 LI-COR#926-32213.

We performed a number of pilot studies designed to increase the concentration of protein from cell lysates. Our goal was to obtain a protein concentration of 616ug/mL to load 20ug into one well (50uL) of the gel to obtain a reasonable blot for β-arrestin-1 and β-arrestin-2.

B. Graph of protein concentration (ug/mL) in cell lysates of $2x10^5$ cells using decreasing lysate volumes (uL RIPA buffer).
C. Graph of protein concentration (ug/mL) in cell lysates of increasing number of cells (double, triple, or quadruple the number of cells) in the same lysate volume (50uL RIPA buffer).
D. Graph of protein concentration (ug/mL) in cell lysates of excessive number of cells in the same lysate volume (50uL RIPA buffer).
E. Graph of blood draw volume (uL whole blood) to total cell count after red blood cell (RBC) lysis. Number of cells in an average blood draw = $30.95x10^6$ cells.

5.3.1 β-arrestin-1 protein is likely present in splenocytes and peripheral blood cells

Using a polyclonal antibody for β-arrestin-1 (Abcam#31868), we were able to visualize β-arrestin-1 among positive control samples (brain cerebellum; Figure 4A, Figure 4C), bulk splenocytes (Figures 4A, 4C), and peripheral blood mononuclear cells (Figure 4B). We were able to visually confirm that the protein band we were blotting at ~47kDa was indeed β-arrestin-1 using a monoclonal antibody for β-arrestin-1 (Abcam#32099; Figure 4C). Because the visualization of β-arrestin-1 was less clear among low abundance splenocyte samples (~8-9ug of total protein), we used the LICOR software to detect the bands of interest and estimate their molecular weight based on the AllBlue protein standard ladder (Figure 4C, 4D). We found that the monoclonal antibody detected a band for β-arrestin-1 at an average molecular weight of 50kDa ± SD 2.62 across three replicate samples, and the polyclonal antibody detected a band for β-arrestin-1 at an average molecular weight of 45.5kDa ± SD 3.65 across three replicate samples (Calculated molecular weight of protein = 47kDa; Parruti et al., 1993; Shenoy et al., 2006).

5.3.2 β-arrestin-2 protein is likely present in splenocytes, unknown in peripheral blood cells

Using a polyclonal antibody for β-arrestin-2 (Thermo#PA1-732), we were able to visualize β-arrestin-2 among positive control samples (brain cerebellum; Figure 5A) and bulk splenocytes (Figure 5A) using high abundance splenocyte samples (20ug of total protein). Due to technical error, we did not visualize β-arrestin-2 in peripheral blood mononuclear cells.

Visualization or quantification of β-arrestin-1 and β-arrestin-2 proteins was not attempted for stimulated samples of interest due to low total protein starting amounts. Stimulated mouse lysates were frozen as described in Section 3.4.3. Triplicate 65uL samples saved for each mouse

for Western Blot (~195uL total). Based on pilot studies, the protein concentrations for these samples are likely in the 100-250ug/mL range, resulting in a total of 19ug – 48ug protein in the entire 195uL sample. The largest wells for the pre-cast mini-PROTEAN gels are 50uL. The maximum amount of sample that can be loaded into a 50uL well is 32.5uL (together with 12.5uL 4x sample buffer and 5uL reducing agent), resulting in a total of 3.25ug - 8.1ug protein per well. We used this amount of protein in the pilot studies for bulk splenocytes (Figures 4A, 4C) and found that our ability to visualize β-arrestin-1 was limited, thus rendering our ability to detect subtle differences in expression to be limited as well.

We performed a number of pilot studies designed to increase the concentration of protein. We found that a concentration of 616ug/mL total protein to load 20ug into one well resulted in a reasonable blot for β-arrestin-1 and β-arrestin-2 (Figures 4A, 4B, 5A). We first reduced the volume of lysate, but found that this did not substantially increase the concentration of total protein (Figure 5B). We then increased the number of cells in 50uL of lysis buffer, but we were not able to obtain sufficient concentration with double, triple, or quadruple the number of cells in the lysate (Figure 5C). Although it may be possible to further increase the number of cells in the lysate (we obtained higher concentrations with a higher number of cells; Figure 5D), both scaling up the stimulation assay and obtaining enough cells for multiple blots per sample would be problematic, especially for peripheral cells (~3.1×10^7 total cells after RBC lysis from an average blood draw; Figure 5E). Thus, visualization and/or quantification the β-arrestin proteins using Western Blot in samples from Cohort 2 was not attempted.

5.4 Aim 1c

Aim 1c assessed whether RSD induced β-AR "signal switching" by measuring differential gene expression on stimulation with isoproterenol among peripheral monocytes from RSD mice compared to controls. (*Note*: We only address CD11b+ cells [monocytes/granulocytes] in the study due to technical issues with obtaining enough $Ly6c^{hi}$ monocytes for the stimulation assay; See Section 3.3.5). Cells for these assessments were harvested immediately after the end of the last cycle of RSD. Gene transcription was amplified using mRNA Illumina NextGen Sequencing, with subsequent grouping of differential gene expression by key transcription factors (e.g., NFkB, CREB, MAPK). *Hypothesis: CD11b+ cells from RSD mice vs. controls would show an upregulation of proinflammatory genes consistent with β-AR "signal switching" (i.e., ↑MAPK and ↓CREB mediated gene transcription). Consistent with the idea that the non-canonical pathway in RSD mice is amplified on stimulation with isoproterenol, the upregulation of proinflammatory genes and utilization of the non-canonical β-AR signaling pathway was predicted to be greater in the isoproterenol-stimulated RSD condition than the non-stimulated condition.*

The mean sequence alignment rate for all samples was 79.3% ± SE .48%, with a range from 71.4% to 83.6%. The duplication rate was 20.9% ± SE .32%, with a range from 17.1% to 24.2%.

5.4.1 RSD_R vs CON_R: Consistent with our hypothesis, RSD promoted a primed and pro-inflammatory gene expression profile in both CD11b+ splenocytes and peripheral cells

5.4.1.1 Spleen

Among CD11b+ splenocytes from RSD and Control mice, there was differential expression of 3,601 mRNA gene transcripts (1,594 upregulated; 2,007 downregulated, $p_{adj} < .05$; Figure 6D). Upregulated genes included many associated with a primed and pro-inflammatory gene expression profile (Figure 7). For example, upregulated genes included those of myeloid origin (*CD14, Ly6c1, Ly6c2, Ly6g, CD33, CD200r1, CD164, Stat3*), those involved in cytokine and chemokine regulatory signaling (*CCR1, IL1b, IL-15, IL18rap (an IL-6 alias*; *IL1rap, IL13ra1*), and genes involved in immune and inflammatory-related signaling (*Ager, Alox5, Mmp9*), including pathogen, Ig or antigen related signaling (*Fcgr1, Fcgr2b, Fcgr3, Fcgr4, Myd88, Irak3, Kif1b, Kif2c, Kif4, Kif11, Kif20a, Kif22, Kifc1, Rab3d, Rab24, Rab32, Tirap*). Upregulated genes also included those involved in cell cycling and proliferation (*Cdc20, Cdca3, Cdca5, Cdca8, Cdkn2d, Cdkn3, Ccna2, Ccnb1, Ccnb2,* Ccnd3, *G0s2, Pbx1, Pbx2, Rnaseh2c, Top2a*), signal transduction (*Aurka, Aurkb, Cebpd, Klf5, Klf7, Lmo4, Rab27a, Rac1, Rac2, Rasgrp4, Socs3*), and extracellular matrix remodeling (*Mmp8*). Among genes involved in glucocorticoid signaling, a gene important in inhibition of GR translocation to the nucleus was significantly upregulated (*Fkbp5*), which is consistent with prior work demonstrating the development of glucocorticoid resistance in these cells (Niraula et al., 2018). Notably, there was no significant difference in expression of the GR gene (*Nr3c1),* which has previously served as a negative control for both gene expression and protein levels in RSD mice (Powell et al., 2013, Bailey 2009, Engler 2008; Quan 2003).

Overall, there were fewer downregulated genes involved in primed and pro-inflammatory signaling profiles among CD11b+ splenocytes from RSD mice vs controls (Figure 7). These downregulated genes included those involved in myeloid and early monocytic lineage (*Cd40, Cd55, Csf1r, Cx3cr1, Cd209a, Fosb*), those involved in cytokine and chemokine regulatory signaling (*Tgfb1, IL12a, Ifng, Pdgfb*), and genes involved in immune, pathogen, Ig, and antigen signaling (*Mef2c, Lta, Ltb, H2-Eb1* (*MHCII protein*), *Rab3ip*). There was also downregulation of a gene involved in cell cycling and proliferation (*Ccdc122*), and a gene involved in signal transduction (*Rasgrp1*).

Using both significantly up and downregulated genes ($p_{adj} < .05$), IPA identified a significant upregulation in pathways involved in pro-inflammatory signaling among RSD mice vs Controls (p's < .005; Figure 6E, Figures 8A-D). These pathways included IL-6 signaling (Z=2.75; Figure 8A), toll-like receptor (TLR) signaling (Z=2.50; Figure 8B), NF-kB signaling (Z=2.21; Figure 8C), and chemokine signaling (Z=1.21; Figure 8D). This evidence suggests that RSD promotes a primed and pro-inflammatory gene expression profile among CD11b+ splenocytes.

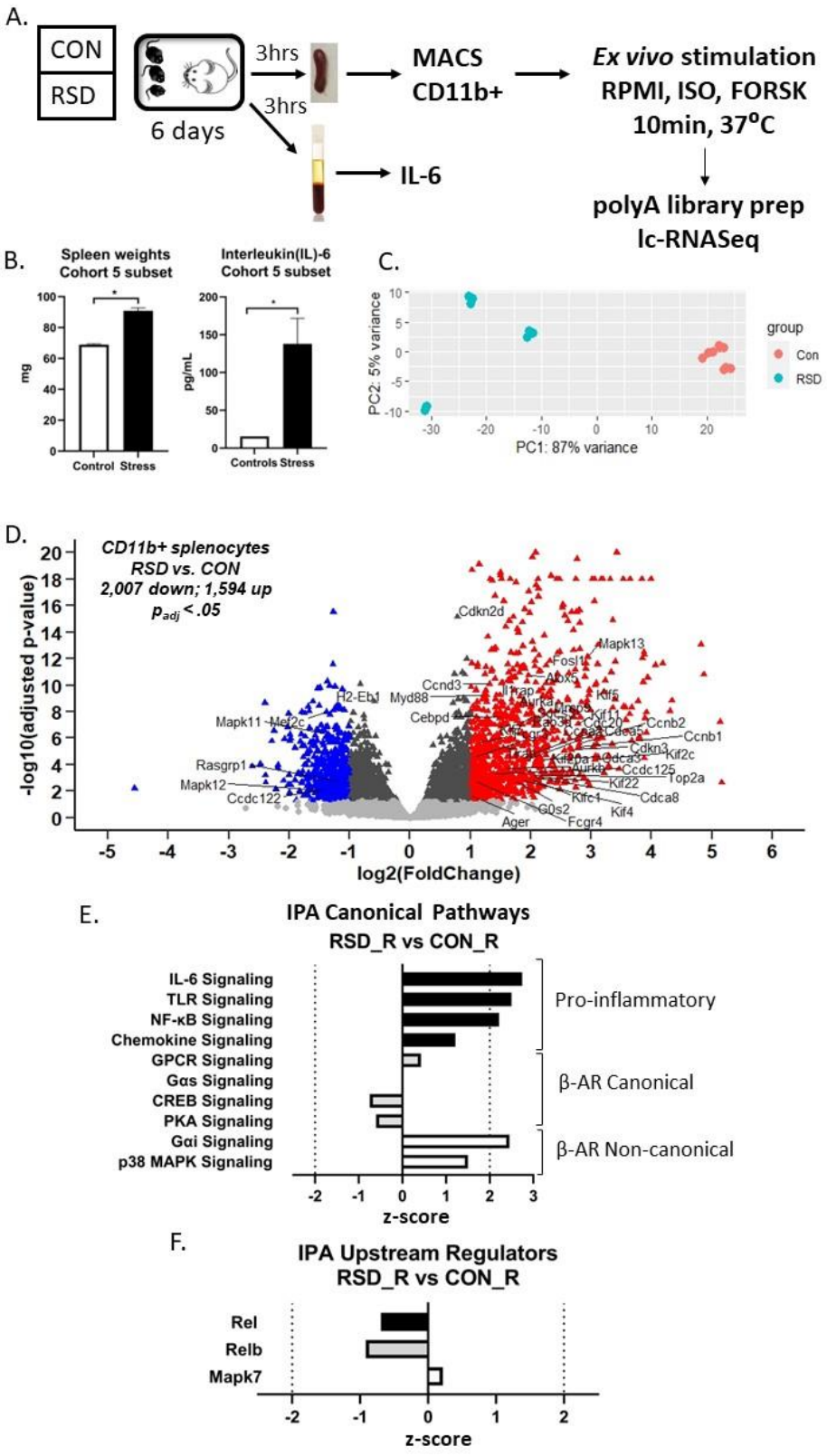


Figure 6. Stress Effect: Splenocytes

The experiment (described in A) resulted in a significant effect of Repeated Social Defeat (RSD) on splenomegaly (B), peripheral pro-inflammatory proteins (B), and differential gene transcription (C, D). Differentially regulated genes included those involved in pro-inflammatory signaling pathways (E, F), as well as β-adrenergic receptor (β-AR) canonical and non-canonical signaling pathways (E, F).

A. Diagram of experiment. Male C56BL/6 mice (6-8 weeks old) underwent repeated social defeat (RSD) for 6 days or were left undisturbed in their cages in the control room (CON) until sacrifice. Mice were sacrificed within three hours of the last round of social defeat. After sacrifice, the spleen and peripheral blood was harvested from the mice for isolation of CD11b+ cells and plasma, respectively. CD11b+ splenocytes were isolated using Miltenyi Biotec CD11b+ Microbeads (Miltenyi Biotec Cat #130-049-60). Levels of IL-6 were measured using a quantitative sandwich enzyme immunoassay kit (BD OptEIA Mouse IL-6 ELISA Cat#555220; BD Biosciences, San Jose, CA), according to the manufacturer's directions. CD11b+ splenocytes were either left unstimulated (RPMI 1640 cell culture media alone) or were subject to stimulation by isoproterenol (ISO), a non-specific β-adrenergic agonist, or forskolin (FORSK), a non-specific activator of adenylate cyclase (AC) for 10 minutes at 37ºC. RNA was then isolated. A subset of these samples (N = 3 in each group CON/RSD) were sent for polyA library preparation (poly-A tail was used to amplify mRNA using oligo(dT) primers for mRNA library generation; the poly-A tail is a long chain of adenosine nucleotides added to 3' end RNA to prevent degradation and mark it for transport to ribosomes to be translated into protein) and lc-RNAseq (mRNA library generation for limiting cell (lc = low input RNA amounts: 10pg-1ng) was performed using the Clontech SMART-Seq HT kit (Takara Bio Inc., Mountain View, CA, Cat #634456) and the Nextera XT DNA Library Prep Kit (Illumina, Inc., San Diego, CA, Cat #FC-131-1096). RNA-Seq = Samples were then sequenced at a depth of 15-21million 2x100bp paired-end clusters on an Illumina NovaSeq6000 platform (Illumina, Inc., San Diego)). hrs = hours; ; IL = interleukin.

B. Quantitative measures of stress in animals experiencing repeated social defeat (RSD/"Stress") include splenomegaly and elevated levels of peripheral pro-inflammatory proteins. The data comparing spleen weights in the subset of mice sent for sequencing is reproduced from Figure 2G (N = 3 mice in each CON/RSD group). The graph comparing interleukin(IL)-6 levels in the subset of mice sent for sequencing (N = 3 mice in each CON/RSD group) represents a subset of the data presented in Figure 2H. **p*< .05

C. Principle Component Analysis (PCA) plot of all genes for all treatment groups (RPMI, ISO, FORSK) and mice (CON (N = 3), RSD (N = 3) included in the analysis. Separation between groups (CON/RSD) accounted for greater variance (87%) than separation between treatment conditions (RPMI, ISO, FORSK; 5%).

D. Volcano plot for all up and down-regulated genes for RSD = RSD_RPMI group (N = 3 mice/samples) vs. CON = CON_RPMI group (N = 3 mice/samples) after CLEAR selection for robust/low-noise transcripts (Walker et al., 2020) and filtering out all genes with an average median count of ≤ 2 (across all samples). Differential gene expression was estimated using the DESeq2 package in R (Love et al., 2014). Genes in dark gray, blue, and red are significantly up or down regulated using a p-adjusted value of .05 (N = 1,594 significantly upregulated genes; N = 2,007 significantly downregulated genes). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg

(Love et al., 2014). Red genes represent a > 1-fold upregulation in RSD mice vs CON samples; Blue genes represent a > 1-fold downregulation in RSD mice vs CON samples.

(E, F). Qiagen Ingenuity Pathway (IPA) Analyses based on significantly up and down regulated genes in (D) using a p-adjusted value of .05, performed using the default settings in IPA with specific selection for species (mus musculus) and cell type (splenocytes).

E. IPA Canonical Pathway analysis for RSD_R = RSD_RPMI (N = 3) vs. CON_R = CON_RPMI (N=3) demonstrates an upregulation in pro-inflammatory gene transcription pathways, a downregulation in Beta-adrenergic receptor (β-AR) canonical signaling pathways, and an upregulation in β-AR Non-canonical signaling pathways.
Interleukin(IL)-6 Signaling: Z-score = 2.746, *p* = .000489
Toll-like Receptor (TLR) Signaling: Z-score = 2.496, *p* = .001738
NF-κB Signaling: Z-score = 2.214, *p* = .000295
Chemokine Signaling: Z-score = 1.213, *p* = .002630
G-Protein Coupled Receptor (GPCR) Signaling: Z-score = .426, *p* = .001318
Gαs Signaling: n/a
cAMP response element binding protein (CREB) Signaling: Z-score: -.75, *p* = .003388
Protein Kinase-A (PKA) Signaling: Z = -.603, *p* = .000741
Gαi Signaling: Z-score = 2.449, *p* = .000295
p38 mitogen-activated protein kinase (MAPK) Signaling: Z-score = 1.706, *p* = .005012

F. IPA Upstream Regulator analysis for RSD_R = RSD_RPMI (N = 3) vs. CON_R = CON_RPMI (N=3) represents a downregulation in activator protein (AP)-1 related gene transcription (REL, REL-B) and an upregulation in non-canonical MAPK7 related gene transcription.
REL: transcription regulator, Activation Z-Score = -0.692, *p* = 0.00595
Genes identified: BACH2, BCL2L1, CD40, CREM, Cxcl9, ICAM1, IFNG, MAFG, MYC, NAB2
RELB: transcription regulator; Activation Z-Score = -0.915, *p* = 0.00925
Genes identified: IFNG, LTA, LTB, LTBR, TNFRSF1A, TNFSF14
MAPK7: kinase, Activation Z-Score = 0.218, *p* = 0.0463
Genes identified: BCL2A1, MCL1

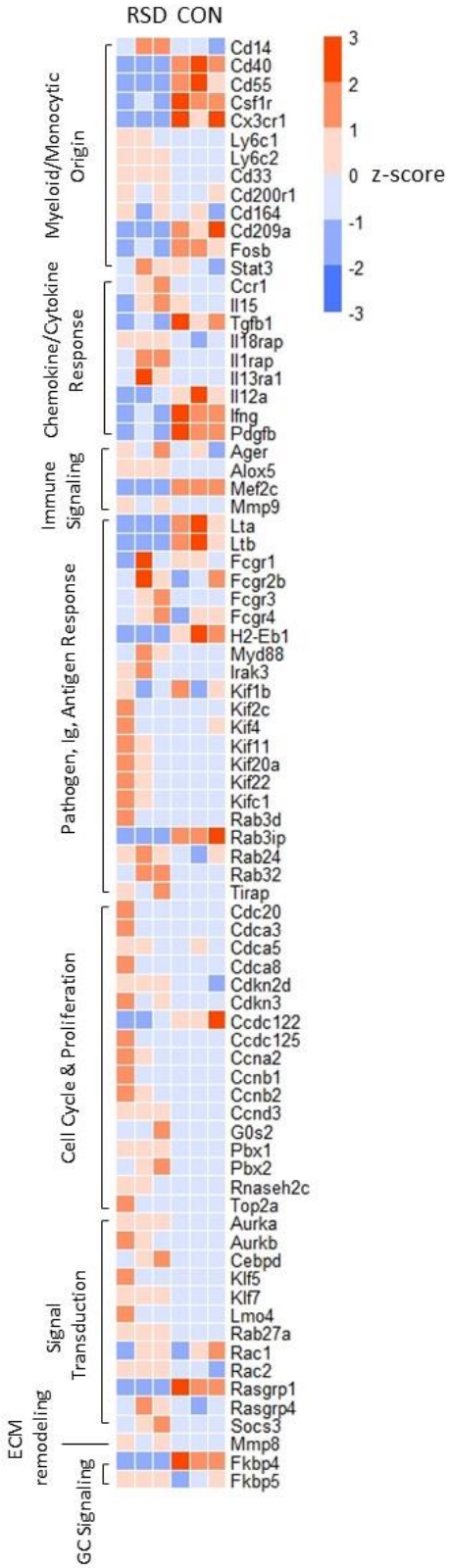


Figure 7. Stress Effect: Splenocytes - Genes previously identified in the Repeated Social Defeat (RSD) literature

Heat map of individual genes previously identified in Repeated Social Defeat (RSD) literature (Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018) demonstrates a significant effect of RSD on gene transcription, including an upregulation in pro-inflammatory signaling, an upregulation in myeloid function, and a downregulation in glucocorticoid signaling.

Notes: Individual gene z-scores depicted in the heat map (see legend) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg in DESeq2 (Love et al., 2014). RSD = RSD_RPMI group (N = 3); CON = Control_RPMI group (N = 3); Each column represents an individual mouse. Ig = Immunoglobin; ECM = extracellular matrix; GC = glucocorticoid.

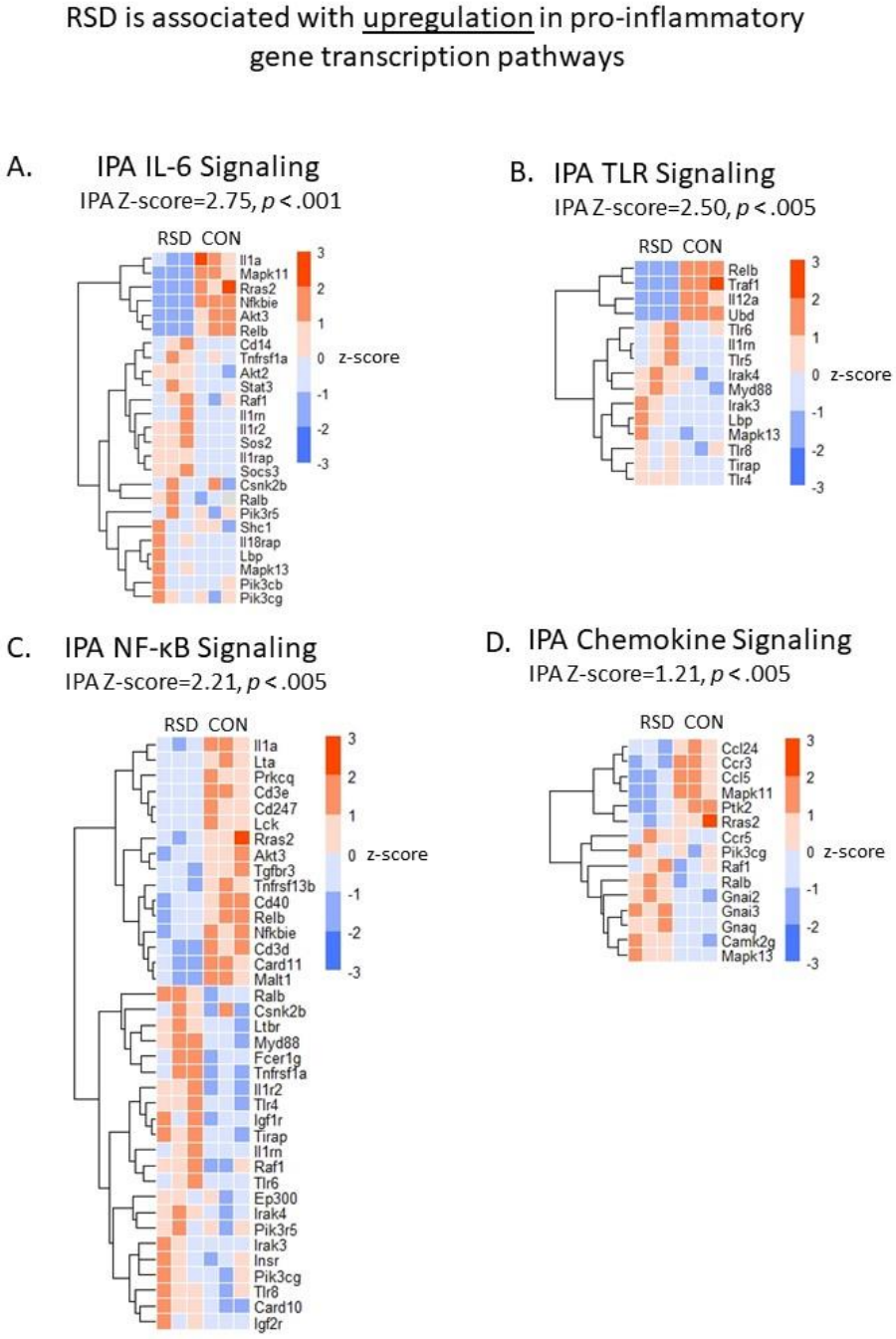


Figure 8. Stress Effect: Splenocytes – Individual genes identified in IPA Canonical Pathway analyses, pro-inflammatory pathways

Heat maps of individual genes identified in IPA Canonical Pathway analyses representing pro-inflammatory pathways (A-D) support the idea that Repeated Social Defeat (RSD) is associated with an upregulation in pro-inflammatory gene transcription compared to control mice.

Notes: Individual gene z-scores depicted in the heat map (see legends) are based on raw counts data for each individual gene, normalized across all genes displayed in each group. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg in DESeq2 (Love et al., 2014). RSD = RSD_RPMI group (N = 3 mice/samples); CON = Control_RPMI group (N = 3 mice/samples). Each column represents an individual mouse. Cluster analysis is based on similarity in individual gene z-score distribution pattern within each group.

- **A.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in interleukin(IL)-6 signaling pathways. Activation score (Z = 2.75, $p < .001$) indicates an overall significant upregulation in IL-6 related genes among untreated CD11b+ cells from RSD mice compared to untreated CD11b+ cells from control mice.
- **B.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in toll-like receptor (TLR) pathways. Activation score (Z = 2.50, $p < .005$) indicates an overall significant upregulation in TLR-related genes among untreated CD11b+ cells from RSD mice compared to untreated CD11b+ cells from control mice.
- **C.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in nuclear factor – kappaBeta (NF-κB) signaling pathways. Activation score (Z = 2.21, $p < .005$) indicates an overall significant upregulation in NF-κB-related genes among untreated CD11b+ cells from RSD mice compared to untreated CD11b+ cells from control mice.
- **D.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in chemokine-related signaling pathways. Activation score (Z = 1.21, $p < .005$) indicates an overall significant upregulation in chemokine signaling related genes among untreated CD11b+ cells from RSD mice compared to untreated CD11b+ cells from control mice.

5.4.1.2 Peripheral Blood

Among CD11b+ cells isolated from the peripheral blood, there was differential expression of 681 mRNA gene transcripts (334 upregulated; 347 downregulated, $p_{adj}<.05$, Figure 9D). Of note, one RSD mouse was removed from the analyses due to significant overlap with gene expression among control mice; while this mouse had a similar spleen weight to the other RSD mice, the wound score was lower. Importantly, removal of this mouse still resulted in significantly greater spleen weight among RSD mice vs controls ($p < .05$). Thus, this and all following analyses in CD11b+ peripheral cells are based on only two RSD mice and three control mice.

Similar to CD11b+ splenocytes, upregulated genes among peripheral CD11b+ cells included many associated with a primed and pro-inflammatory gene expression profile (Figure

10A). For example, upregulated genes included those of myeloid origin (*Cd14*[5†], *Ly6c2*[†], *Ly6g*[†], *Cd38*) those involved in immune, pathogen, Ig, or antigen related signaling (*Fcgr1*[†], *Fcgr2b*[†], *Fcrg4*[†], *Rab3d*[†]), genes involved in cell cycling and proliferation (*Ccnd3*[†]), signal transduction (*Klf5*[†], *Klf7*[†]), and extracellular matrix remodeling (*Mmp8*[†], *Timp2*). Similar to what was observed in splenocytes, a gene important in inhibition of GR translocation to the nucleus was significantly upregulated among CD11b+ peripheral cells (*Fkbp5*[†]), consistent with the development of glucocorticoid resistance. There was no significant difference in expression of the GR gene (*Nr3c1*) among CD11b+ peripheral cells, which was observed in splenocytes and has previously served as a negative control for both gene expression and protein levels in RSD (Powell et al., 2013, Bailey 2009, Engler 2008; Quan 2003).

Downregulated genes among peripheral CD11b+ cells from RSD mice vs controls (Figure 10A) included those involved in myeloid and early monocytic lineage (*Cd40*[†], *Csf1r*[†], *Cx3cr1*[†], *CD300a*), cytokine and chemokine regulatory signaling (*Pdgfb* [†], *Tgfbr1*), immune and inflammatory-related signaling (*Mef2c*[†]), cell cycling and proliferation (*Mdm1*), and signal transduction (*Rasgrp1*[†]).

In contrast to the findings in splenocytes, IPA did not identify any significant pathways involved in pro-inflammatory signaling among significantly up and downregulated genes from CD11b+ peripheral cells (Figure 9E). This is likely due to the smaller number of differentially regulated genes. Indeed, many of the individual genes involved in the IPA pathways identified among splenocytes were differentially regulated in the same direction among peripheral CD11b+ cells (Figures 10B-E). When significantly up and downregulated genes were assessed using

[5] † Indicates that this gene was expressed in the same direction (i.e., upregulated or downregulated) in the same analysis (RSD vs CON) among CD11b+ splenocytes

Panther overrepresentation analysis, there was an enrichment in genes involved in inflammation mediated by chemokine and cytokine signaling (7.75-fold, $p = .0002$, $FDR = .008$; identified genes: *Arpc3, Ccr3, Ptk2b, Fpr2, Ccrl2, Gna11, Ccl2, Grap2, Pak4, Cxcr4, Nfatc2, Fpr2, Itga4, Cx3cr1, Nfkbie, Ifngr1, Ccr5, Itgb2I*). Although the collective evidence is less strong than among CD11b+ splenocytes, the evidence presented here suggests that RSD promotes a primed and pro-inflammatory gene expression profile among CD11b+ peripheral cells.

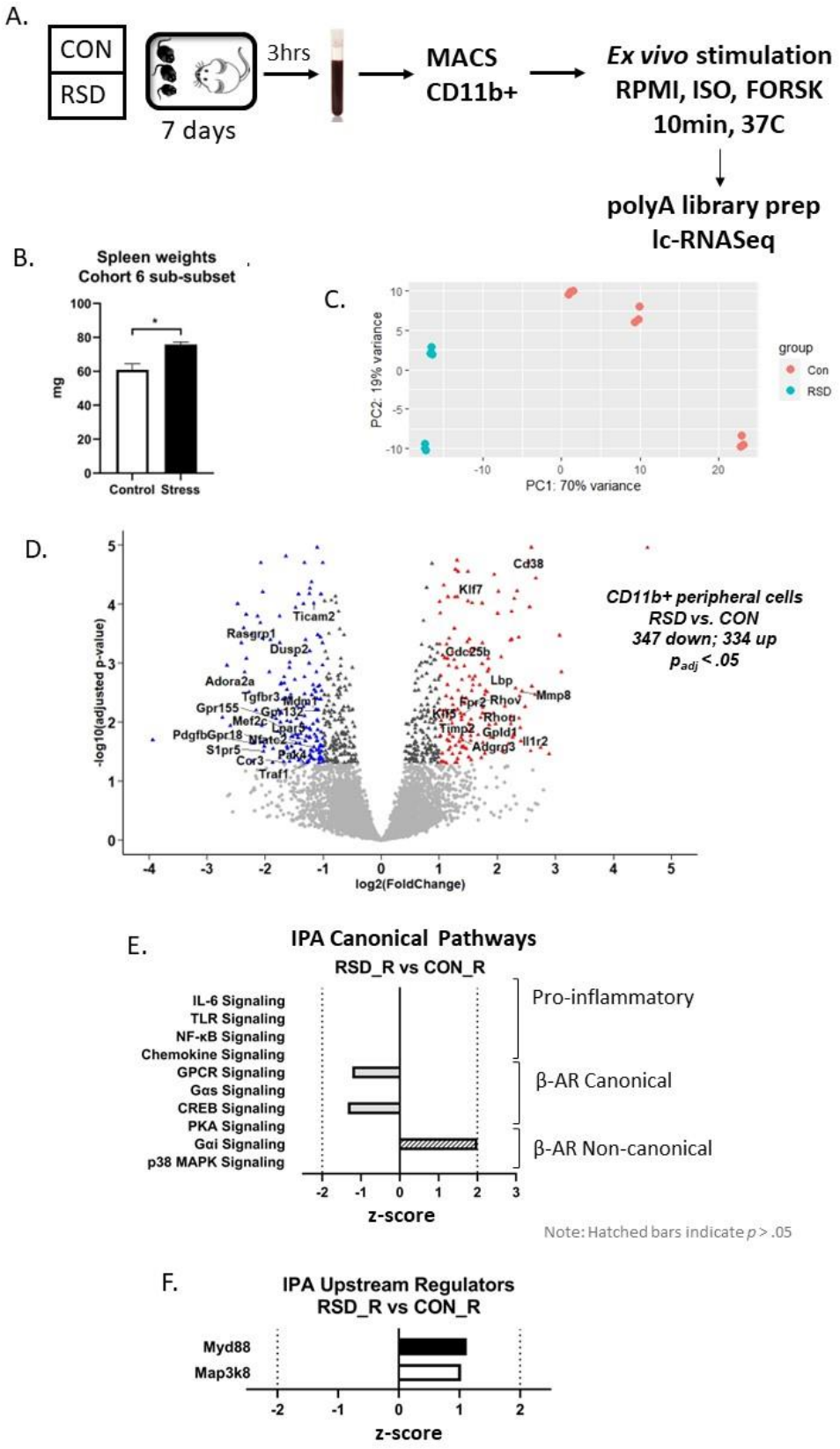


Figure 9. Stress Effect: Peripheral Cells

The experiment (described in A) resulted in a significant effect of Repeated Social Defeat (RSD) on splenomegaly (B) and differential gene transcription (C, D). Differentially regulated genes included those involved in β-adrenergic receptor (β-AR) canonical and non-canonical signaling pathways (E, F).

A. Diagram of experiment. Male C56BL/6 mice (6-8 weeks old) underwent repeated social defeat (RSD) for 6 days or were left undisturbed in their cages in the control room (CON) until sacrifice. Mice were sacrificed within three hours (hrs) of the last round of social defeat. After sacrifice, the peripheral blood was harvested from the mice for isolation of CD11b+ cells. CD11b+ peripheral cells were isolated using Miltenyi Biotec CD11b+ Microbeads (Miltenyi Biotec Cat #130-049-60). CD11b+ peripheral cells were either left unstimulated (RPMI 1640 cell culture media alone) or were subject to stimulation by isoproterenol (ISO), a non-specific β-adrenergic agonist, or forskolin (FORSK), a non-specific activator of adenylate cyclase (AC) for 10 minutes at 37ºC. RNA was then isolated. A subset of these samples (N = 3 in each group CON/RSD) were sent for polyA library preparation (poly-A tail was used to amplify mRNA using oligo(dT) primers for mRNA library generation; the poly-A tail is a long chain of adenosine nucleotides added to 3' end RNA to prevent degradation and mark it for transport to ribosomes to be translated into protein) and lc-RNAseq (mRNA library generation for limiting cell (lc = low input RNA amounts: 10pg-1ng) was performed using the Clontech SMART-Seq HT kit (Takara Bio Inc., Mountain View, CA, Cat #634456) and the the Nextera XT DNA Library Prep Kit (Illumina, Inc., San Diego, CA, Cat #FC-131-1096). RNA-Seq = Samples were then sequenced at a depth of 15-21million 2x100bp paired-end clusters on an Illumina NovaSeq6000 platform (Illumina, Inc., San Diego)).

B. A quantitative measure of stress in animals experiencing repeated social defeat (RSD/"Stress") includes splenomegaly. The sub-subset of data displayed here represents the mice for the samples used in the analyses (N = 2 RSD, N = 3 CON). Specifically, one RSD mouse that was sent for sequencing was removed from the analyses due to significant overlap with gene expression among control mice. The data comparing spleen weights in the subset of mice sent for sequencing can be seen in Figure 2J. **p*< .05

C. Principle Component Analysis (PCA) plot of all genes for all treatment groups (RPMI, ISO, FORSK) and mice (CON (N = 3), RSD (N = 2)) included in the analysis. Separation between groups (CON/RSD) accounted for greater variance (70%) than separation between treatment conditions (RPMI, ISO, FORSK; 19%).

D. Volcano plot for all up and down-regulated genes for RSD = RSD_RPMI group (N = 2 mice/samples) vs. CON = CON_RPMI group (N = 3 mice/samples) after CLEAR selection for robust/low-noise transcripts (Walker et al., 2020) and filtering out all genes with an average median count of ≤ 2 (across all samples). Differential gene expression was estimated using the DESeq2 package in R (Love et al., 2014). Genes in dark gray, blue, and red are significantly up or down-regulated using a *p*-adjusted value of .05 (N = 334 upregulated genes; N = 347 downregulated genes). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg (Love et al., 2014). Red genes represent a > 1 fold upregulation in RSD vs. CON samples; Blue genes represent a > 1 fold downregulation in RSD vs. CON samples. Light gray genes were not significantly up or down-regulated.

(E, F). Qiagen Ingenuity Pathway (IPA) Analyses based on significantly up and down-regulated genes using a *p*-adjusted value of .05, performed using the default settings in IPA with specific selection for species (mus musculus) and cell type (peripheral mononuclear cells & leukocytes).

E. IPA Canonical Pathway analysis for RSD_R = RSD_RPMI (N = 2) vs. CON_R = CON_RPMI (N=3) demonstrates a downregulation in beta-adrenergic receptor (β-AR) canonical signaling pathways and an upregulation in β-AR non-canonical signaling pathways.
Interleukin(IL)-6 Signaling: n/a
Toll-like Receptor (TLR) Signaling: n/a
NF-κB Signaling: n/a
Chemokine Signaling: n/a
G-Protein Coupled Receptor (GPCR) Signaling: Z-score = -1.225, *p* = .000550
$G\alpha_s$ Signaling: n/a
cAMP-response element bindingprotein (CREB) Signaling: Z-score:-1.342, *p*=.000851
Protein Kinase-A (PKA) Signaling: Z-score = 0, *p* = 1.00
$G\alpha_i$ Signaling: Z-score = 2.000, *p* = .097724
p38 mitogen-activated protein kinase (MAPK) Signaling: n/a

F. IPA Upstream Regulator analysis for RSD_RPMI (N = 2) vs. CON_RPMI (N=3) found an upregulation in a pro-inflammatory pathway (MYD88) and upregulation in non-canonical gene transcription regulated by MAP3K8.
MYD88: other; Activation Z-Score = 1.12, *p* = 3.89E-04
Genes identified:
Ccl2, CD38, EGR1, FPR1, FPR2, IRF8, ITGAX, MMP8, NFATC2, Saa3, TFEC
MAP3K8: kinase, Activation Z-Score = 1.026, *p* = 4.22E-03
Genes identified: ADORA3, CCR5, CIITA, EGR1, GAB1, SESN1, SLCO4A1

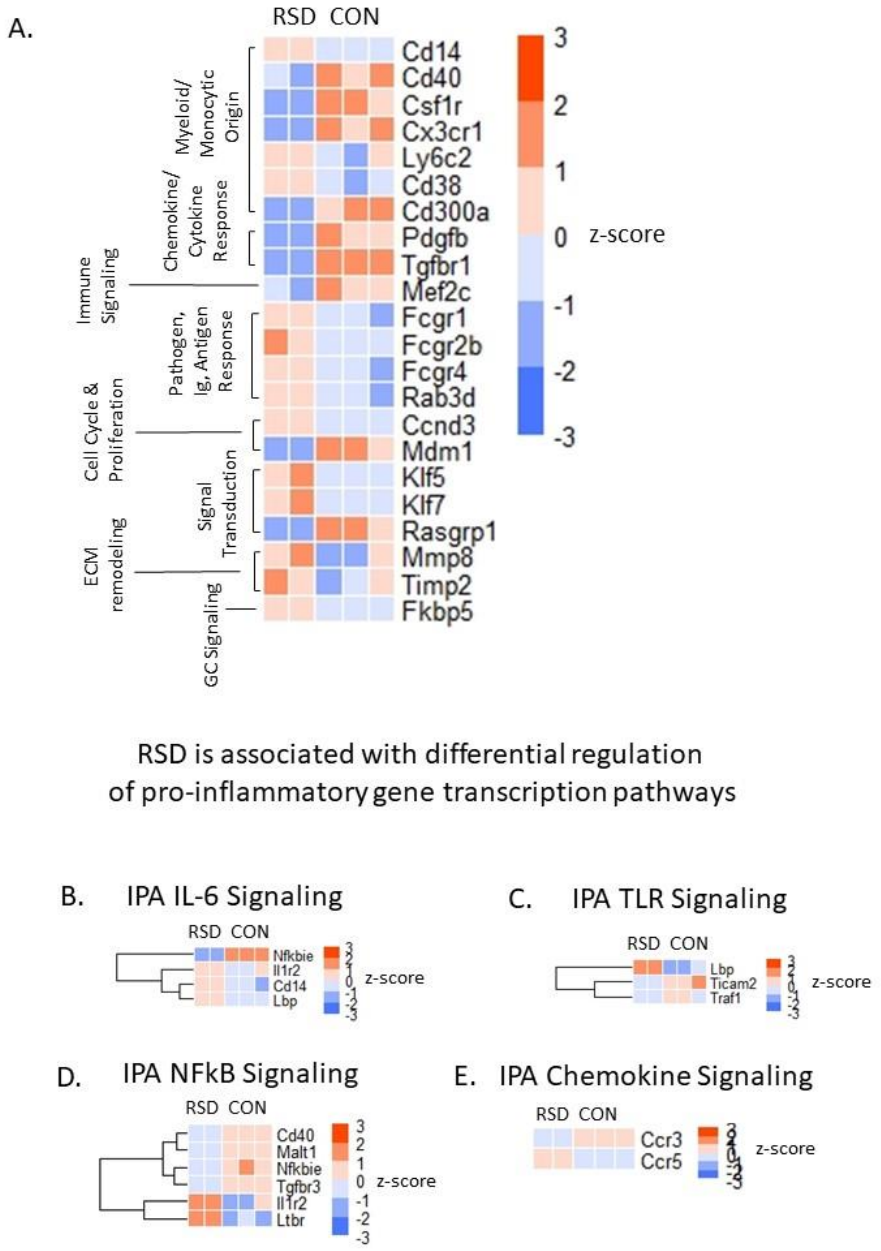


Figure 10. Stress Effect: Peripheral Cells - Genes previously identified in Repeated Social Defeat (RSD) literature & individual genes identified in IPA Canonical Pathway analyses, pro-inflammatory pathways

Heat map of individual genes previously identified in Repeated Social Defeat (RSD) literature (A; Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018) demonstrates a significant effect of RSD on gene transcription, including an upregulation in pro-inflammatory signaling, an upregulation in myeloid function, and a downregulation in glucocorticoid signaling. Heat maps of individual genes identified in IPA Canonical Pathway analyses representing pro-inflammatory pathways (B-E) suggest association with alterations in pro-inflammatory gene transcription compared to control mice, although these pathways were not significant.

Notes: Individual gene z-scores depicted in the heat maps (see legends) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg (Love et al., 2014). RSD = RSD_RPMI group (N = 2); CON = Control_RPMI group (N = 3); Each column represents an individual mouse.

- **A.** Heat map of individual genes previously identified in RSD literature (Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018) demonstrates a significant effect of RSD on gene transcription, including an upregulation in pro-inflammatory signaling, an upregulation in myeloid function, and a downregulation in glucocorticoid signaling. *Notes*: Ig = Immunoglobin; ECM = extracellular matrix; GC = glucocorticoid.
- **B.** Heat map of individual genes identified in IPA Canonical Pathway analyses representing interleukin(IL)-6 pro-inflammatory signaling pathways. Cluster analysis is based on similarity in individual gene z-score distribution pattern within each group. No IPA Z-score was calculated for this pathway, although a number of individual genes were identified.
- **C.** Heat map of individual genes identified in IPA Canonical Pathway analyses representing toll-like receptor (TLR) pro-inflammatory signaling pathways. Cluster analysis is based on similarity in individual gene z-score distribution pattern within each group. No IPA Z-score was calculated for this pathway, although a number of individual genes were identified.
- **D.** Heat map of individual genes identified in IPA Canonical Pathway analyses representing nuclear factor – kappaBeta (NF-κB; nuclear factor kappa-light-chain-enhancer) pro-inflammatory signaling pathways. Cluster analysis is based on similarity in individual gene z-score distribution pattern within each group. No IPA Z-score was calculated for this pathway, although a number of individual genes were identified.
- **E.** Heat map of individual genes identified in IPA Canonical Pathway analyses representing chemokine-related pro-inflammatory signaling pathways. No IPA Z-score was calculated for this pathway, although two individual genes were identified.

5.4.2 RSD_R vs CON_R: Consistent with our hypothesis, RSD promotes differential gene transcription consistent with β-AR "signal switching."

5.4.2.1 Spleen.

A number of genes identified as differentially expressed in CD11b+ splenocytes from RSD mice vs Controls ($p_{adj} < .05$) were consistent with a pattern of signaling through the proposed non-canonical β-adrenergic pathway rather than the canonical β-adrenergic pathway (Figure 6E, Figure 11A; See Figure 1 for diagram of these pathways). For example, among IPA-identified pathways, there was an upregulation in p38 MAPK pathway utilization (Z=1.71, p =.005; Figure 6E, Figure

12C), as well as activation of pathways utilizing Ga_i in GPCR signaling (Z=2.45, $p < .005$; Figure 6E, Figure 13C). Findings among individual genes of interest ($p_{adj} < .05$) also supported a pattern of signaling through the non-canonical β-adrenergic pathway. For example, *Arrb2,* a gene encoding a scaffolding protein involved in non-canonical β-adrenergic receptor signaling, β-arrestin 2, was significantly upregulated among RSD mice vs controls (Figure 11A, Figure 11E; See Figure 1 for diagram of these pathways). Also consistent with a pattern of signaling through the proposed non-canonical β-adrenergic receptor pathway was the upregulation of a number of individual genes involved in signal transduction via AP-1 pathways (*Fosl1, Fosl2, Ap1m1, Ap1s1, Ap1g1*).

Together with a shift toward non-canonical β-adrenergic signaling would be an expected *downregulation* in β-adrenergic *canonical* signaling. Evidence consistent with a downregulation in signaling through the β-adrenergic canonical pathway included a significant downregulation in IPA-identified CREB-mediated signaling pathways (Z= -.75, $p < .005$; Figure 6E, Figure 12A), as well as a downregulation in signaling through PKA (Z= -.60, $p < .001$; Figure 6E, Figure 12B). Among individual genes of interest ($p_{adj} < .05$), a gene encoding a β-adrenergic receptor was also significantly downregulated among RSD mice vs Controls (*Adrb1*: Figure 11A, Figure 11B); this evidence is consistent with a downregulation of the β-adrenergic receptor in the context of chronic sympathetic activation via RSD and could also indicate a downregulation in β-adrenergic *canonical* signaling more generally.

Other significant IPA pathways of interest involved in β-adrenergic signaling included an upregulation in GPCR signaling broadly (Z=.43, $p < .005$; Figure 6E, Figure 13A). This finding could be consistent with signaling through either pathway. Specifically, an upregulation in GPCR signaling could be evidence for the use of β-adrenergic canonical signaling pathways, although

non-canonical β-adrenergic signaling may still utilize GPCR signaling in signal transduction via coupling with Ga_i rather than Ga_s (See Figure 1 for diagram of these pathways). Indeed, an upregulation in Ga_i-mediated GPCR signaling was observed as a part of these analyses (Ga_i signaling: Z= 2.45, $p < .005$; Figure 6E, Figure 13C), and thus it may be more likely that an upregulation in GPCR signaling broadly is suggestive of a "switch" to the use of β-adrenergic canonical signaling pathways.

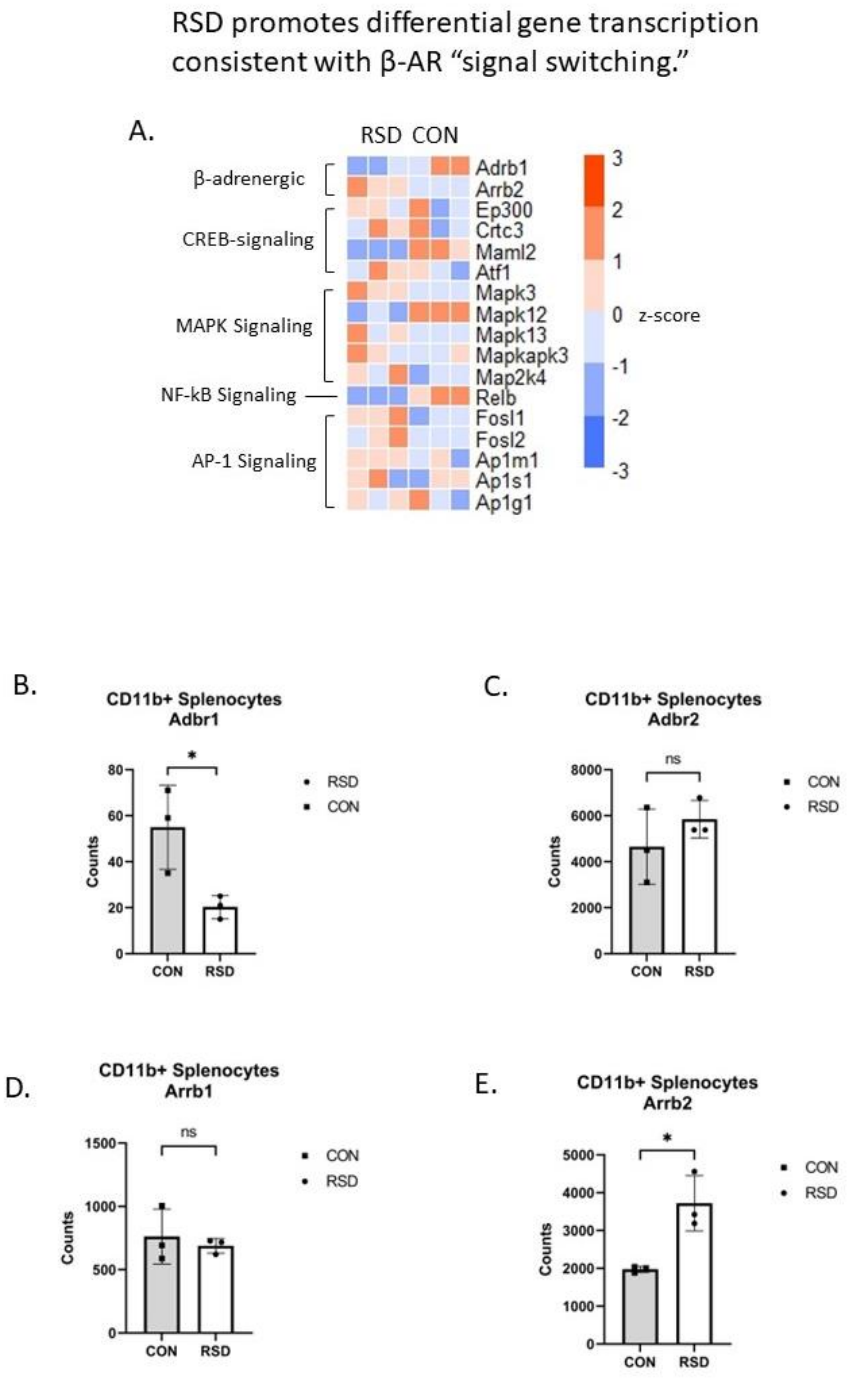


Figure 11. Stress Effect: Splenocytes – Individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching."

Repeated Social Defeat (RSD) promotes differential gene transcription consistent with β-adrenergic receptor (β-AR) "signal switching" (A-E), including a downregulation in canonical β-AR signaling and an upregulation in non-canonical β-AR signaling.

A. Heat map of individual genes identified a-priori as involved in β-adrenergic receptor (β-AR) "signal switching" support a downregulation in canonical β-AR signaling and an upregulation in non-canonical β-AR signaling. Individual gene z-scores depicted in the heat map (see legend) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg in DESeq2 (Love et al., 2014). Each column represents an individual mouse. *Notes*: RSD = RSD_RPMI group (N = 3 mice/samples); CON = RSD_RPMI group (N = 3 mice/samples). CREB Signaling = genes involved in cAMP response element binding protein (CREB) signaling; MAPK Signaling = genes involved in mitogen-activated protein kinase (MAPK) signaling; NF-κB Signaling = genes identified in nuclear factor – kappaBeta (NF-κB) pro-inflammatory signaling; AP-1 Signaling = genes involved in activator protein-1 (AP-1) pro-inflammatory signaling.

(B-E). Graphs of individual gene counts for particular genes of interest identified a-priori as involved in β-adrenergic receptor (β-AR) "signal switching." *Notes:* $*p_{adj} < .05$, ns = not significant. Individual points in each column represent raw gene counts from an individual mouse. RSD = RSD_RPMI group (N = 3 mice/samples); CON = Control_RPMI group (N = 3 mice/samples). CD11b+ = cell surface protein marker indicating mouse monocytes and granulocytes.

B. RSD resulted in a significantly lower expression of Adbr1, the gene encoding the β1-adrenergic receptor, among untreated samples compared to untreated samples from control mice.

C. There was no significant difference in gene expression of Adbr2, the gene encoding the β2-adrenergic receptor, among untreated samples from RSD mice compared to untreated samples from control mice.

D. There was no significant difference in gene expression of Arrb1, the gene encoding the β-arrestin-1 protein, among untreated samples from RSD mice compared to untreated samples from control mice. β-arrestin-1 is a key signaling protein involved in canonical β-adrenergic receptor signaling.

E. RSD resulted in a significantly greater expression of Arrb2 , the gene encoding the β-arrestin-2 protein, among untreated samples compared to untreated samples from control mice. β-arrestin-2 is a key signaling protein involved in non-canonical β-adrenergic receptor signaling.

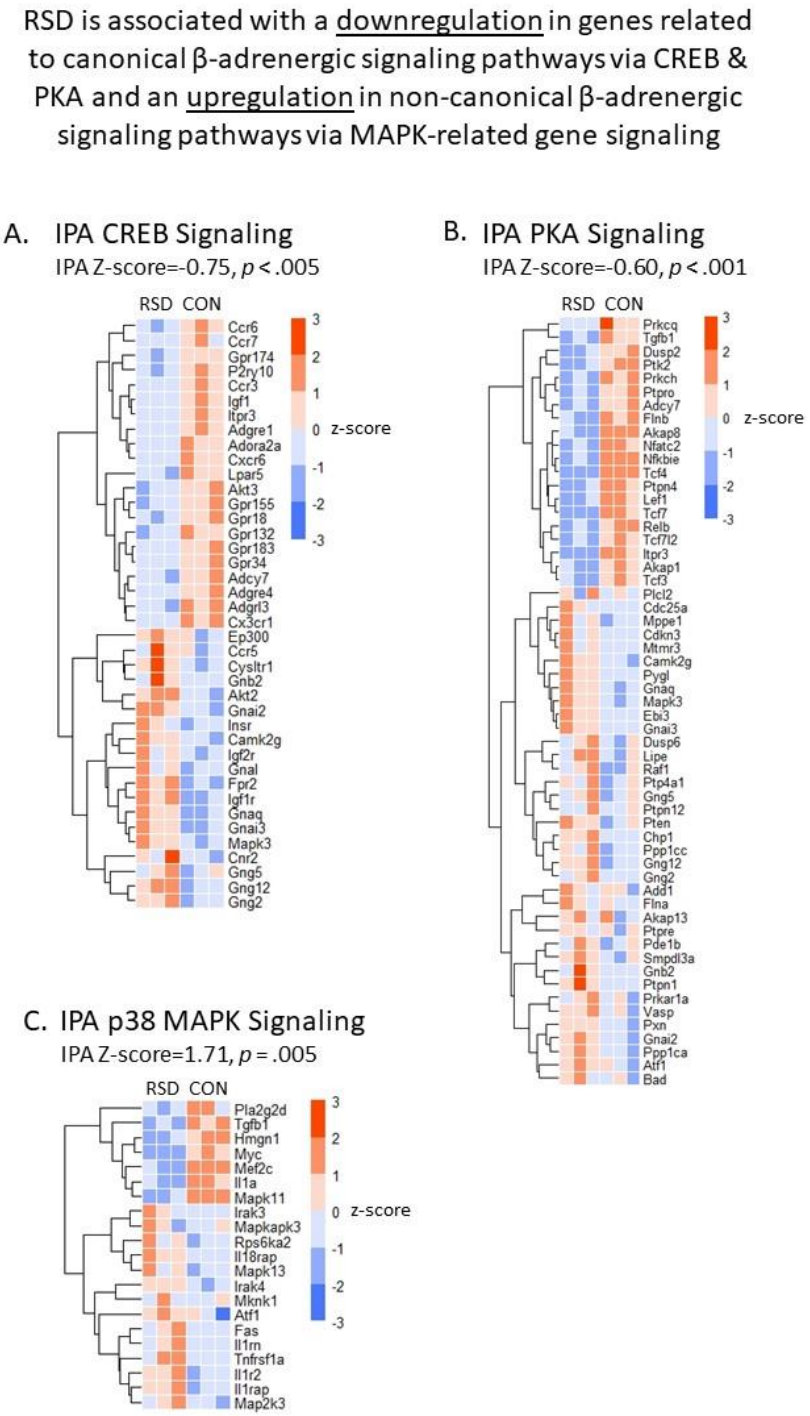


Figure 12. Stress Effect: Splenocytes – Individual genes identified in IPA Canonical Pathway analyses, protein kinase-A (PKA), cAMP response element binding (CREB), and mitogen-activated protein kinase (MAPK) pathways

Repeated Social Defeat (RSD) promotes differential regulation of genes related to a downregulation in canonical β-adrenergic signaling pathways via CREB (A) and PKA (B) and an upregulation in non-canonical β-adrenergic signaling pathways via MAPK-related gene transcription (C).

Notes: Individual gene z-scores depicted in the heat map (see legends) are based on raw counts data for each individual gene, normalized across all genes displayed in each group. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg in DESeq2 (Love et al., 2014). RSD = RSD_RPMI group (N = 3 mice/samples); CON = Control_RPMI group (N = 3 mice/samples). Each column represents an individual mouse. Cluster analysis is based on similarity in individual gene z-score distribution pattern within each group.

A. Heat map of individual genes identified in IPA Canonical Pathway analyses involved in cAMP response element binding (CREB) protein signaling pathways. Activation score (Z = -0.60, $p < .001$) indicates an overall significant downregulation in CREB-related genes among untreated CD11b+ cells from RSD mice compared to untreated CD11b+ cells from control mice.

B. Heat map of individual genes identified in IPA Canonical Pathway analyses involved in protein kinase-A (PKA) signaling pathways. Activation score (Z = -0.75, $p < .005$) indicates an overall significant downregulation in PKA-related genes among untreated CD11b+ cells from RSD mice compared to untreated CD11b+ cells from control mice.

C. Heat map of individual genes identified in IPA Canonical Pathway analyses involved in mitogen-activated protein kinase (MAPK) signaling pathways. Activation score (Z = 1.71, $p = .005$) indicates an overall significant upregulation in p38 MAPK-related genes among untreated CD11b+ cells from RSD mice compared to untreated CD11b+ cells from control mice.

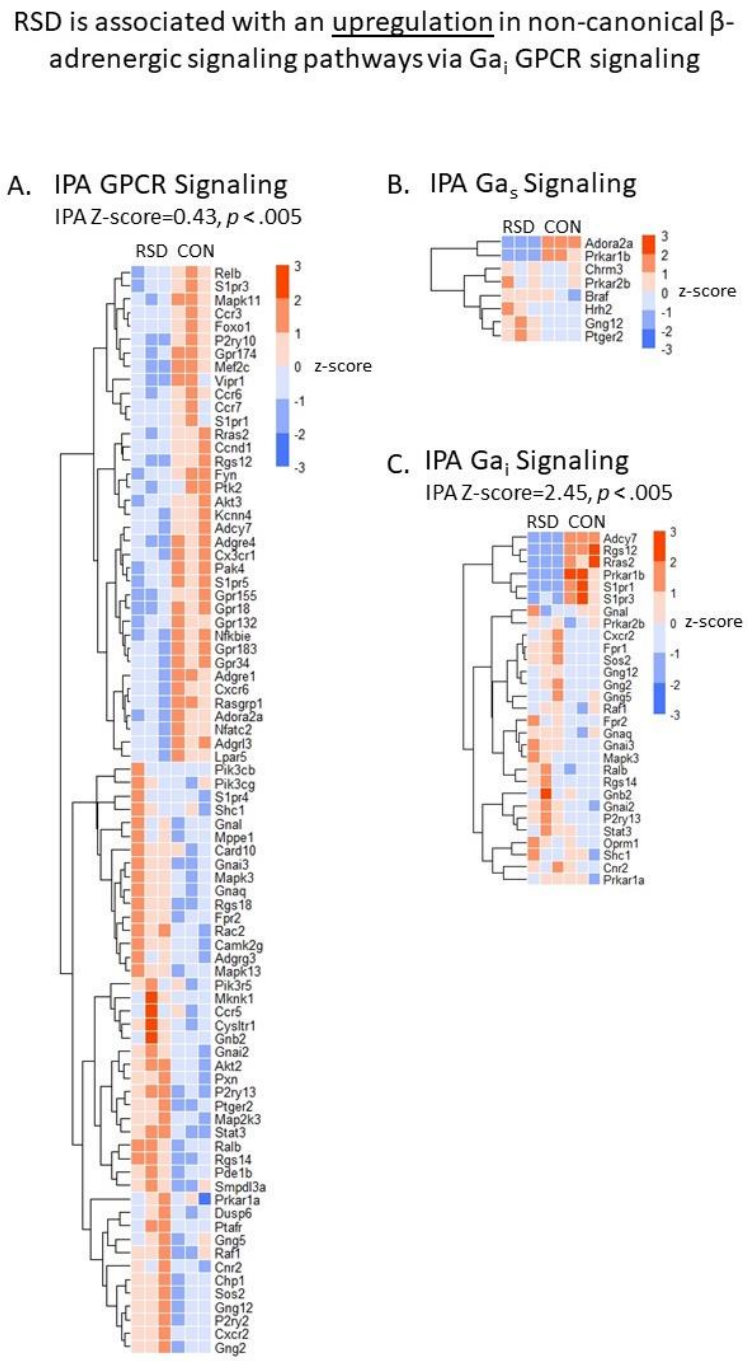


Figure 13. IPA Canonical Pathway analyses involving G-protein coupled receptor (GPCR) signaling pathways (A-C) show that Repeated Social Defeat (RSD) promotes differential gene transcription consistent with an upregulation in non-canonical β-adrenergic signaling

IPA Canonical Pathway analyses involving G-protein coupled receptor (GPCR) signaling pathways (A-C) show that Repeated Social Defeat (RSD) promotes differential gene transcription consistent with an upregulation in non-canonical β-adrenergic signaling pathways via Ga$_i$ GPCR signaling (A,C).

Notes: Heat map of individual genes identified in IPA Canonical Pathway analyses involved in G-protein coupled receptor (GPCR) signaling pathways. Individual gene z-scores depicted in the heat map (see legends) are based on raw counts data for each individual gene, normalized across all genes displayed in each group. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg in DESeq2 (Love et al., 2014). RSD = RSD_RPMI group (N = 3 mice/samples); CON = Control_RPMI group (N = 3 mice/samples). Each column represents an individual mouse. Cluster analysis is based on similarity in individual gene z-score distribution pattern within each group.

- **A.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in G-protein coupled receptor (GPCR) signaling pathways. The activation score (Z = 0.43, p < .005) indicates an overall significant upregulation in GPCR-related genes among untreated CD11b+ cells from RSD mice compared to untreated CD11b+ cells from control mice.
- **B.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in G-protein-alpha-s ($G\alpha_s$) GPCR signaling pathways. G-protein alpha (Gα) is a subunit of the G-protein complex that dissociates from the GPCR after ligand binding. Subunit (s) indicates the subtype of G-protein alpha; Ga_s signaling is involved in canonical β-adrenergic receptor signaling. No IPA Z-score was calculated for this pathway, although a number of individual genes were identified.
- **C.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in G-protein-alpha-s ($G\alpha_i$) GPCR signaling pathways. G-protein alpha (Gα) is a subunit of the G-protein complex that dissociates from the GPCR after ligand binding. Subunit (i) indicates the subtype of G-protein alpha; Ga_i signaling is involved in non-canonical β-adrenergic receptor signaling. The activation score (Z = 2.45, p < .005) indicates an overall significant upregulation in Ga_i GPCR-related genes among untreated CD11b+ cells from RSD mice compared to untreated CD11b+ cells from control mice.

5.4.2.2 Peripheral Blood

Among CD11b+ cells isolated from peripheral blood, there were some genes identified as differentially expressed in RSD mice vs Controls ($p_{adj} < .05$) that were consistent with a pattern of downregulation of signaling via the canonical β-adrenergic pathway. Specifically, IPA-identified pathways of interest included a significant downregulation in CREB-mediated signaling (Z= -1.27, $p < .005$; Figure 9E and 15A). In contrast with splenocytes, there was a *downregulation* in GPCR signaling broadly among IPA-identified pathways (Z= -1.51, $p < .005$; Figure 9E and 15B). While this could be evidence for a downregulation in canonical signaling pathways or a switch to non-canonical β-adrenergic receptor *independent* signaling cascades via MAPK, together with

evidence for a significant downregulation in CREB-mediated signaling, it may be that a downregulation in GPCR signaling broadly can be attributed to a downregulation in β-adrenergic receptor canonical signaling pathways.

Among genes identified as differentially expressed in RSD mice vs Controls ($p_{adj} < .05$), there was also evidence suggestive of an upregulation in signaling through the proposed *non-canonical* β-adrenergic pathway. Specifically, IPA identified an upregulation in pathways using Ga_i in GPCR signaling (Z = 2.00; Figure 9E, Figure 15F). Although the magnitude of this activation score was relatively large, it was not considered statistically significant ($p = .09$). Stronger evidence was observed among IPA-identified upstream regulators of gene transcription; specifically, there was a significant activation of genes regulated by *Map3k8* (Z = .218, $p < .05$; Figure 9F), consistent with the upregulation in MAPK/ERK signaling pathways as a part of non-canonical β-adrenergic signaling. In an exploratory analysis among individual genes of interest[6] (using a $p < .05$ criteria), there was a significant upregulation in *Arrb2,* a gene encoding a scaffolding protein involved in non-canonical β-adrenergic receptor signaling, β-arrestin-2 (Figure 14A, Figure 14E), which was also observed among splenocytes. Also consistent with an upregulation in signaling through non-canonical β-adrenergic receptor pathways in peripheral cells was the upregulation of a gene involved in signal transduction via MAPK-pathways (*Mapk13*; Figure 14A) and a gene involved in signaling through the pro-inflammatory transcription factor AP-1 (*Fosl2*; Figure 14A). Two genes that were downregulated (*Ikbkb*, *Chuk*; Figure 14A) encoded proteins important in the inhibition of IkB, a protein that is responsible for sequestration

[6] There were no individual genes of interest that were identified a-priori among β-adrenergic signaling pathways using the $p_{adj} < .05$ criteria. Thus, in exploratory analyses, we examined whether there was significant differential regulation among genes identified a-priori using a non-adjusted $p < .05$ (Figure 14A).

of NFkB in the cytoplasm; thus, downregulation of these genes would contribute to pro-inflammatory state of CD11b+ peripheral cells through an upregulation in NFkB-signaling.

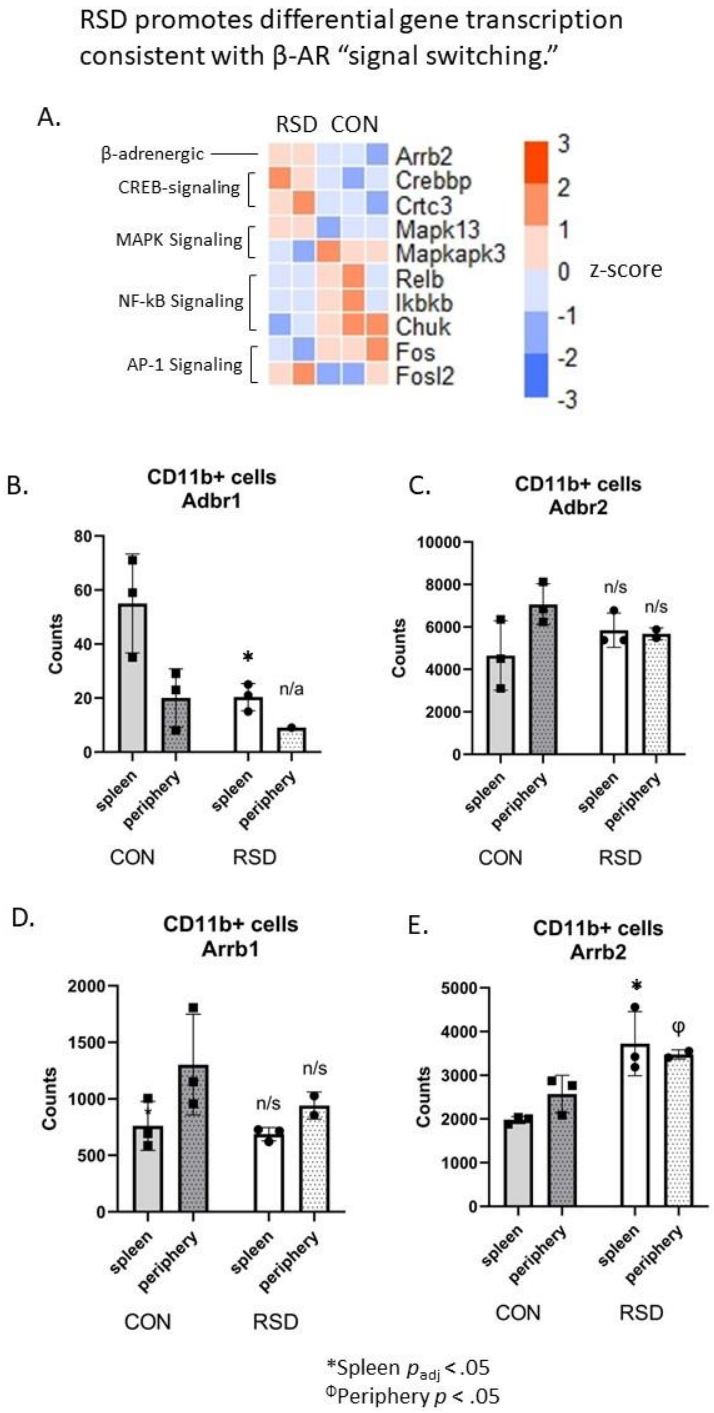


Figure 14. Stress Effect: Peripheral Cells – Individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching"

Repeated Social Defeat (RSD) promotes differential gene transcription consistent with β-adrenergic receptor (β-AR) "signal switching" (A-E), including a downregulation in canonical β-AR signaling and an upregulation in non-canonical β-AR signaling.

Notes: RSD = RSD_RPMI group (N = 2 mice/samples); CON = CON_RPMI group (N = 3 mice/samples).

A. Heat map of individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching" support a downregulation in canonical β-AR signaling and an upregulation in non-canonical β-AR signaling. Individual gene z-scores depicted in the heat map (see legend) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p < .05$ (DESeq2), not adjusted for multiple comparisons. Each column represents an individual mouse. CREB Signaling = genes involved in cAMP response element binding protein (CREB) signaling; MAPK Signaling = genes involved in mitogen-activated protein kinase (MAPK) signaling; NF-κB Signaling = genes identified in nuclear factor – kappaBeta (NF-κB) pro-inflammatory signaling; AP-1 Signaling = genes involved in activator protein-1 (AP-1) pro-inflammatory signaling.

(B-E). Graphs of individual gene counts for particular genes of interest identified a-priori as involved in β-AR "signal switching." *Notes:* spleen = splenocytes, periphery = peripheral cells. All cells were CD11b+, representing mouse monocytes and granulocytes. $*p_{adj} < .05$, $^{\Phi} p < .05$ (not adjusted for multiple comparisons), n/s = not significant, n/a = statistical comparison was not possible due to N = 1 sample in the RSD group. Individual points in each column (squares, circles) represent raw gene counts from an individual mouse.

B. RSD resulted in a significantly lower expression of Adrb1 (the gene encoding the β1-adrenergic receptor) among untreated CD11b+ splenocytes compared to untreated CD11b+ splenocytes from control mice. Although differential gene expression analysis was unable to be performed due to missing data for in the RSD group, the available counts were consistent with a lower expression of Adrb1 in untreated CD11b+ peripheral cells from RSD mice vs. untreated CD11b+ peripheral cells from control mice.

C. There was no significant difference in gene expression of Adrb2, the gene encoding the β2-adrenergic receptor, among untreated CD11b+ splenocytes or peripheral cells from RSD mice compared to untreated CD11b+ splenocytes or peripheral cells from control mice.

D. There was no significant difference in gene expression of Arrb1, the gene encoding the β-arrestin-1 protein, among untreated CD11b+ splenocytes or peripheral cells from RSD mice compared to untreated CD11b+ splenocytes or peripheral cells from control mice. β-arrestin-1 is a key signaling protein involved in canonical β-adrenergic receptor signaling.

E. RSD resulted in a significantly greater expression of Arrb2, the gene encoding the β-arrestin-2 protein, among untreated CD11b+ splenocytes using a p-value < .05 after adjustment for multiple comparisons (*), and among untreated CD11b+ peripheral cells using a p-value < .05 NOT adjusted for multiple comparisons ($^{\Phi}$), compared to untreated CD11b+ splenocytes and peripheral cells from control mice. β-arrestin-2 is a key signaling protein involved in non-canonical β-adrenergic receptor signaling.

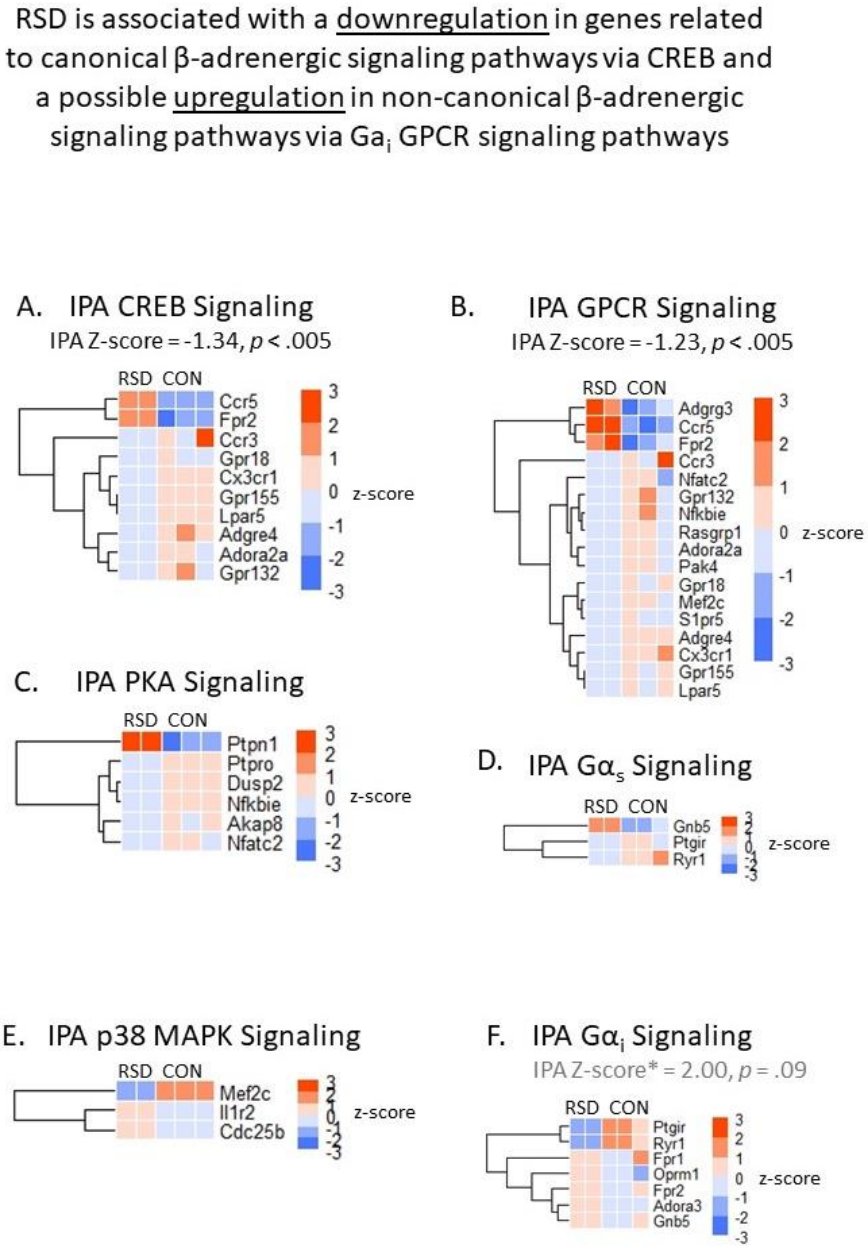


Figure 15. Stress Effect: Peripheral Cells – Individual genes identified in IPA Canonical Pathway analyses, protein kinase-A (PKA), cAMP response element binding (CREB) protein, mitogen-activated protein kinase (MAPK), and G-protein coupled receptor (GPCR) signaling pathways.

Heat maps of individual genes identified in IPA Canonical Pathway analyses (A-F) suggest that Repeated Social Defeat (RSD) is associated with a downregulation in genes related to canonical ß-adrenergic signaling pathways via CREB (A) and a possible upregulation in non-canonical ß-adrenergic signaling pathways via Ga$_i$ GPCR signaling (F).

Notes: Individual gene z-scores depicted in the heat maps (see legends) are based on raw counts data for each individual gene, normalized across all genes displayed in each group. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg (Love et al.,

2014). RSD = RSD_RPMI group (N = 2 mice/samples); CON = Control_RPMI group (N = 3 mice/samples). Each column represents an individual mouse. Cluster analysis is based on similarity in individual gene z-score distribution pattern within each group.

A. Heat map of individual genes identified in IPA Canonical Pathway analyses involved in cAMP response element binding (CREB) protein signaling pathways. The activation score ($Z = -1.34$, $p < .005$) indicates an overall significant downregulation in CREB-related genes among untreated CD11b+ cells from RSD mice compared to untreated CD11b+ cells from control mice.

B. Heat map of individual genes identified in IPA Canonical Pathway analyses involved in G-protein coupled receptor (GPCR) signaling pathways. The activation score ($Z = -1.23$, $p < .005$) indicates an overall significant downregulation in GPCR-related signaling genes among untreated CD11b+ cells from RSD mice compared to untreated CD11b+ cells from control mice.

C. Heat map of individual genes identified in IPA Canonical Pathway analyses involved in protein kinase-A (PKA) signaling pathways. No IPA Z-score was calculated for this pathway, although a number of individual genes were identified.

D. Heat map of individual genes identified in IPA Canonical Pathway analyses involved in G-protein-alpha-s ($G\alpha_s$) GPCR signaling pathways. G-protein alpha ($G\alpha$) is a subunit of the G-protein complex that dissociates from the GPCR after ligand binding. Subunit (s) indicates the subtype of G-protein alpha; Ga_s signaling is involved in canonical β-adrenergic receptor signaling. No IPA Z-score was calculated for this pathway, although a number of individual genes were identified.

E. Heat map of individual genes identified in IPA Canonical Pathway analyses involved in mitogen-activated protein kinase (MAPK) signaling pathways. No IPA Z-score was calculated for this pathway, although a number of individual genes were identified.

F.Heat map of individual genes identified in IPA Canonical Pathway analyses involved in G-protein-alpha-i ($G\alpha_i$) GPCR signaling pathways. G-protein alpha ($G\alpha$) is a subunit of the G-protein complex that dissociates from the GPCR after ligand binding. Subunit (i) indicates the subtype of G-protein alpha; Ga_i signaling is involved in non-canonical β-adrenergic receptor signaling. The activation score ($Z = 2.00$, $p = .09$) indicates an overall non-significant upregulation in Ga_i GPCR-related genes among untreated CD11b+ cells from RSD mice compared to untreated CD11b+ cells from control mice.

5.4.2.3 Similarities and differences between spleen and blood

Differential gene expression that was similar between CD11b+ cells isolated from splenocytes and peripheral blood included key genes involved in β-adrenergic pathways of interest. Many of these genes were consistent with the use of non-canonical β-adrenergic pathways together with a downregulation in canonical β-adrenergic pathways. The strongest evidence is the IPA-identified pathways that were significant for both CD11b+ splenocytes and CD11b+ peripheral

cells using genes that were differentially regulated with the $p_{adj} < .05$ criteria. Specifically, there was an overall downregulation in CREB-mediated signaling pathways ($Z = -1.34$, $p < .005$; Figure 6E, Figure 9E); this pattern is consistent with a downregulation in canonical β-adrenergic receptor signaling. There was also a significant activation of pathways utilizing Ga_i in GPCR signaling among CD11b+ splenocytes (Figure 6E); while this pathway was also identified as activated among CD11b+ peripheral cells (Figure 9E), it was not statistically significant. Activation pathways utilizing Ga_i in GPCR signaling would be consistent with an upregulation in signaling through non-canonical β-adrenergic pathways.

Among individual genes that were identified in CD11b+ splenocytes using a $p_{adj} < .05$ criterion and CD11b+ peripheral blood cells using a non-adjusted *p*-value < .05 criterion, a gene encoding the scaffolding protein β-arrestin 2 (*Arrb2*) was significantly upregulated (Figure 14E). Using the same criteria, we also observed an upregulation in the *Mapk13* gene in both cell types (Figure 11A, Figure 14A). This is particularly notable given that the *Mapk13* gene encodes a protein that is one of the four major p38 MAPKs that respond to extracellular stimuli, and it directly activates downstream transcription factors (Hu et al., 1999; Wang, Diener et al., 1997). We also observed an upregulation in a gene important in AP-1 pro-inflammatory signaling, *Fosl2,* among both CD11b+ splenocytes using a $p_{adj} < .05$ criterion (Figure 11A) and CD11b+ peripheral blood cells using a non-adjusted p-value < .05 criterion (Figure 14A). Upregulation of all three of these genes would be consistent with a shift in signaling through non-canonical β-adrenergic pathways.

When considering the downregulation of key genes among β-adrenergic pathways of interest, we observed a significant downregulation in the β-adrenergic receptor gene *Adrb1* among CD11b+ splenocytes (Figure 11B). Although differential gene expression was unable to be performed among peripheral blood cells due to missing data for two animals, the available counts

were consistent with a lower expression of this gene from RSD mice vs. controls (see Figure 14B). Downregulation of the β-adrenergic receptor gene transcription is consistent with a downregulation in canonical β-adrenergic signaling pathways due to chronic sympathetic activation in the context of chronic stress (Bellinger & Lorton, 2018).

5.4.3 Stimulation of CD11b+ cells from control mice with forskolin and isoproterenol may result in 1) differential regulation of genes consistent with canonical signaling pathways for forskolin and isoproterenol and 2) a pattern of pro-inflammatory signaling

In examining the forskolin and isoproterenol treatment effect in CD11b+ splenocytes and peripheral cells, there were very few differentially expressed genes using $p_{adj} < .05$ criteria. Thus, the data were examined using an exploratory $p < .05$ criteria to detect differentially regulated genes across treatment groups.

5.4.3.1 Control CD11b+ Splenocytes: Forskolin vs RPMI.

For CD11b+ splenocytes from control mice, there was differential expression of 249 mRNA gene transcripts (151 upregulated; 98 downregulated, $p < .05$; Figure 16E) between those stimulated with forskolin compared to RPMI controls. Separation between biological replicates (i.e., animals) accounted for greater variance (67%, Figure 16D) than the separation between treatment conditions (i.e., forskolin vs RPMI; 20%, Figure 16D), suggesting that differences between groups was greater than between treatment conditions (i.e., within-groups).

Forskolin is a cell-permeable compound that activates adenylate cyclase (AC) independently of GPCR activation, resulting in production of cAMP and activation of subsequent

downstream pathways (e.g., PKA signaling; see Figure 1). Unfortunately, IPA and Panther over-representation analysis did not yield any notable significant results, likely due to the small number of differentially regulated genes. However, among individual genes of interest consistent with forskolin stimulation (Figure 16F), there was an upregulation in genes involved in PKA-signaling (*Flna* and *Pik3cg*). There was also downregulation of a gene involved in the inhibition of intracellular cAMP accumulation (*Ffar2*; Kimura et al., 2020); thus, downregulation of this gene would be consistent with allowing the accumulation of cAMP as a result of forskolin stimulation.

Among inflammatory genes, there was an upregulation in several broadly pro-inflammatory genes including *Fos*, *Pik3cg*, and *Mapk7*, and downregulation of *Grb2*, *Mx1*, and *Tnf*.

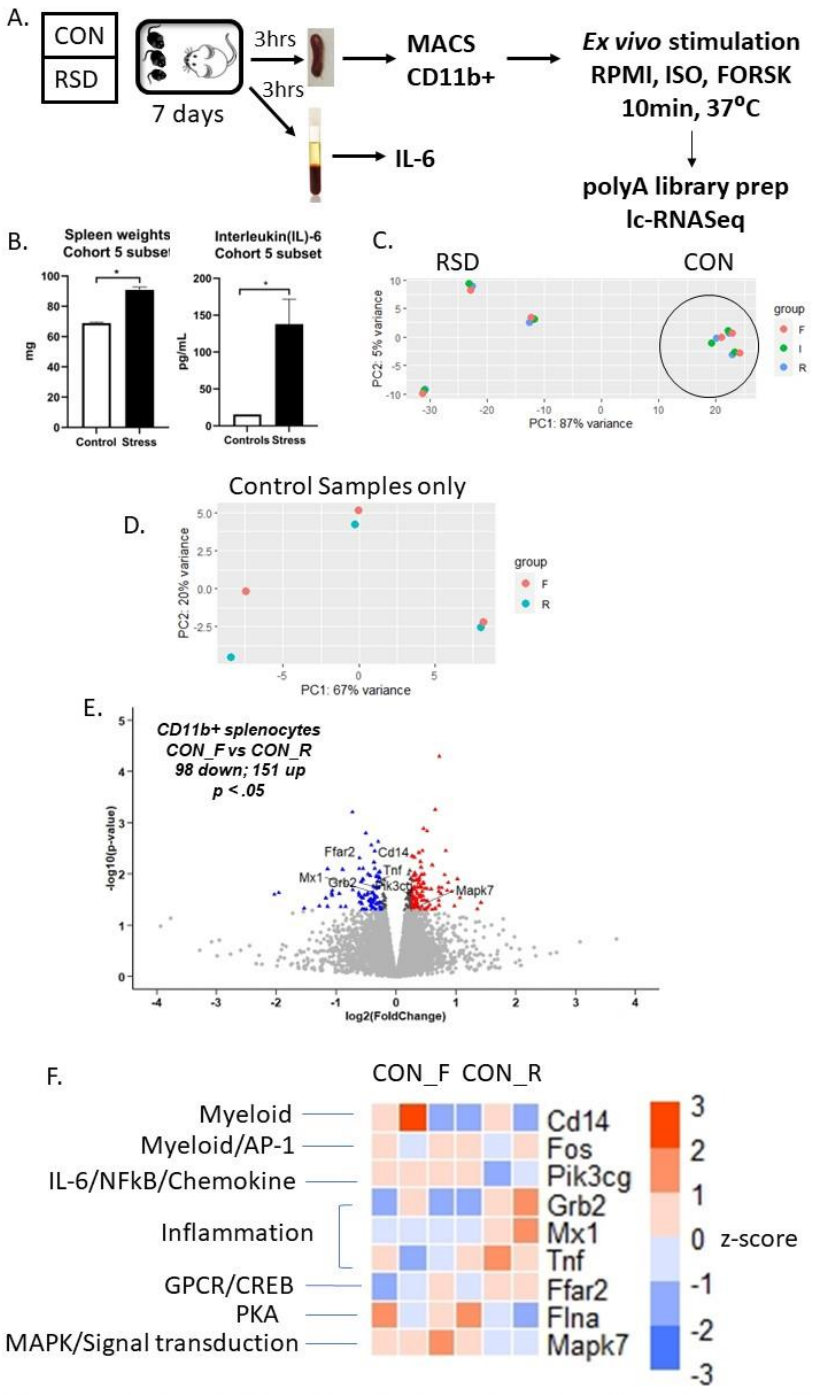


Figure 16. Treatment Effect: Control Splenocytes – Forskolin (F) vs. RPMI 1640 media (R)

The experiment (described in A) resulted in a significant effect of Repeated Social Defeat (RSD) on splenomegaly (B), peripheral pro-inflammatory proteins (B), and differential gene transcription (C). When examining the effect of forskolin treatment on CD11b+ splenocytes from control mice only (D, E, F), there was sparse evidence of differential regulation of genes consistent with canonical signaling pathways for forskolin (F), and sparse evidence for a pattern of pro-inflammatory signaling (F).

Notes: The canonical signaling pathway for forskolin involves β-adrenergic receptor (β-AR) independent activation of adenylate cyclase (AC), followed by downstream activation of cyclic adenosine monophosphate (cAMP), protein kinase–A (PKA), and cAMP response element binding (CREB)-mediated gene transcription.

A. Diagram of experiment. Male C56BL/6 mice (6-8 weeks old) underwent repeated social defeat (RSD) for 6 days or were left undisturbed in their cages in the control room (CON) until sacrifice. Mice were sacrificed within three hours of the last round of social defeat. After sacrifice, the spleen and peripheral blood was harvested from the mice for isolation of CD11b+ cells and plasma, respectively. CD11b+ splenocytes were isolated using Miltenyi Biotec CD11b+ Microbeads (Miltenyi Biotec Cat #130-049-60). Levels of IL-6 were measured using a quantitative sandwich enzyme immunoassay kit (BD OptEIA Mouse IL-6 ELISA Cat#555220; BD Biosciences, San Jose, CA), according to the manufacturer's directions. CD11b+ splenocytes were either left unstimulated (RPMI 1640 cell culture media alone) or were subject to stimulation by isoproterenol (ISO), a non-specific β-adrenergic agonist, or forskolin (FORSK), a non-specific activator of adenylate cyclase (AC) for 10 minutes at 37ºC. RNA was then isolated. A subset of these samples (N = 3 in each group CON/RSD) were sent for polyA library preparation (poly-A tail was used to amplify mRNA using oligo(dT) primers for mRNA library generation; the poly-A tail is a long chain of adenosine nucleotides added to 3' end RNA to prevent degradation and mark it for transport to ribosomes to be translated into protein) and lc-RNAseq (mRNA library generation for limiting cell (lc = low input RNA amounts: 10pg-1ng) was performed using the Clontech SMART-Seq HT kit (Takara Bio Inc., Mountain View, CA, Cat #634456) and the Nextera XT DNA Library Prep Kit (Illumina, Inc., San Diego, CA, Cat #FC-131-1096). RNA-Seq = Samples were then sequenced at a depth of 15-21million 2x100bp paired-end clusters on an Illumina NovaSeq6000 platform (Illumina, Inc., San Diego)). hrs = hours; IL = interleukin.
B. Quantitative measures of stress in animals experiencing repeated social defeat (RSD/"Stress") include splenomegaly and elevated levels of peripheral pro-inflammatory proteins. The data comparing spleen weights in the subset of mice sent for sequencing is reproduced from Figure 2G (N = 3 mice in each CON/RSD group). The graph comparing interleukin(IL)-6 levels in the subset of mice sent for sequencing (N = 3 mice in each CON/RSD group) represents a subset of the data presented in Figure 2H. *$p < .05$
C. Principle Component Analysis (PCA) plot of all genes for all treatment groups (RPMI, ISO, FORSK) and mice (CON (N = 3), RSD (N = 3) included in the analysis. Separation between groups (CON/RSD) accounted for greater variance (87%) than separation between treatment conditions (RPMI, ISO, FORSK; 5%). This analysis focused only on samples from the CON group, indicated by the black circle.
D. Principle Component Analysis (PCA) plot of all genes for the forskolin (F) and RPMI (R) treatment groups using CD11b+ splenocytes from control mice. Separation between biological replicates (i.e., animals) accounted for greater variance (67%) than the separation between treatment conditions (i.e., forskolin vs RPMI; 20%).
E. Volcano plot for all up and down-regulated genes for CON_F = CON_FORSK group (N = 3 mice/samples) vs. CON_R = CON_RPMI group (N = 3 mice/samples) after CLEAR selection for robust/low-noise transcripts (Walker et al., 2020) and filtering out all genes with an average median count of ≤ 2 (across all samples). Differential gene expression was

estimated using the DESeq2 package in R (Love et al., 2014). This analysis is based on a secondary pairwise comparison between treatment groups (FORSK vs. RPMI) from a primary within & between groups regression analysis in DESeq2. Genes in dark gray, blue, and red are significantly up or down regulated using a p-value of .05, which was NOT adjusted for multiple comparisons (N = 151 significantly upregulated genes; N = 98 significantly downregulated genes). Red genes represent a > .5 fold upregulation in CON_F vs. CON_R samples; Blue genes represent a > .5 fold downregulation in CON_F vs. CON_R samples.

F. Heat map of differentially expressed genes based on 1) individual genes previously identified in RSD literature (Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018), 2) individual genes identified in prior IPA Canonical Pathway analyses involved in pro-inflammatory pathways, 3) individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching," and 4) individual genes from intracellular signaling pathways identified in prior IPA Canonical Pathway analyses including protein kinase-A (PKA), cAMP response element binding (CREB) protein, mitogen-activated protein kinase (MAPK), and G-protein coupled receptor (GPCR) signaling pathways. Of note, there was an upregulation in genes involved in PKA-signaling (*Flna* and *Pik3cg*) and downregulation of a gene involved in the inhibition of intracellular cAMP accumulation (*Ffar2).* Among inflammatory genes, there was an upregulation in several broadly pro-inflammatory genes including *Fos, Pik3cg, and Mapk7,* and downregulation of *Grb2, Mx1, and Tnf. Notes*: Individual gene z-scores depicted in the heat map (see legend) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p < .05$ (DESeq2), NOT adjusted for multiple comparisons (Love et al., 2014). CON_F = Control_FORSK group (N = 3); CON_R = Control_RPMI group (N = 3); Each column represents an individual mouse. Ig = Immunoglobin; ECM = extracellular matrix; GC = glucocorticoid, IL-6 = interleukin-6; TLR = toll-like receptor; NF-κB = nuclear factor – kappaBeta; AP-1 = genes involved in activator protein-1 (AP-1) pro-inflammatory signaling.

5.4.3.2 Control CD11b+ Splenocytes: Isoproterenol vs RPMI

Among CD11b+ splenocytes from control mice, there was differential expression of 356 mRNA gene transcripts (232 upregulated; 124 downregulated, $p < .05$; Figure 17B) between those stimulated with isoproterenol compared to RPMI controls. Separation between biological replicates (i.e., animals) accounted for greater variance (64%, Figure 17A) than the separation between treatment conditions (i.e., isoproterenol vs RPMI; 19%, Figure 17A), suggesting that differences between groups was greater than between treatment conditions (i.e., within-groups).

Isoproterenol typically stimulates canonical β-adrenergic signaling pathways through GPCR mediated activation of cAMP and PKA intracellular signaling cascades. Among

differentially regulated genes consistent with isoproterenol stimulation (Figure 17C), there was a downregulation in a β-adrenergic receptor gene (*Adbr2*; Figure 17C, 18B). There was also a downregulation in genes involved in PKA-related signaling (*Dusp6, Ptp4a1, Tcf4, Tgfb1*; Figure 17C). This pattern of differential gene transcription may align with canonical isoproterenol signaling pathways in that initial β-adrenergic signaling associates with an internalization of the receptor and thus a decrease in signaling via PKA.

Among inflammatory-related genes, Panther overrepresentation analysis revealed an enrichment in expression of TLR-signaling genes (7.02-fold, $p = .0003$, *FDR* = .011: *Tlr2, Irak1, Ptgs2, Cd14, Nfkbia, Jun*) and an enrichment in expression of genes involved in inflammation mediated by chemokine and cytokine signaling (4.38-fold; $p < .0001$, *FDR* < .0001: *IL1b, Ptgs2, Cish, Ccrl2, Ccr1, Ccl7, Fpr1, Rgs1, Myh10, Ccl2, Nfkbia, Grk6, Junb, Jund, Jun, Ccl3, C5ar1, Grb2*). Differential regulation among individual genes of interest generally supported this pattern of pro-inflammatory activation (Figure 17C).

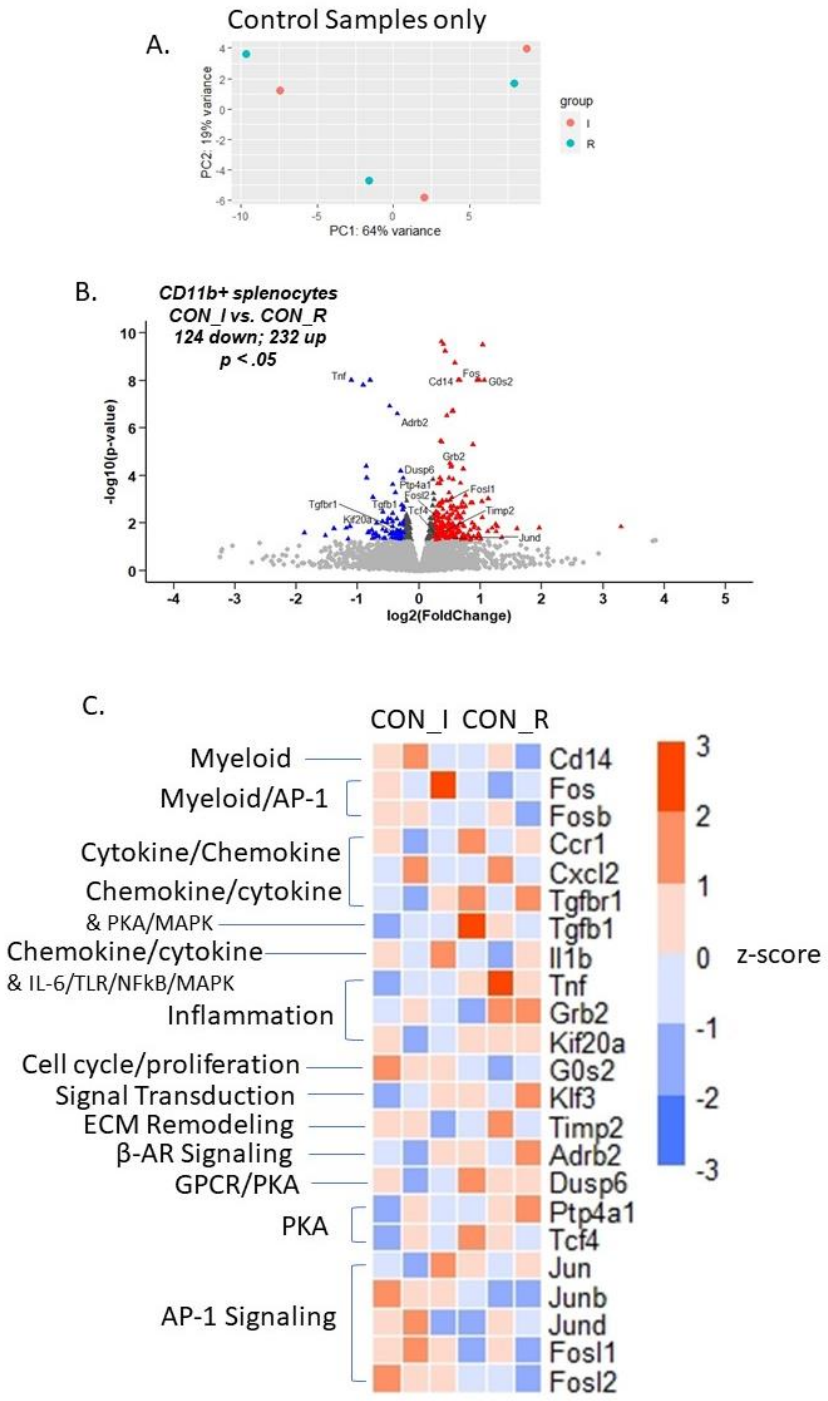


Figure 17. Treatment Effect: Control Splenocytes – Isoproterenol (I) vs. RPMI 1640 media (R)

Isoproterenol treatment of CD11b+ splenocytes from control mice only (A, B, C) resulted in initial evidence of differential regulation of genes consistent with canonical signaling pathways for isoproterenol stimulation and pro-inflammatory signaling (C).

Notes: The canonical signaling pathway for isoproterenol, a non-specific β-adrenergic receptor (β-AR) agonist, involves G-protein dependent activation of G-protein-alpha-S ($G\alpha_s$), followed by adenylate cyclase (AC) and downstream activation of cyclic adenosine monophosphate (cAMP), protein kinase–A (PKA), and cAMP response element binding (CREB)-mediated gene transcription.

A. Principle Component Analysis (PCA) plot of all genes for the isoproterenol (I) and RPMI (R) treatment groups only using CD11b+ splenocytes from control mice. Separation between biological replicates (i.e., animals) accounted for greater variance (64%) than the separation between treatment conditions (i.e., isoproterenol vs RPMI; 19%).

B. Volcano plot for all up and down-regulated genes for CON_I = CON_ISO group (N = 3 mice/samples) vs. CON_R = CON_RPMI group (N = 3 mice/samples) after CLEAR selection for robust/low-noise transcripts (Walker et al., 2020) and filtering out all genes with an average median count of ≤ 2 (across all samples). Differential gene expression was estimated using the DESeq2 package in R (Love et al., 2014). This analysis is based on a secondary pairwise comparison between treatment groups (ISO vs. RPMI) from a primary within & between groups regression analysis in DESeq2. Genes in dark gray, blue, and red are significantly up or down regulated using a p-value of .05, which was NOT adjusted for multiple comparisons (N = 232 significantly upregulated genes; N = 124 significantly downregulated genes). Red genes represent a > .5-fold upregulation in CON_I vs. CON_R samples; Blue genes represent a > .5-fold downregulation in CON_I vs. CON_R samples.

C. Heat map of differentially expressed genes based on 1) individual genes previously identified in RSD literature (Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018), 2) individual genes identified in prior IPA Canonical Pathway analyses involved in pro-inflammatory pathways, 3) individual genes identified *a-priori* as involved in β-AR "signal switching," and 4) individual genes from intracellular signaling pathways identified in prior IPA Canonical Pathway analyses including protein kinase-A (PKA), cAMP response element binding (CREB) protein, mitogen-activated protein kinase (MAPK), and G-protein coupled receptor (GPCR) signaling pathways. Of note, there was a downregulation in a β-adrenergic receptor gene (*Adbr2*) and a downregulation in genes involved in PKA-related signaling (*Dusp6, Ptp4a1, Tcf4, Tgfb1*). Differential regulation among individual pro-inflammatory genes of interest generally supported a pattern of pro-inflammatory activation. *Notes*: Individual gene z-scores depicted in the heat map (see legend) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p < .05$ (DESeq2), NOT adjusted for multiple comparisons (Love et al., 2014). CON_I = Control_ISOgroup (N = 3); CON_R = Control_RPMI group (N = 3); Each column represents an individual mouse. Ig = Immunoglobin; ECM = extracellular matrix; GC = glucocorticoid, IL-6 = interleukin-6; TLR = toll-like receptor; NF-κB = nuclear factor – kappaBeta; AP-1 = genes involved in activator protein-1 (AP-1) pro-inflammatory signaling.

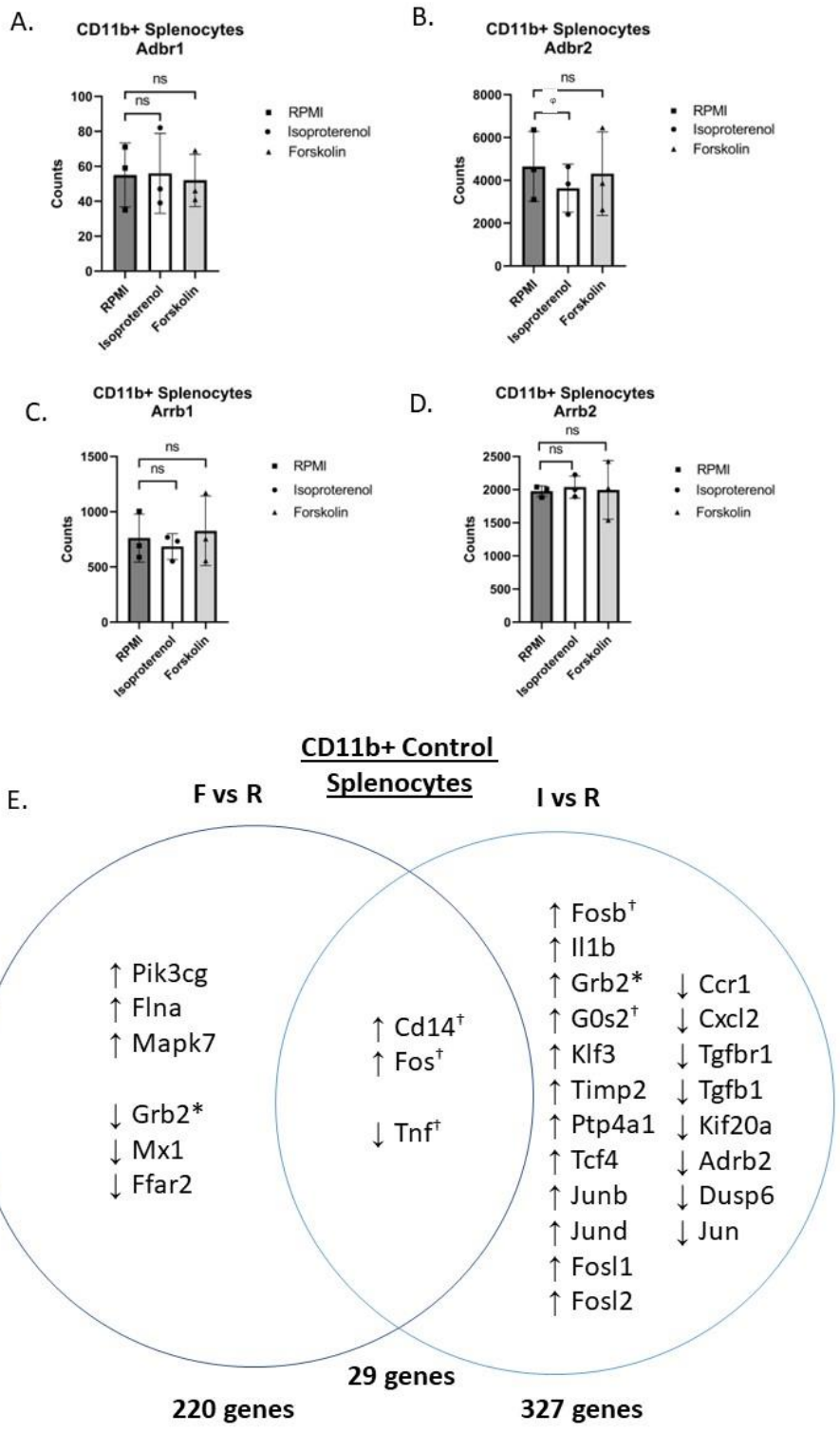


Figure 18. Treatment Effect: Control Splenocytes – Individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching;" shared and unique genes of interest across treatment conditions

Across particular genes of interest identified a-priori as involved in β-adrenergic receptor (β-AR) "signal switching" (A-D), isoproterenol stimulation resulted in significantly lower gene expression of Adrb2, the gene encoding the β2-adrenergic receptor (B) in CD11b+ splenocytes from control mice. Among shared genes of interest to forskolin and isoproterenol stimulation in CD11b+ splenocytes from control mice (E), there was an upregulation in myeloid related genes (CD14, Fos) and a downregulation in the pro-inflammatory gene Tnf. Unique genes of interest to forskolin and isoproterenol stimulation were broadly supportive of expected intracellular signaling patterns (E).

Notes: The canonical signaling pathway for forskolin involves β-adrenergic receptor (β-AR) independent activation of adenylate cyclase (AC), followed by downstream activation of cyclic adenosine monophosphate (cAMP), protein kinase–A (PKA), and cAMP response element binding (CREB)-mediated gene transcription. The canonical signaling pathway for isoproterenol, a non-specific β-adrenergic receptor (β-AR) agonist, involves G-protein dependent activation of G-protein-alpha-S ($G\alpha_s$), followed by activation of AC, and downstream activation of cAMP, PKA, and CREB-mediated gene transcription.

(A-D). Graphs of individual gene counts for particular genes of interest identified *a-priori* as involved in β-AR "signal switching" among CD11b+ splenocytes from control mice only. *Notes:* $*p_{adj} < .05$, $^{\Phi}p < .05$ (not adjusted for multiple comparisons), ns = not significant. Individual points in each column (squares, circles, triangles) represent raw gene counts from an individual mouse. RPMI = unstimulated group, cells incubated in RPMI 1640 cell culture media alone for 10 min at 37ºC, Isoproterenol = cells were stimulated with 10uM isoproterenol, a non-specific β-adrenergic agonist via incubation for 10 min at 37ºC, Forskolin = cells were stimulated with 10uM forskolin, a non-specific activator of adenylate cyclase (AC), via incubation for 10 min at 37ºC. CD11b+ = cell surface protein marker indicating mouse monocytes and granulocytes.

- **A.** There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ splenocytes from control mice on gene expression of Adrb1, the gene encoding the β1-adrenergic receptor.
- **B.** Isoproterenol stimulation of CD11b+ splenocytes from control mice resulted in significantly lower gene expression of Adrb2, the gene encoding the β2-adrenergic receptor, compared to unstimulated (RPMI) cells using a p-value < .05, unadjusted for multiple comparisons. There was no significant effect of forskolin stimulation of CD11b+ splenocytes from control mice on gene expression of Adrb2.
- **C.** There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ splenocytes from control mice on gene expression of Arrb1, the gene encoding the β-arrestin-1 protein. β-arrestin-1 is a key signaling protein involved in canonical β-adrenergic receptor signaling.
- **D.** There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ splenocytes from control mice on gene expression of Arrb2 , the gene encoding the β-arrestin-2 protein. β-arrestin-2 is a key signaling protein involved in non-canonical β-adrenergic receptor signaling.

- **E.** Shared and unique genes of interest to forskolin and/or isoproterenol stimulation in CD11b+ splenocytes from control mice. Total number of shared and unique genes are indicated in bold below the Venn-diagram. Genes of interest indicated within the diagram

were identified from 1) individual genes previously identified in RSD literature (Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018), 2) individual genes identified in prior IPA Canonical Pathway analyses involved in pro-inflammatory pathways, 3) individual genes identified *a-priori* as involved in β-AR "signal switching," and 4) individual genes from intracellular signaling pathways identified in prior IPA Canonical Pathway analyses including protein kinase-A (PKA), cAMP response element binding (CREB) protein, mitogen-activated protein kinase (MAPK), and G-protein coupled receptor (GPCR) signaling pathways. All genes displayed were significantly differentially regulated using $p < .05$ (DESeq2), NOT adjusted for multiple comparisons. *Notes*: R = RPMI, F = forskolin, I = isoproterenol. *expressed in both treatment comparisons (i.e., F vs R and I vs R) but in opposite directions. †also expressed among CD11b+ peripheral cells from Control mice.

5.4.3.3 Control CD11b+ Splenocytes: Summary

In general, exploratory analyses found patterns of differential regulation across CD11b+ splenocytes from control mice to be broadly supportive of expected intracellular signaling patterns consistent with forskolin or isoproterenol stimulation, including an upregulation in pro-inflammatory signaling. Shared and unique genes of interest to forskolin and/or isoproterenol stimulation in CD11b+ splenocytes from control mice are listed in Figure 18E.

5.4.3.4 Control CD11b+ Peripheral Cells: Forskolin vs RPMI

Among CD11b+ peripheral cells from control mice, there was differential expression of 80 mRNA gene transcripts (61 upregulated; 19 downregulated, $p < .05$; Figure 19E) between those stimulated with forskolin compared to RPMI controls. Separation between biological replicates (i.e., animals) accounted for greater variance (80%, Figure 19D) than the separation between treatment conditions (i.e., forskolin vs RPMI; 15%, Figure 19D), suggesting that differences between groups was greater than between treatment conditions (i.e., within-groups).

Panther Over-representation analysis found that many of the differentially regulated genes may be involved in upregulation of cell cycling (12.39-fold enrichment), although this pathway was not statistically significant ($p > .05$). Among individual genes that were differentially regulated

across treatment conditions ($p < .05$), there were no genes identified as a part of our pathways of interest.

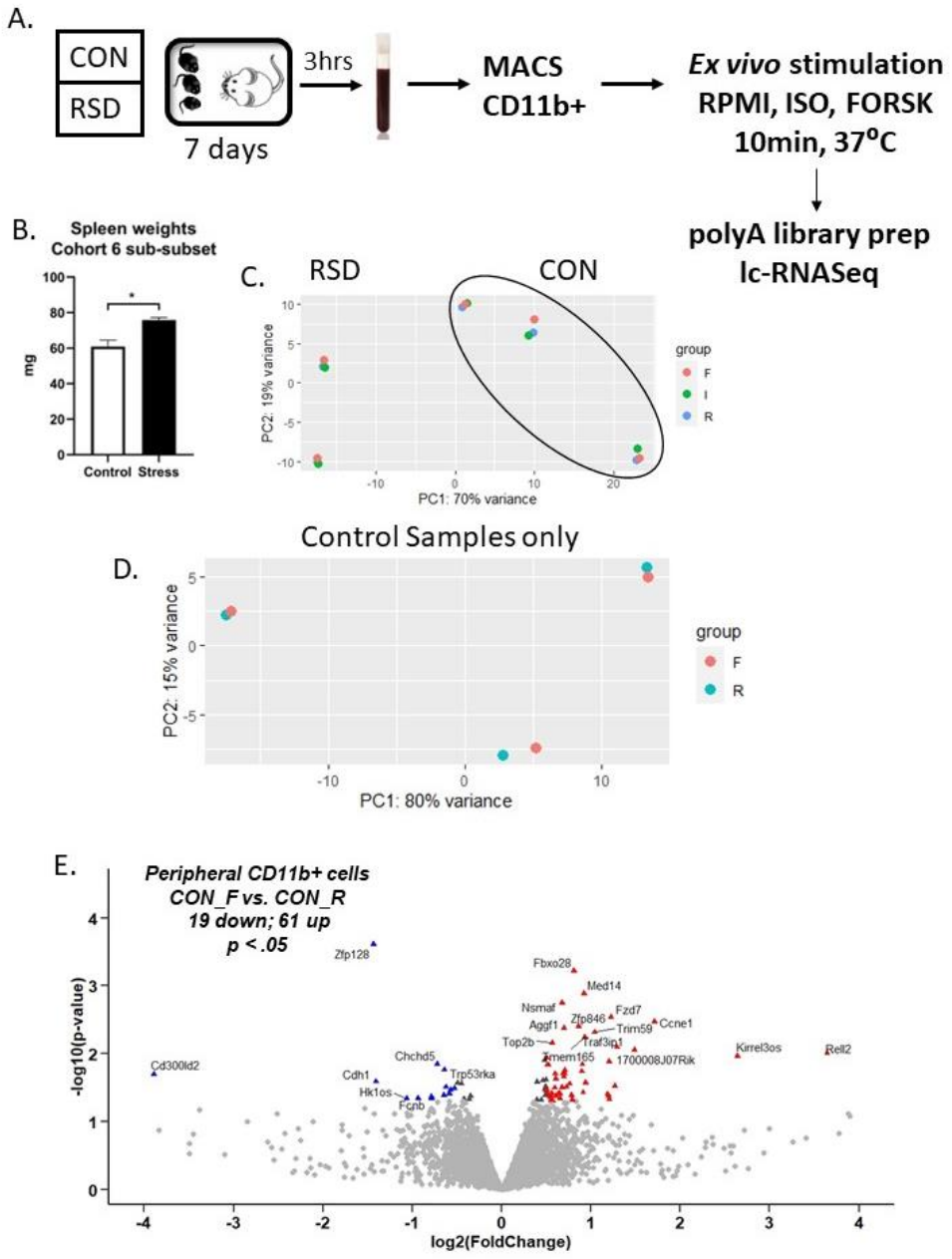


Figure 19. Treatment Effect: Control Peripheral Cells – Forskolin (F) vs. RPMI 1640 media (R)

The experiment (described in A) resulted in a significant effect of Repeated Social Defeat (RSD) on splenomegaly (B) and differential gene transcription (C). When examining the effect of forskolin treatment on CD11b+ peripheral cells from control mice only (D, E), there was sparse to no evidence of differential regulation of genes consistent with canonical signaling pathways for forskolin or pro-inflammatory signaling pathways.

Notes: Among individual genes that were differentially regulated across treatment conditions (*E*), there were no genes identified as a part of our pathways of interest.

A. Diagram of experiment. Male C56BL/6 mice (6-8 weeks old) underwent repeated social defeat (RSD) for 6 days or were left undisturbed in their cages in the control room (CON) until sacrifice. Mice were sacrificed within three hours (hrs) of the last round of social defeat. After sacrifice, the peripheral blood was harvested from the mice for isolation of CD11b+ cells. CD11b+ peripheral cells were isolated using Miltenyi Biotec CD11b+ Microbeads (Miltenyi Biotec Cat #130-049-60). CD11b+ peripheral cells were either left unstimulated (RPMI 1640 cell culture media alone) or were subject to stimulation by isoproterenol (ISO), a non-specific β-adrenergic agonist, or forskolin (FORSK), a non-specific activator of adenylate cyclase (AC) for 10 minutes at 37ºC. RNA was then isolated. A subset of these samples (N = 3 in each group CON/RSD) were sent for polyA library preparation (poly-A tail was used to amplify mRNA using oligo(dT) primers for mRNA library generation; the poly-A tail is a long chain of adenosine nucleotides added to 3' end RNA to prevent degradation and mark it for transport to ribosomes to be translated into protein) and lc-RNAseq (mRNA library generation for limiting cell (lc = low input RNA amounts: 10pg-1ng) was performed using the Clontech SMART-Seq HT kit (Takara Bio Inc., Mountain View, CA, Cat #634456) and the the Nextera XT DNA Library Prep Kit (Illumina, Inc., San Diego, CA, Cat #FC-131-1096). RNA-Seq = Samples were then sequenced at a depth of 15-21million 2x100bp paired-end clusters on an Illumina NovaSeq6000 platform (Illumina, Inc., San Diego)).

B. A quantitative measure of stress in animals experiencing repeated social defeat (RSD/"Stress") includes splenomegaly. The sub-subset of data displayed here represents the mice for the samples used in the analyses (N = 2 RSD, N = 3 CON). Specifically, one RSD mouse that was sent for sequencing was removed from the analyses due to significant overlap with gene expression among control mice. The data comparing spleen weights in the subset of mice sent for sequencing can be seen in Figure 2J. **p*< .05

C. Principle Component Analysis (PCA) plot of all genes for all treatment groups (RPMI, ISO, FORSK) and mice (CON (N = 3), RSD (N = 2)) included in the differential gene analysis. Separation between groups (CON/RSD) accounted for greater variance (70%) than separation between treatment conditions (RPMI, ISO, FORSK; 19%). This analysis focused only on samples from the CON group, indicated by the black circle.

D. Principle Component Analysis (PCA) plot of all genes for the forskolin (F) and RPMI (R) treatment groups using CD11b+ peripheral cells from Control mice. Separation between biological replicates (i.e., animals) accounted for greater variance (80%) than the separation between treatment conditions (i.e., forskolin vs RPMI; 15%).

E. Volcano plot for all up and down-regulated genes for CON_F = CON_FORSK group (N = 3 mice/samples) vs. CON_R = CON_RPMI group (N = 3 mice/samples) after CLEAR selection for robust/low-noise transcripts (Walker et al., 2020) and filtering out all genes with an average median count of ≤ 2 (across all samples). Differential gene expression was estimated using the DESeq2 package in R (Love et al., 2014). This analysis is based on a secondary pairwise comparison between treatment groups (FORSK vs. RPMI) from a primary within & between groups regression analysis in DESeq2. Genes in dark gray, blue, and red are significantly up or down regulated using a p-value of .05, which was NOT adjusted for multiple comparisons (N = 61 significantly upregulated genes; N = 19

significantly downregulated genes). Red genes represent a > .5 fold upregulation in CON_F vs. CON_R samples; Blue genes represent a > .5 fold downregulation in CON_F vs. CON_R samples.

5.4.3.5 Control CD11b+ Peripheral Cells: Isoproterenol vs RPMI

Among CD11b+ peripheral cells from control mice, there was differential expression of 75 mRNA gene transcripts (50 upregulated; 25 downregulated, $p < .05$; Figure 20B) between those stimulated with isoproterenol compared to RPMI controls. Separation between biological replicates (i.e., animals) accounted for greater variance (78%, Figure 20A) than the separation between treatment conditions (i.e., isoproterenol vs RPMI; 17%, Figure 20A), suggesting that differences between groups was greater than between treatment conditions (i.e., within-groups).

Using Panther Overrepresentation analysis, there was a significant enrichment in inflammatory-related genes mediated by chemokine and cytokine signaling pathways (6.61-fold enrichment, $p = .001$), however, this pathway was not significant after adjustment for multiple comparisons (FDR > .05). Among individual genes of interest ($p < .05$; Figure 20C), there was an upregulation in several broadly pro-inflammatory genes including *Cd14, Fos, Fosb*, and a downregulation in the inflammatory-related gene *Tnf*.

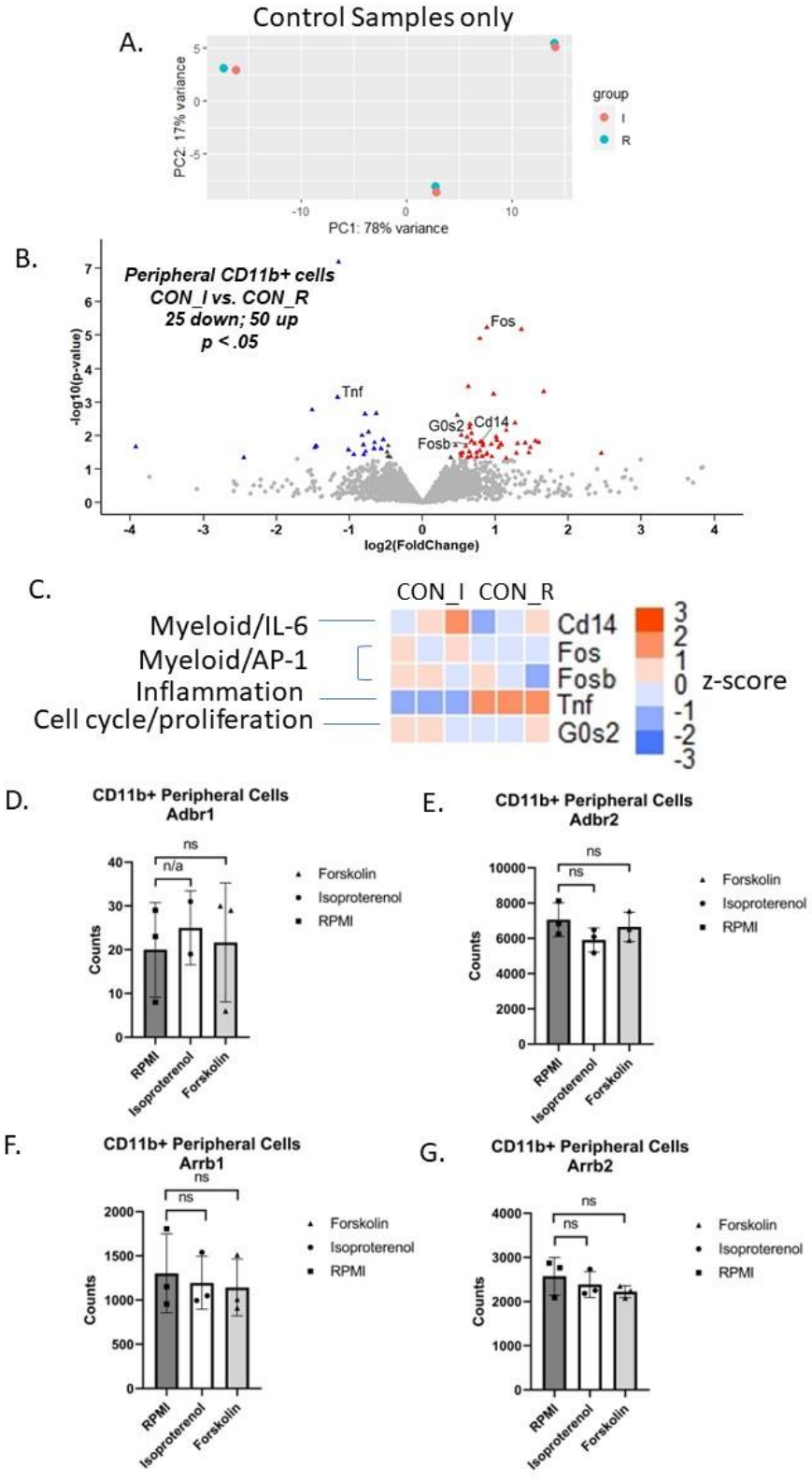


Figure 20. Treatment Effect: Control Peripheral Cells – Isoproterenol (I) vs. RPMI 1640 media (R); individual genes identified a-priori as involved in β-adrenergic receptor (β-AR) "signal switching."

Isoproterenol stimulation of CD11b+ peripheral cells from control (CON) mice only (A, B, C) resulted in inconclusive/lack of evidence for differential regulation of genes consistent with canonical signaling pathways for isoproterenol, and initial evidence suggesting an upregulation in pro-inflammatory signaling (C). Individual gene counts for particular genes of interest identified a-priori as involved in β-adrenergic receptor (β-AR) "signal switching" (D-G) were not significantly different between treatment conditions for CD11b+ peripheral cells from control mice.

Notes: The canonical signaling pathway for isoproterenol, a non-specific β-adrenergic receptor (β-AR) agonist, involves G-protein dependent activation of G-protein-alpha-S ($G\alpha_s$), followed by adenylate cyclase (AC) and downstream activation of cyclic adenosine monophosphate (cAMP), protein kinase–A (PKA), and cAMP response element binding (CREB)-mediated gene transcription.

- **A.** Principle Component Analysis (PCA) plot of all genes for the isoproterenol (I) and RPMI (R) treatment groups only using CD11b+ peripheral cells from control mice. Separation between biological replicates (i.e., animals) accounted for greater variance (78%) than the separation between treatment conditions (i.e., isoproterenol vs RPMI; 17%).
- **B.** Volcano plot for all up and down-regulated genes for CON_I = CON_ISO group (N = 3 mice/samples) vs. CON_R = CON_RPMI group (N = 3 mice/samples) after CLEAR selection for robust/low-noise transcripts (Walker et al., 2020) and filtering out all genes with an average median count of ≤ 2 (across all samples). Differential gene expression was estimated using the DESeq2 package in R (Love et al., 2014). This analysis is based on a secondary pairwise comparison between treatment groups (ISO vs. RPMI) from a primary within & between groups regression analysis in DESeq2. Genes in dark gray, blue, and red are significantly up or down regulated using a p-value of .05, which was NOT adjusted for multiple comparisons (N = 50 significantly upregulated genes; N = 25 significantly downregulated genes). Red genes represent a > .5-fold upregulation in CON_I vs. CON_R samples; Blue genes represent a > .5-fold downregulation in CON_I vs. CON_R samples.
- **C.** Heat map of differentially expressed genes based on 1) individual genes previously identified in RSD literature (Cole et al., 2010; Powell et al., 2013; Niruala, Witcher, et al., 2018), 2) individual genes identified in prior IPA Canonical Pathway analyses involved in pro-inflammatory pathways, 3) individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching," and 4) individual genes from intracellular signaling pathways identified in prior IPA Canonical Pathway analyses including protein kinase-A (PKA), cAMP response element binding (CREB) protein, mitogen-activated protein kinase (MAPK), and G-protein coupled receptor (GPCR) signaling pathways. Consistent with initial evidence for an upregulation in pro-inflammatory signaling, there was an upregulation in several broadly pro-inflammatory genes including *Cd14, Fos, Fosb*, and a downregulation in the inflammatory-related gene *Tnf* among isoproterenol stimulated CD11b+peripheral cells from control mice (CON_I) compared to unstimulated (RPMI) CD11b+peripheral cells from control mice (CON_R). *Notes*: Individual gene z-scores depicted in the heat map (see legend) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p < .05$ (DESeq2), NOT adjusted for multiple comparisons. CON_I = CON_ISO group (N = 3); CON_R = CON_RPMI group (N = 3); Each column

represents an individual mouse. IL-6 = interleukin-6; AP-1 = activator protein-1 (AP-1), a pro-inflammatory transcriptional activator.

(D-G). Graphs of individual gene counts for particular genes of interest identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching" among CD11b+ peripheral cells from control mice only. *Notes:* $*p_{adj} < .05$, $^{\Phi}p < .05$ (not adjusted for multiple comparisons), ns = not significant. Individual points in each column (squares, circles, triangles) represent raw gene counts from an individual mouse. RPMI = unstimulated group, cells incubated in RPMI 1640 cell culture media alone for 10 min at 37ºC, Isoproterenol = cells were stimulated with 10uM isoproterenol, a non-specific β-adrenergic agonist via incubation for 10 min at 37ºC, Forskolin = cells were stimulated with 10uM forskolin, a non-specific activator of adenylate cyclase (AC), via incubation for 10 min at 37ºC. CD11b+ = cell surface protein marker indicating mouse monocytes and granulocytes.

D. There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ peripheral cells from control mice on gene expression of Adrb1, the gene encoding the β1-adrenergic receptor.

E. There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ peripheral cells from control mice on gene expression of Adrb2, the gene encoding the β2-adrenergic receptor.

F. There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ peripheral cells from control mice on gene expression of Arrb1, the gene encoding the β-arrestin-1 protein. β-arrestin-1 is a key signaling protein involved in canonical β-adrenergic receptor signaling.

G. There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ peripheral cells from control mice on gene expression of Arrb2 , the gene encoding the β-arrestin-2 protein. β-arrestin-2 is a key signaling protein involved in non-canonical β-adrenergic receptor signaling.

5.4.3.6 Control CD11b+ Peripheral Cells: Summary

In general, exploratory analyses found patterns of differential regulation across CD11b+ peripheral cells to be possibly suggestive of an upregulation in pro-inflammatory signaling, with little information about intracellular signaling patterns as a result of forskolin and/or isoproterenol stimulation. Shared and unique genes of interest to forskolin and/or isoproterenol stimulation in CD11b+ peripheral cells from control mice are listed in Figure 21.

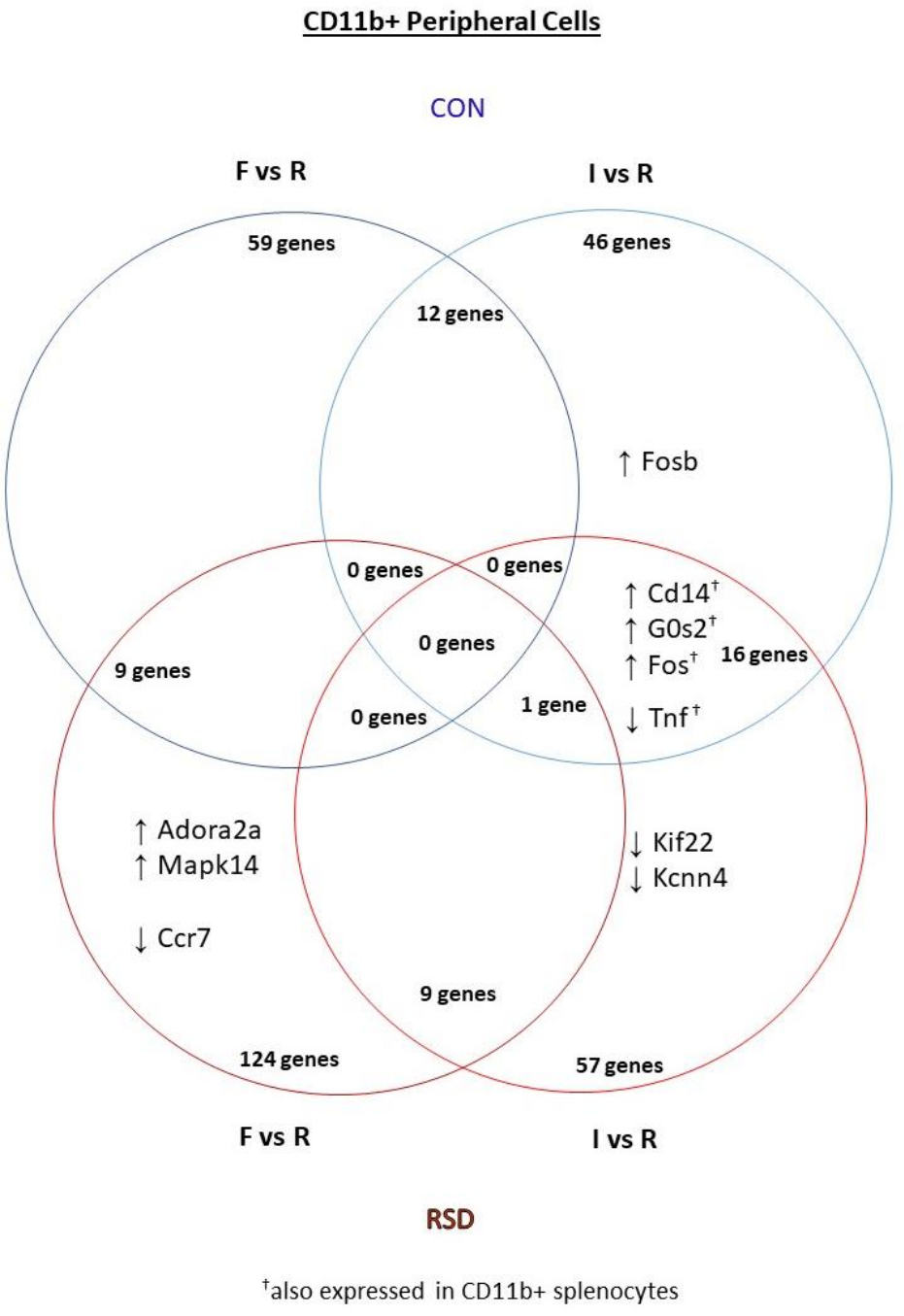


Figure 21. Treatment Effect: Peripheral Cells – Shared and unique genes of interest

Among shared and unique genes of interest to forskolin and isoproterenol stimulation of CD11b+ peripheral cells from control (CON) and Repeated Social Defeat (RSD) mice, evidence was possibly suggestive of an upregulation in pro-inflammatory signaling, with little information about intracellular signaling patterns as a result of forskolin and/or isoproterenol stimulation. Among CD11b+ peripheral cells from control mice, there were few unique genes of interest to forskolin and isoproterenol stimulation and few shared genes with CD11b+ peripheral cells from RSD mice.

Notes: Shared and unique genes of interest to forskolin and/or isoproterenol stimulation in CD11b+ peripheral cells from control (CON) and RSD mice. Total number of shared and unique genes are indicated in bold within the Venn-diagram. Genes of interest indicated within the diagram were identified from 1) individual genes previously identified in RSD literature (Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018), 2) individual genes identified in prior IPA Canonical Pathway analyses involved in pro-inflammatory pathways, 3) individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching," and 4) individual genes from intracellular signaling pathways identified in prior IPA Canonical Pathway analyses including protein kinase-A (PKA), cAMP response element binding (CREB) protein, mitogen-activated protein kinase (MAPK), and G-protein coupled receptor (GPCR) signaling pathways. All genes displayed were significantly differentially regulated using $p < .05$ (DESeq2), NOT adjusted for multiple comparisons. *Notes*: R = RPMI, F = forskolin, I = isoproterenol. †also expressed among CD11b+ splenocytes.

5.4.4 Stimulation of CD11b+ cells from RSD mice with forskolin and isoproterenol resulted in 1) inconclusive evidence and/or lack of evidence for signaling through identified pathways after stimulation with forskolin and isoproterenol, and 2) mixed evidence regarding pro-inflammatory signaling

Across both CD11b+ splenocytes and peripheral cells from RSD mice, there were few differentially expressed genes using a $p_{adj} < .05$ criteria. Thus, similar to control mice, the data were examined using an exploratory $p < .05$ criteria to detect differentially regulated genes across treatment groups.

5.4.4.1 RSD CD11b+ Splenocytes: Forskolin vs RPMI

Among CD11b+ splenocytes from RSD mice, there was differential expression of 84 mRNA gene transcripts (40 upregulated; 44 downregulated, $p < .05$; Figure 22E) between those stimulated with forskolin compared to RPMI controls. Separation between biological replicates (i.e., animals) accounted for greater variance (75%, Figure 22D) than the separation between

treatment conditions (i.e., forskolin vs RPMI; 19%, Figure 22D), suggesting that differences between groups was greater than between treatment conditions (i.e., within-groups).

Panther Overexpression analysis found an enrichment in Ras-mediated signaling among CD11b+ splenocytes from RSD mice (3.98-fold enrichment, p = .043: *Kras, Pik3cg, Grb2*), although this finding was not significant after adjustment for multiple comparisons (FDR > .05). This pathway can be involved in GPCR to NFkB signaling (and non-canonical β-adrenergic signaling), although this is not consistent with typical forskolin signaling pathways.

Individual genes of interest that were differentially regulated between treatment conditions were broadly suggestive of an *attenuation* in pro-inflammatory signaling (Figure 22F). This included two inflammatory genes that were downregulated: *Tnf,* a gene encoding a pro-inflammatory cytokine expressed primarily in monocytes, and *Mpo*, a gene encoding a protein important in the microbicidal activity of neutrophils (Furtmüller et al., 1998; Figure 22F). There was also downregulation in a gene that encodes a protein known to promote NFkB activation (*Card11*; Bertin et al., 2001; Figure 22F).

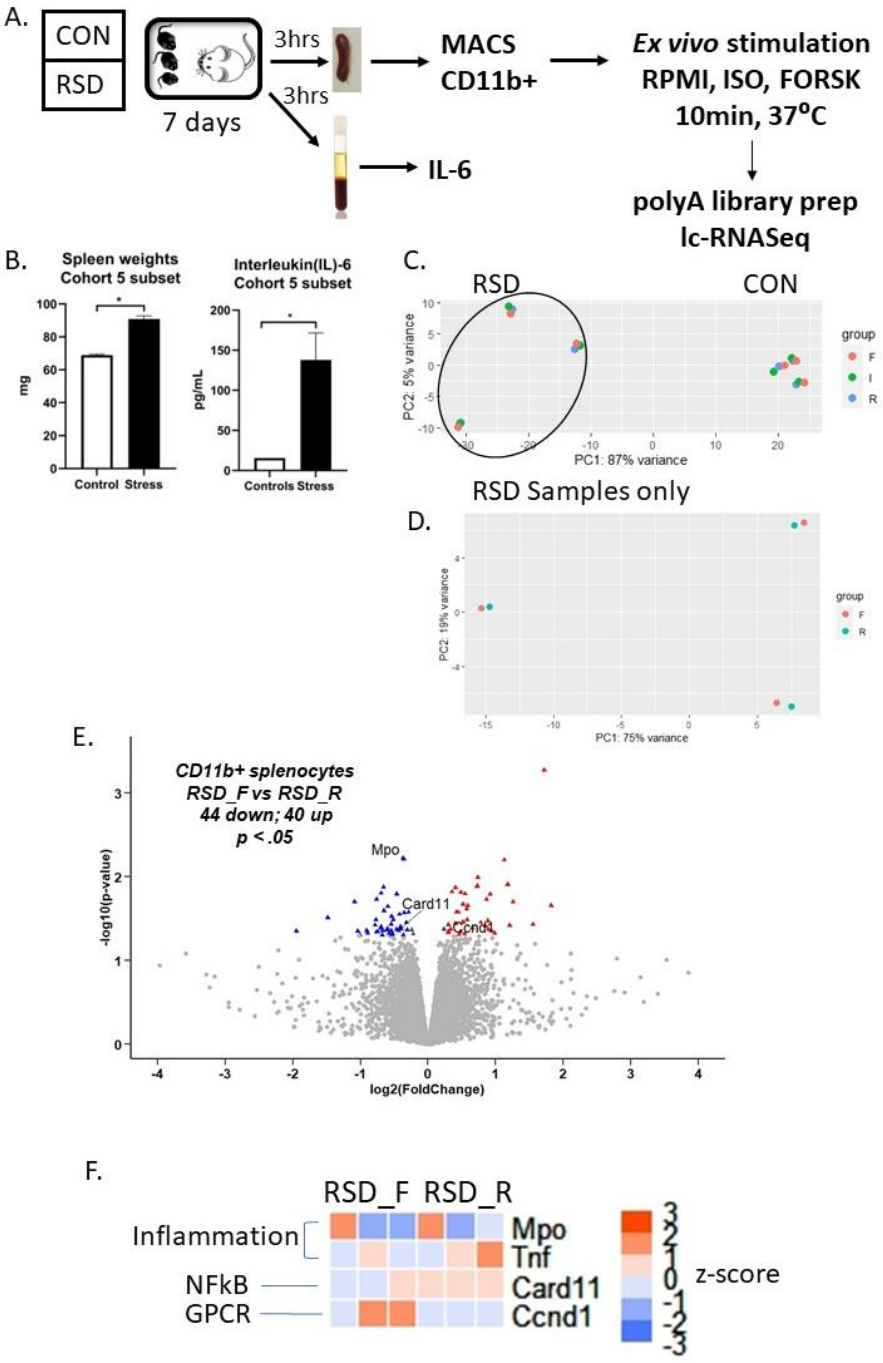


Figure 22. Treatment Effect: Repeated Social Defeat (RSD) Splenocytes – Forskolin (F) vs. RPMI 1640 media (R)

The experiment (described in A) resulted in a significant effect of Repeated Social Defeat (RSD) on splenomegaly (B), peripheral pro-inflammatory proteins (B), and differential gene transcription (C). When examining the effect of forskolin treatment on CD11b+ splenocytes from Repeated Social Defeat (RSD) mice only (D, E, F), there was inconclusive/lack of evidence for differential regulation of genes consistent with canonical signaling pathways for forskolin, and initial evidence for an attenuation in pro-inflammatory signaling (F).

Notes: The canonical signaling pathway for forskolin involves β-adrenergic receptor (β-AR) independent activation of adenylate cyclase (AC), followed by downstream activation of cyclic adenosine monophosphate (cAMP), protein kinase–A (PKA), and cAMP response element binding (CREB)-mediated gene transcription.

A. Diagram of experiment. Male C56BL/6 mice (6-8 weeks old) underwent repeated social defeat (RSD) for 6 days or were left undisturbed in their cages in the control room (CON) until sacrifice. Mice were sacrificed within three hours of the last round of social defeat. After sacrifice, the spleen and peripheral blood was harvested from the mice for isolation of CD11b+ cells and plasma, respectively. CD11b+ splenocytes were isolated using Miltenyi Biotec CD11b+ Microbeads (Miltenyi Biotec Cat #130-049-60). Levels of IL-6 were measured using a quantitative sandwich enzyme immunoassay kit (BD OptEIA Mouse IL-6 ELISA Cat#555220; BD Biosciences, San Jose, CA), according to the manufacturer's directions. CD11b+ splenocytes were either left unstimulated (RPMI 1640 cell culture media alone) or were subject to stimulation by isoproterenol (ISO), a non-specific β-adrenergic agonist, or forskolin (FORSK), a non-specific activator of adenylate cyclase (AC) for 10 minutes at 37ºC. RNA was then isolated. A subset of these samples (N = 3 in each group CON/RSD) were sent for polyA library preparation (poly-A tail was used to amplify mRNA using oligo(dT) primers for mRNA library generation; the poly-A tail is a long chain of adenosine nucleotides added to 3' end RNA to prevent degradation and mark it for transport to ribosomes to be translated into protein) and lc-RNAseq (mRNA library generation for limiting cell (lc = low input RNA amounts: 10pg-1ng) was performed using the Clontech SMART-Seq HT kit (Takara Bio Inc., Mountain View, CA, Cat #634456) and the Nextera XT DNA Library Prep Kit (Illumina, Inc., San Diego, CA, Cat #FC-131-1096). RNA-Seq = Samples were then sequenced at a depth of 15-21million 2x100bp paired-end clusters on an Illumina NovaSeq6000 platform (Illumina, Inc., San Diego)). hrs = hours; ; IL = interleukin.

B. Quantitative measures of stress in animals experiencing repeated social defeat (RSD/"Stress") include splenomegaly and elevated levels of peripheral pro-inflammatory proteins. The data comparing spleen weights in the subset of mice sent for sequencing is reproduced from Figure 2G (N = 3 mice in each CON/RSD group). The graph comparing interleukin(IL)-6 levels in the subset of mice sent for sequencing (N = 3 mice in each CON/RSD group) represents a subset of the data presented in Figure 2H. *$p < .05$

C. Principle Component Analysis (PCA) plot of all genes for all treatment groups (RPMI, ISO, FORSK) and mice (CON (N = 3), RSD (N = 3) included in the analysis. Separation between groups (CON/RSD) accounted for greater variance (87%) than separation between treatment conditions (RPMI, ISO, FORSK; 5%). This analysis focused only on samples from the RSD group, indicated by the black circle.

D. Principle Component Analysis (PCA) plot of all genes for the forskolin (F) and RPMI (R) treatment groups using CD11b+ splenocytes from RSD mice. Separation between biological replicates (i.e., animals) accounted for greater variance (75%) than the separation between treatment conditions (i.e., forskolin vs RPMI; 19%).

E. Volcano plot for all up and down-regulated genes for RSD_F = RSD_FORSK group (N = 3 mice/samples) vs. RSD_R = RSD_RPMI group (N = 3 mice/samples) after CLEAR selection for robust/low-noise transcripts (Walker et al., 2020) and filtering out all genes with an average median count of ≤ 2 (across all samples). Differential gene expression was

estimated using the DESeq2 package in R (Love et al., 2014). This analysis is based on a secondary pairwise comparison between treatment groups (FORSK vs. RPMI) from a primary within & between groups regression analysis in DESeq2. Genes in dark gray, blue, and red are significantly up or down regulated using a p-value of .05, which was NOT adjusted for multiple comparisons (N = 40 significantly upregulated genes; N = 44 significantly downregulated genes). Red genes represent a > .5 fold upregulation in RSD_F vs. RSD_R samples; Blue genes represent a > .5 fold downregulation in RSD_F vs. RSD_R samples.

F. Heat map of differentially expressed genes based on 1) individual genes previously identified in RSD literature (Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018), 2) individual genes identified in prior IPA Canonical Pathway analyses involved in pro-inflammatory pathways, 3) individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching," and 4) individual genes from intracellular signaling pathways identified in prior IPA Canonical Pathway analyses including protein kinase-A (PKA), cAMP response element binding (CREB) protein, mitogen-activated protein kinase (MAPK), and G-protein coupled receptor (GPCR) signaling pathways. Consistent with an attenuation in pro-inflammatory signaling, two inflammatory genes that were downregulated included *Tnf*, a gene encoding a pro-inflammatory cytokine expressed primarily in monocytes, and *Mpo*, a gene encoding a protein important in the microbicidal activity of neutrophils. There was also downregulation in a gene that encodes a protein known to promote NFkB activation (*Card11*). *Notes*: Individual gene z-scores depicted in the heat map (see legend) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p < .05$ (DESeq2), NOT adjusted for multiple comparisons (Love et al., 2014). CON_F = Control_FORSK group (N = 3); CON_R = Control_RPMI group (N = 3); Each column represents an individual mouse. NF-κB = nuclear factor – kappaBeta.

5.4.4.2 RSD CD11b+ Splenocytes: Isoproterenol vs RPMI

Among CD11b+ splenocytes from RSD mice, there was differential expression of 110 mRNA gene transcripts (79 upregulated; 31 downregulated, $p < .05$; Figure 23B) between those stimulated with isoproterenol compared to RPMI controls. Separation between biological replicates (i.e., animals) accounted for greater variance (68%, Figure 23A) than the separation between treatment conditions (i.e., forskolin vs RPMI; 24%, Figure 23A), suggesting that differences between groups was greater than between treatment conditions (i.e., within-groups).

Panther Overexpression analysis did not yield any notable significant results. Among individual genes of interest, there was some evidence of an upregulation in pro-inflammatory

signaling including upregulation in the myeloid gene *Fos*. In contrast, evidence for an attenuation in pro-inflammatory signaling included downregulation of the inflammatory gene *Tnf*.

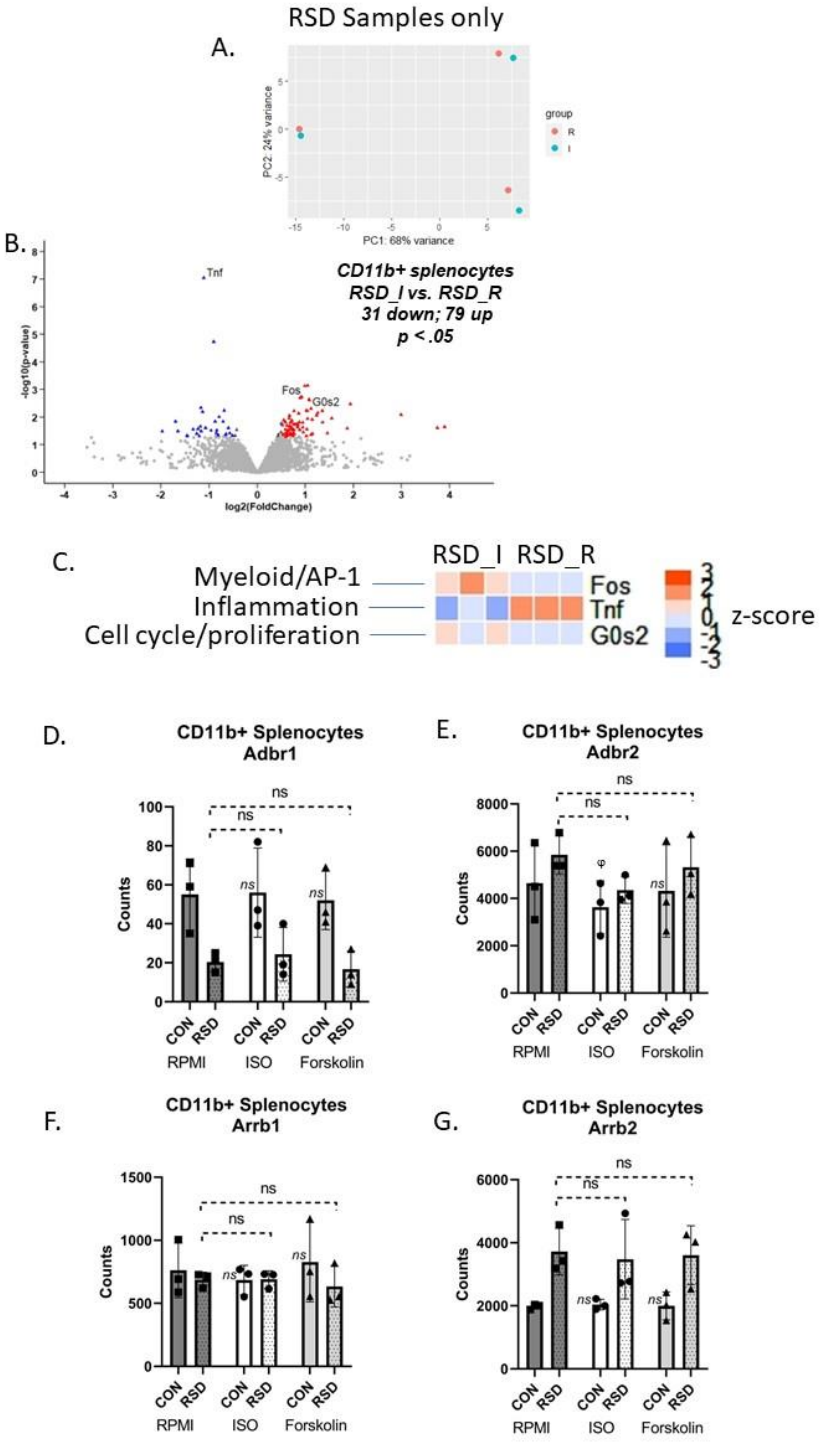


Figure 23. Treatment Effect: RSD Splenocytes – Isoproterenol (I) vs. RPMI 1640 media (R); individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching."

Isoproterenol stimulation of CD11b+ splenocytes from Repeated Social Defeat (RSD) mice only (A, B, C) resulted in inconclusive/lack of evidence for differential regulation of genes consistent with canonical or non-canonical signaling pathways for isoproterenol, and mixed evidence regarding pro-inflammatory signaling (C). Individual gene counts for particular genes of interest identified a-priori as involved in β-adrenergic receptor (β-AR) "signal switching" (D-G) were not significantly different between treatment conditions for CD11b+ splenocytes from RSD mice.

Notes: The canonical signaling pathway for isoproterenol, a non-specific β-adrenergic receptor (β-AR) agonist, involves G-protein dependent activation of G-protein-alpha-S ($G\alpha_s$), followed by adenylate cyclase (AC) and downstream activation of cyclic adenosine monophosphate (cAMP), protein kinase–A (PKA), and cAMP response element binding (CREB)-mediated gene transcription. The non-canonical signaling pathway involves possible engagement of G-protein-alpha-I ($G\alpha_i$), followed by mitogen activated protein kinase (MAPK) mediated signaling, including upregulation of pro-inflammatory NF-κB and activator protein (AP)-1 related gene transcription.

- **A.** Principle Component Analysis (PCA) plot of all genes for the isoproterenol (I) and RPMI (R) treatment groups only using CD11b+ splenocytes from RSD mice. Separation between biological replicates (i.e., animals) accounted for greater variance (68%) than the separation between treatment conditions (i.e., isoproterenol vs RPMI; 24%).
- **B.** Volcano plot for all up and down-regulated genes for RSD_I = RSD_ISO group (N = 3 mice/samples) vs. RSD_R = RSD_RPMI group (N = 3 mice/samples) after CLEAR selection for robust/low-noise transcripts (Walker et al., 2020) and filtering out all genes with an average median count of ≤ 2 (across all samples). Differential gene expression was estimated using the DESeq2 package in R (Love et al., 2014). This analysis is based on a secondary pairwise comparison between treatment groups (ISO vs. RPMI) from a primary within & between groups regression analysis in DESeq2. Genes in dark gray, blue, and red are significantly up or down regulated using a p-value of .05, which was NOT adjusted for multiple comparisons (N = 79 significantly upregulated genes; N = 31 significantly downregulated genes). Red genes represent a > .5-fold upregulation in RSD_I vs. RSD_R samples; Blue genes represent a > .5-fold downregulation in RSD_I vs. RSD_R samples.
- **C.** Heat map of differentially expressed genes based on 1) individual genes previously identified in RSD literature (Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018), 2) individual genes identified in prior IPA Canonical Pathway analyses involved in pro-inflammatory pathways, 3) individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching," and 4) individual genes from intracellular signaling pathways identified in prior IPA Canonical Pathway analyses including protein kinase-A (PKA), cAMP response element binding (CREB) protein, mitogen-activated protein kinase (MAPK), and G-protein coupled receptor (GPCR) signaling pathways. Consistent with mixed evidence for pro-inflammatory signaling, there was an upregulation in the myeloid gene *Fos*, and a downregulation of the inflammatory gene *Tnf*. *Notes*: Individual gene z-scores depicted in the heat map (see legend) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p < .05$ (DESeq2), NOT adjusted for multiple comparisons. RSD_I = RSD_ISO group (N = 3); RSD_R = RSD_RPMI group (N = 3); Each column represents an individual mouse. NF-κB = nuclear factor – kappaBeta.

(D-G). Graphs of individual gene counts for particular genes of interest identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching" among CD11b+ splenocytes from control (CON) and Repeated Social Defeat (RSD) mice. *Notes:* $*p_{adj} < .05$, $^{\Phi}p < .05$ (not adjusted for multiple comparisons), ns = not significant. Individual points in each column (squares, circles, triangles) represent raw gene counts from an individual mouse. RPMI = unstimulated group, cells incubated in RPMI 1640 cell culture media alone for 10 min at 37ºC, ISO = cells were stimulated with 10uM isoproterenol, a non-specific β-adrenergic agonist via incubation for 10 min at 37ºC, Forskolin = cells were stimulated with 10uM forskolin, a non-specific activator of adenylate cyclase (AC), via incubation for 10 min at 37ºC. CD11b+ = cell surface protein marker indicating mouse monocytes and granulocytes.

- **D.** There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ splenocytes from control or RSD mice on gene expression of Adrb1, the gene encoding the β1-adrenergic receptor.
- **E.** Isoproterenol stimulation of CD11b+ splenocytes from control mice, but not RSD mice, resulted in significantly lower gene expression of Adrb2, the gene encoding the β2-adrenergic receptor, compared to unstimulated (RPMI) cells using a p-value < .05, unadjusted for multiple comparisons. There was no significant effect of forskolin stimulation of CD11b+ splenocytes from control or RSD mice on gene expression of Adrb2.
- **F.** There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ splenocytes from control or RSD mice on gene expression of Arrb1, the gene encoding the β-arrestin-1 protein. β-arrestin-1 is a key signaling protein involved in canonical β-adrenergic receptor signaling.
- **G.** There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ splenocytes from control or RSD mice on gene expression of Arrb2, the gene encoding the β-arrestin-2 protein. β-arrestin-2 is a key signaling protein involved in non-canonical β-adrenergic receptor signaling.

5.4.4.3 RSD CD11b+ Splenocytes: Summary

In general, exploratory analyses of differentially regulated genes across CD11b+ splenocytes from RSD mice yielded some evidence that could be suggestive of an attenuation in pro-inflammatory signaling and a lack of evidence regarding intracellular signaling patterns as a result of forskolin and/or isoproterenol stimulation. Shared and unique genes of interest to forskolin and/or isoproterenol stimulation in CD11b+ splenocytes from RSD mice are listed in Figure 24.

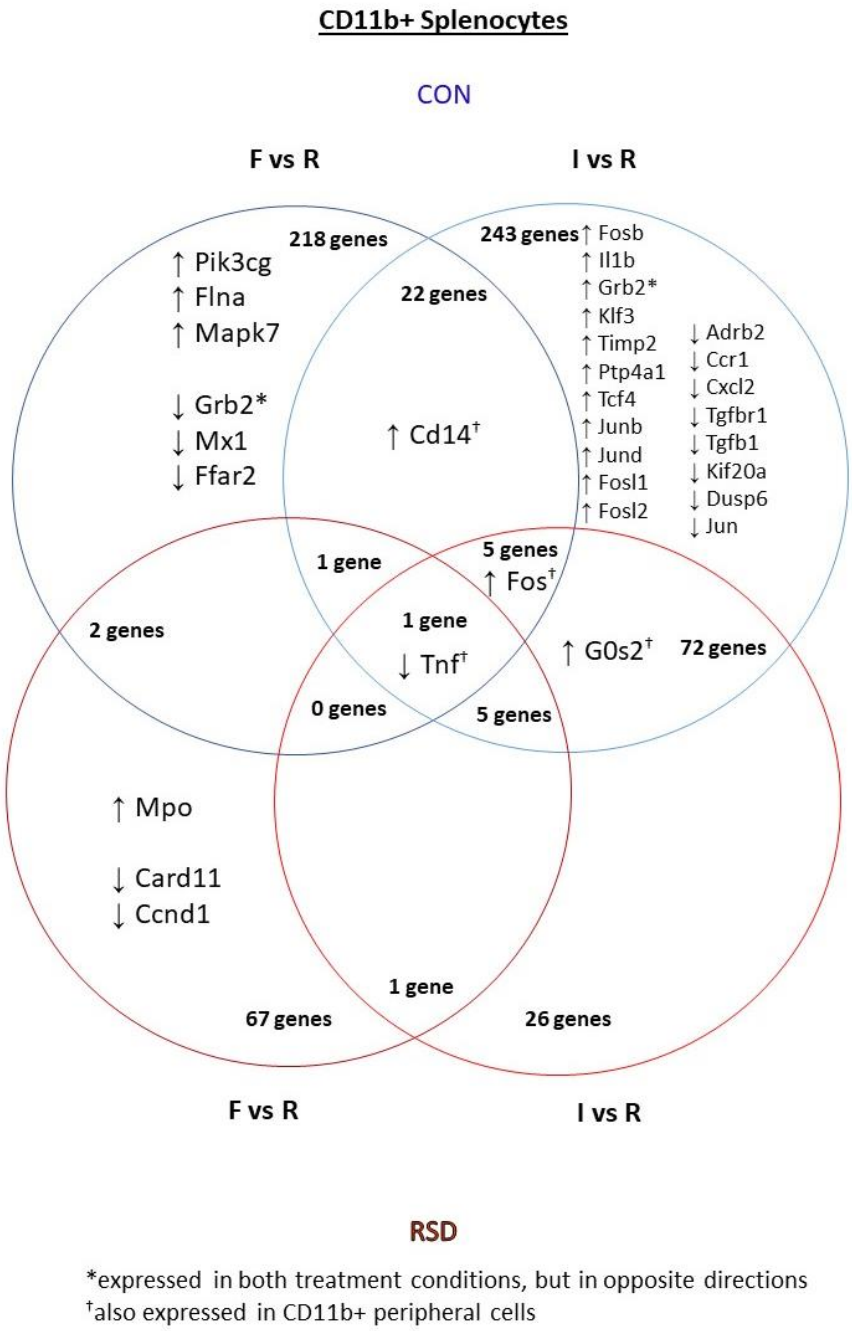


Figure 24. Treatment Effect: Splenocytes – Shared and unique genes of interest

Among shared genes of interest to forskolin and isoproterenol stimulation in CD11b+ splenocytes from control (CON) and Repeated Social Defeat (RSD) mice, there was a downregulation in the pro-inflammatory gene Tnf. Unique genes of interest to forskolin and isoproterenol stimulation among CD11b+ splenocytes from control mice were broadly supportive of expected intracellular signaling patterns. Among CD11b+ splenocytes from RSD mice, there were few unique genes of interest to forskolin and isoproterenol stimulation and few shared genes with CD11b+ splenocytes from control mice.

Notes: Shared and unique genes of interest to forskolin and/or isoproterenol stimulation in CD11b+ splenocytes from control (CON) and RSD mice. Total number of shared and unique genes are indicated in bold within the Venn-diagram. Genes of interest indicated within the diagram were identified from 1) individual genes previously identified in RSD literature (Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018), 2) individual genes identified in prior IPA Canonical Pathway analyses involved in pro-inflammatory pathways, 3) individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching," and 4) individual genes from intracellular signaling pathways identified in prior IPA Canonical Pathway analyses including protein kinase-A (PKA), cAMP response element binding (CREB) protein, mitogen-activated protein kinase (MAPK), and G-protein coupled receptor (GPCR) signaling pathways. All genes displayed were significantly differentially regulated using $p < .05$ (DESeq2), NOT adjusted for multiple comparisons. *Notes*: R = RPMI, F = forskolin, I = isoproterenol. *expressed in both treatment comparisons (i.e., F vs R and I vs R) but in opposite directions. †also expressed among CD11b+ peripheral cells from Control mice.

5.4.4.4 RSD CD11b+ Peripheral Cells: Forskolin vs RPMI

For CD11b+ peripheral cells from RSD mice, there was differential expression of 143 mRNA gene transcripts (99 upregulated; 44 downregulated, $p < .05$; Figure 25E) between those stimulated with forskolin compared to RPMI controls. Separation between biological replicates (i.e., animals) accounted for greater variance (89%, Figure 25D) than the separation between treatment conditions (i.e., forskolin vs RPMI; 7%, Figure 25D), suggesting that differences between groups was greater than between treatment conditions (i.e., within-groups).

Panther Overexpression analysis did not yield any notable significant results. Among individual genes of interest ($p < .05$; Figure 25F), there was initial support for intracellular signaling via forskolin-stimulated pathways. Specifically, there was downregulation of a gene encoding a protein involved in GPCR signaling (*Ccr7*), and upregulation of a gene encoding a protein that works to increase intracellular cAMP levels (*Adora2a*). There was also upregulation in a gene known to encode a protein important in mediating cellular responses to extracellular stimuli via activation of the transcription factors CREB and NFkB (*Mapk14*). These findings are broadly consistent with forskolin stimulated signaling. These genes could also suggest an

upregulation in pro-inflammatory signaling. Specifically, *Ccr7* is known to be involved in chronic inflammatory pathogenesis and *Mapk14* is broadly pro-inflammatory.

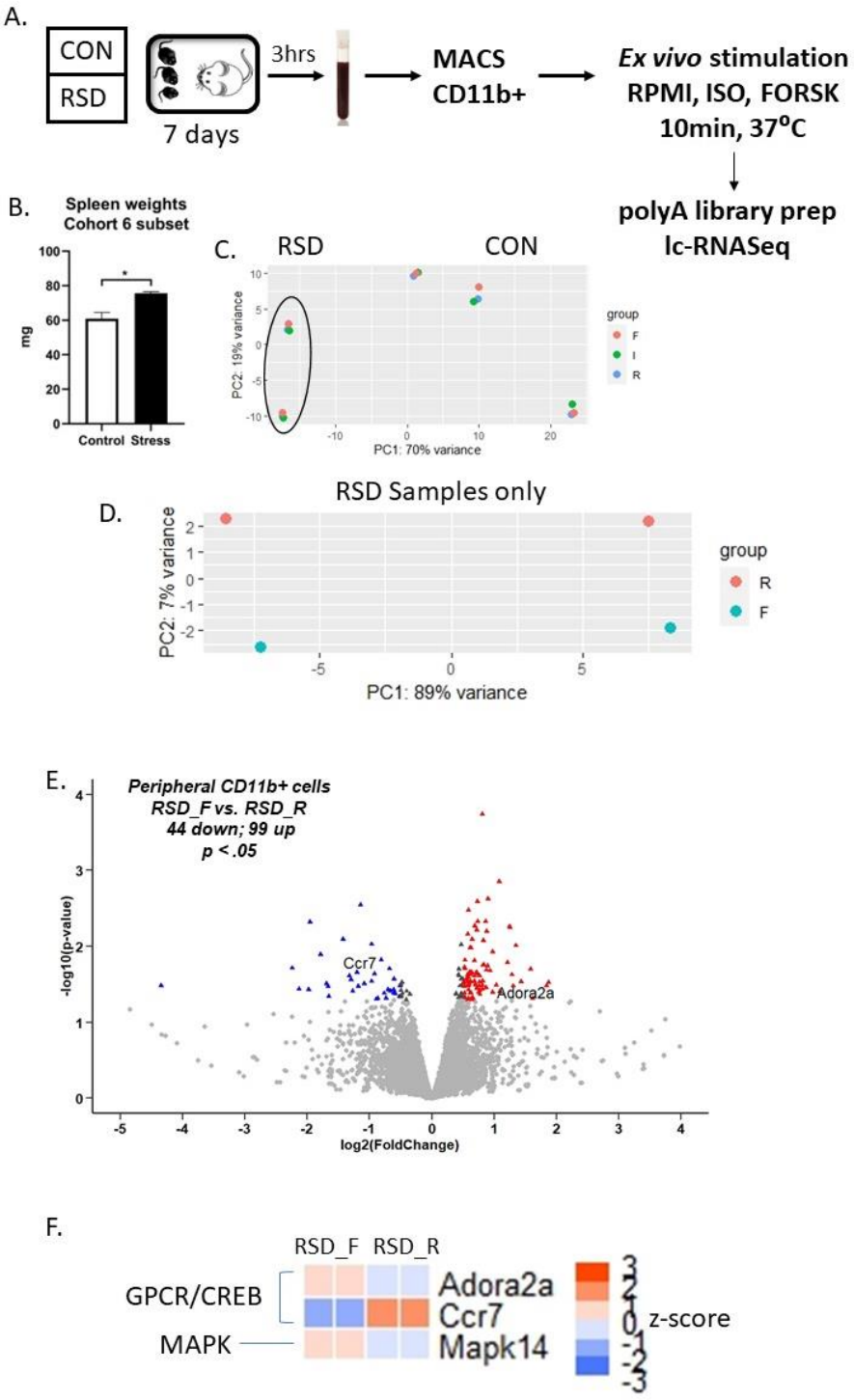


Figure 25. Treatment Effect: Repeated Social Defeat (RSD) Peripheral Cells – Forskolin (F) vs. RPMI 1640 media (R)

The experiment (described in A) resulted in a significant effect of Repeated Social Defeat (RSD) on splenomegaly (B) and differential gene transcription (C). When examining the effect of forskolin treatment on CD11b+ peripheral cells from RSD mice only (D, E, F), there was initial evidence of differential regulation of genes consistent with canonical signaling pathways for forskolin and an upregulation in pro-inflammatory signaling pathways.

Notes: The canonical signaling pathway for forskolin involves β-adrenergic receptor (β-AR) independent activation of adenylate cyclase (AC), followed by downstream activation of cyclic adenosine monophosphate (cAMP), protein kinase–A (PKA), and cAMP response element binding (CREB)-mediated gene transcription.

- **A.** Diagram of experiment. Male C56BL/6 mice (6-8 weeks old) underwent repeated social defeat (RSD) for 6 days or were left undisturbed in their cages in the control room (CON) until sacrifice. Mice were sacrificed within three hours (hrs) of the last round of social defeat. After sacrifice, the peripheral blood was harvested from the mice for isolation of CD11b+ cells. CD11b+ peripheral cells were isolated using Miltenyi Biotec CD11b+ Microbeads (Miltenyi Biotec Cat #130-049-60). CD11b+ peripheral cells were either left unstimulated (RPMI 1640 cell culture media alone) or were subject to stimulation by isoproterenol (ISO), a non-specific β-adrenergic agonist, or forskolin (FORSK), a non-specific activator of adenylate cyclase (AC) for 10 minutes at 37ºC. RNA was then isolated. A subset of these samples (N = 3 in each group CON/RSD) were sent for polyA library preparation (poly-A tail was used to amplify mRNA using oligo(dT) primers for mRNA library generation; the poly-A tail is a long chain of adenosine nucleotides added to 3' end RNA to prevent degradation and mark it for transport to ribosomes to be translated into protein) and lc-RNAseq (mRNA library generation for limiting cell (lc = low input RNA amounts: 10pg-1ng) was performed using the Clontech SMART-Seq HT kit (Takara Bio Inc., Mountain View, CA, Cat #634456) and the the Nextera XT DNA Library Prep Kit (Illumina, Inc., San Diego, CA, Cat #FC-131-1096). RNA-Seq = Samples were then sequenced at a depth of 15-21million 2x100bp paired-end clusters on an Illumina NovaSeq6000 platform (Illumina, Inc., San Diego)).
- **B.** A quantitative measure of stress in animals experiencing repeated social defeat (RSD/"Stress") includes splenomegaly. The sub-subset of data displayed here represents the mice for the samples used in the analyses (N = 2 RSD, N = 3 CON). Specifically, one RSD mouse that was sent for sequencing was removed from the analyses due to significant overlap with gene expression among control mice. The data comparing spleen weights in the subset of mice sent for sequencing can be seen in Figure 2J. *$p < .05$
- **C.** Principle Component Analysis (PCA) plot of all genes for all treatment groups (RPMI, ISO, FORSK) and mice (CON (N = 3), RSD (N = 2)) included in the differential gene analysis. Separation between groups (CON/RSD) accounted for greater variance (70%) than separation between treatment conditions (RPMI, ISO, FORSK; 19%). This analysis focused only on samples from the RSD group, indicated by the black circle.
- **D.** Principle Component Analysis (PCA) plot of all genes for the forskolin (F) and RPMI (R) treatment groups using CD11b+ peripheral cells from RSD mice (N = 2). Separation between biological replicates (i.e., animals) accounted for greater variance (89%) than the separation between treatment conditions (i.e., forskolin vs RPMI; 7%).

E. Volcano plot for all up and down-regulated genes for RSD_F = RSD_FORSK group (N = 2 mice/samples) vs. RSD_R = RSD_RPMI group (N = 2 mice/samples) after CLEAR selection for robust/low-noise transcripts (Walker et al., 2020) and filtering out all genes with an average median count of ≤ 2 (across all samples). Differential gene expression was estimated using the DESeq2 package in R (Love et al., 2014). This analysis is based on a secondary pairwise comparison between treatment groups (FORSK vs. RPMI) from a primary within & between groups regression analysis in DESeq2. Genes in dark gray, blue, and red are significantly up or down regulated using a p-value of .05, which was NOT adjusted for multiple comparisons (N = 99 significantly upregulated genes; N = 44 significantly downregulated genes). Red genes represent a > .5 fold upregulation in RSD_F vs. RSD_R samples; Blue genes represent a > .5 fold downregulation in RSD_F vs. RSD_R samples.

F. Heat map of differentially expressed genes based on 1) individual genes previously identified in RSD literature (Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018), 2) individual genes identified in prior IPA Canonical Pathway analyses involved in pro-inflammatory pathways, 3) individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching," and 4) individual genes from intracellular signaling pathways identified in prior IPA Canonical Pathway analyses including protein kinase-A (PKA), cAMP response element binding (CREB) protein, mitogen-activated protein kinase (MAPK), and G-protein coupled receptor (GPCR) signaling pathways. Consistent with intracellular signaling via forskolin-stimulated pathways and an upregulation in pro-inflammatory gene transcription, there was downregulation of a gene encoding a protein involved in GPCR signaling and chronic inflammatory pathogenesis (*Ccr7*), and upregulation of a gene encoding a protein that works to increase intracellular cAMP levels (*Adora2a*). There was also upregulation in a gene known to encode a protein important in mediating cellular responses to extracellular stimuli via activation of the transcription factors CREB and NFkB, which is also broadly pro-inflammatory (*Mapk14*). *Notes*: Individual gene z-scores depicted in the heat map (see legend) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p < .05$ (DESeq2), NOT adjusted for multiple comparisons. RSD_F = RSD_FORSK group (N = 2); RSD_R = RSD_RPMI group (N = 2); Each column represents an individual mouse.

5.4.4.5 RSD CD11b+ Peripheral Cells: Isoproterenol vs RPMI

Among CD11b+ peripheral cells from RSD mice, there was differential expression of 82 mRNA gene transcripts (39 upregulated; 43 downregulated, $p < .05$; Figure 26B) between those stimulated with isoproterenol compared to RPMI controls. Separation between biological replicates (i.e., animals) accounted for greater variance (84%, Figure 26A) than the separation between treatment conditions (i.e., forskolin vs RPMI; 10%, Figure 26A), suggesting that differences between groups was greater than between treatment conditions (i.e., within-groups).

Panther overrepresentation analysis found that there was an enrichment in pathways involved in pro-inflammatory signaling. Specifically, there was an enrichment in inflammation mediated by chemokine and cytokine signaling (5.57-fold enrichment, p = .002: *Ptgs2, Ccrl2, Ccl7, Ccl2, Ccl3*), TLR signaling (10.72-fold enrichment, p = .016: *Ptgs2, Cd14*), and interleukin signaling (6.65-fold enrichment, p = .038: *Cdkn1a, Fos*). However, these findings were not significant after adjustment for multiple comparisons (FDR > .05). Individual genes of interest provided mixed findings with regard to pro-inflammatory signaling (Figure 26C). Specifically, the myeloid and broadly pro-inflammatory genes *Cd14* and *Fos* were upregulated and the inflammatory gene *Tnf* was downregulated. Other differentially regulated genes were either only tangentially related to inflammatory signaling or not directly related to intracellular signaling patterns as a result of forskolin and/or isoproterenol stimulation.

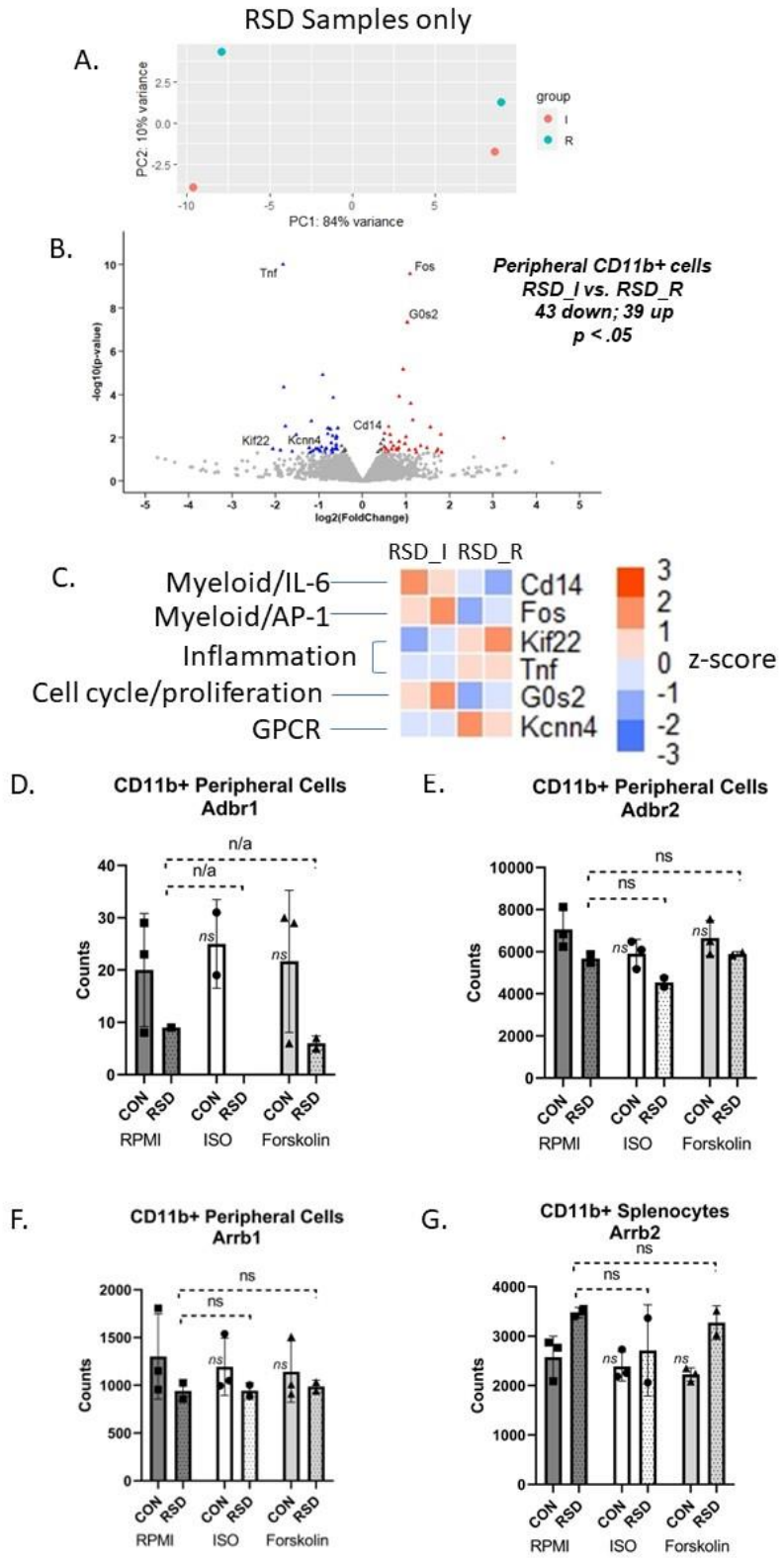


Figure 26. Treatment Effect: Repeated Social Defeat (RSD) Peripheral Cells – Isoproterenol (I) vs. RPMI 1640 media (R); individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching"

Isoproterenol stimulation of CD11b+ peripheral cells from Repeated Social Defeat (RSD) mice only (A, B, C) resulted in inconclusive/lack of evidence for differential regulation of genes consistent with canonical or non-canonical signaling pathways for isoproterenol, and mixed evidence regarding pro-inflammatory signaling (C). Individual gene counts for particular genes of interest identified a-priori as involved in β-adrenergic receptor (β-AR) "signal switching" (D-G) were not significantly different between treatment conditions for CD11b+ peripheral cells from RSD mice.

Notes: The canonical signaling pathway for isoproterenol, a non-specific β-adrenergic receptor (β-AR) agonist, involves G-protein dependent activation of G-protein-alpha-S ($G\alpha_s$), followed by adenylate cyclase (AC) and downstream activation of cyclic adenosine monophosphate (cAMP), protein kinase–A (PKA), and cAMP response element binding (CREB)-mediated gene transcription. The non-canonical signaling pathway involves possible engagement of G-protein-alpha-I ($G\alpha_i$), followed by mitogen activated protein kinase (MAPK) mediated signaling, including upregulation of pro-inflammatory NF-κB and activator protein (AP)-1 related gene transcription.

- **A.** Principle Component Analysis (PCA) plot of all genes for the isoproterenol (I) and RPMI (R) treatment groups only using CD11b+ peripheral cells from RSD mice. Separation between biological replicates (i.e., animals) accounted for greater variance (84%) than the separation between treatment conditions (i.e., isoproterenol vs RPMI; 10%).
- **B.** Volcano plot for all up and down-regulated genes for RSD_I = RSD_ISO group (N = 2 mice/samples) vs. RSD_R = RSD_RPMI group (N = 2 mice/samples) after CLEAR selection for robust/low-noise transcripts (Walker et al., 2020) and filtering out all genes with an average median count of ≤ 2 (across all samples). Differential gene expression was estimated using the DESeq2 package in R (Love et al., 2014). This analysis is based on a secondary pairwise comparison between treatment groups (ISO vs. RPMI) from a primary within & between groups regression analysis in DESeq2. Genes in dark gray, blue, and red are significantly up or down regulated using a p-value of .05, which was NOT adjusted for multiple comparisons (N = 39 significantly upregulated genes; N = 43 significantly downregulated genes). Red genes represent a > .5-fold upregulation in RSD_I vs. RSD_R samples; Blue genes represent a > .5-fold downregulation in RSD_I vs. RSD_R samples.
- **C.** Heat map of differentially expressed genes based on 1) individual genes previously identified in RSD literature (Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018), 2) individual genes identified in prior IPA Canonical Pathway analyses involved in pro-inflammatory pathways, 3) individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching," and 4) individual genes from intracellular signaling pathways identified in prior IPA Canonical Pathway analyses including protein kinase-A (PKA), cAMP response element binding (CREB) protein, mitogen-activated protein kinase (MAPK), and G-protein coupled receptor (GPCR) signaling pathways. Consistent with mixed evidence for pro-inflammatory signaling, myeloid and broadly pro-inflammatory genes *Cd14* and *Fos* were upregulated and the inflammatory gene *Tnf* was downregulated. *Notes*: Individual gene z-scores depicted in the heat map (see legend) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p < .05$ (DESeq2), NOT adjusted for multiple comparisons. RSD_I = RSD_ISO group (N = 2); RSD_R = RSD_RPMI group (N = 2); Each column represents an individual mouse. IL-6 =

interleukin-6; AP-1 = activator protein-1 (AP-1), a pro-inflammatory transcriptional activator.

(D-G). Graphs of individual gene counts for particular genes of interest identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching" among CD11b+ peripheral cells from control (CON) and Repeated Social Defeat (RSD) mice. *Notes:* *$p_{adj} < .05$, $^{\Phi}p < .05$ (not adjusted for multiple comparisons), ns = not significant. Individual points in each column (squares, circles, triangles) represent raw gene counts from an individual mouse. RPMI = unstimulated group, cells incubated in RPMI 1640 cell culture media alone for 10 min at 37ºC, ISO = cells were stimulated with 10uM isoproterenol, a non-specific β-adrenergic agonist via incubation for 10 min at 37ºC, Forskolin = cells were stimulated with 10uM forskolin, a non-specific activator of adenylate cyclase (AC), via incubation for 10 min at 37ºC. CD11b+ = cell surface protein marker indicating mouse monocytes and granulocytes.

D. There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ peripheral cells from control mice on gene expression of Adrb1, the gene encoding the β1-adrenergic receptor. For RSD mice, statistical analyses could not be performed due to missing data (N = 1 RSD_RPMI; N = 0 RSD_ISO).

E. There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ peripheral cells from control or RSD mice on gene expression of Adrb2, the gene encoding the β2-adrenergic receptor.

F. There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ peripheral cells from control or RSD mice on gene expression of Arrb1, the gene encoding the β-arrestin-1 protein. β-arrestin-1 is a key signaling protein involved in canonical β-adrenergic receptor signaling.

G. There was no significant effect of isoproterenol or forskolin stimulation of CD11b+ peripheral cells from control or RSD mice on gene expression of Arrb2, the gene encoding the β-arrestin-2 protein. β-arrestin-2 is a key signaling protein involved in non-canonical β-adrenergic receptor signaling.

5.4.4.6 RSD CD11b+ Peripheral Cells: Summary

In general, exploratory analyses among CD11b+ peripheral cells from RSD mice found evidence consistent with forskolin-stimulated intracellular signaling but no information about intracellular signaling patterns as a result of isoproterenol-simulation. Exploratory analyses were also broadly suggestive of a pro-inflammatory signaling profile as a consequence of forskolin stimulation, with mixed evidence for pro-inflammatory signaling as a consequence of isoproterenol stimulation. Shared and unique genes of interest to forskolin and/or isoproterenol stimulation in CD11b+ splenocytes from RSD mice are listed in Figure 21.

5.4.5 RSD_I vs CON_R: Contrary to our hypothesis, stimulation of RSD CD11b+ cells with isoproterenol may *attenuate* pro-inflammatory signaling in the short term (i.e., 10 min)

To examine the effects of *ex vivo* isoproterenol stimulation on CD11b+ cells from RSD mice, we compared both stimulated and unstimulated CD11b+ cells from RSD mice to unstimulated CD11b+ cells from control mice, which served as a baseline for gene expression. In this way, we were able to examine differences in differential gene transcription due to isoproterenol stimulation alone in CD11b+ cells from RSD mice.

5.4.5.1 Spleen.

Among isoproterenol stimulated CD11b+ splenocytes from RSD mice compared to unstimulated CD11b+ splenocytes from Control mice (RSD_I vs CON_R comparison), there was differential expression of 3,624 mRNA gene transcripts (2,012 upregulated; 1,612 downregulated, $p_{adj} < .05$; Figure 27D). Upregulated genes included many associated with a primed and pro-inflammatory gene expression profile (Figure 27E, Figure 28), although this profile was somewhat attenuated compared to differential expression among unstimulated CD11b+ splenocytes from RSD mice vs unstimulated CD11b+ splenocytes from Control mice (RSD_R vs CON_R comparison; Section 5.4.1.1). For example, using both significantly up and downregulated genes (Figure 27D), IPA identified a significant upregulation in pathways involved in pro-inflammatory signaling in the RSD_I vs CON_R comparison (Figure 27E). These pathways included IL-6 signaling (Z=1.60; Figure 29A), toll-like receptor (TLR) signaling (Z=2.33; Figure 29B), and NF-kB signaling (Z=.728; Figure 29C). However, the magnitude of the Z-scores for the activation of these pro-inflammatory signaling pathways were all quantitatively *lower* than the Z-scores for

these pathways in the RSD_R vs CON_R comparison (Figure 6E, reproduced as light gray bars in Figure 27E), suggesting lower activation of these pathways among stimulated cells. This provides some support for the idea that isoproterenol stimulation of CD11b+ cells from RSD mice may attenuate pro-inflammatory signaling. Adding additional support to the idea of an attenuation in pro-inflammatory signaling, there was difference in the magnitude of change for a number of individual pro-inflammatory genes ($p_{adj} < .05$) that were differentially expressed in the RSD_I vs CON_R comparison vs the RSD_R vs CON_R comparison. For example, the cytokine and chemokine regulatory signaling gene *Tgfb1* had a .39-fold greater downregulation (i.e., log fold change) among stimulated CD11b+ splenocytes from the RSD_I vs CON_R comparison vs. unstimulated CD11b+ splenocytes from the RSD_R vs CON_R comparison. The protein encoded by *Tgfb1* can modulate expression and activation of other regulatory signaling proteins including IFN-γ and TNF (Sumiyoshi et al., 2003); thus, greater downregulation in this gene could relate to greater downregulation in peripheral inflammatory signaling via IFN-γ and/or TNF among isoproterenol stimulated cells from RSD mice. Indeed, there was a significant downregulation in the *Tnf* gene in the RSD_I vs CON_R comparison that was not observed in the RSD_R vs CON_R comparison. Among other pro-inflammatory genes, the gene *Lta* demonstrated a .54-fold greater downregulation in isoproterenol stimulated CD11b+ splenocytes in the RSD_I vs CON_R comparison vs. unstimulated CD11b+ splenocytes in the RSD_R vs CON_R comparison. *Lta* encodes a protein that is part of the TNF family (Grimstad, 2016); thus, greater downregulation of *Lta* adds additional evidence to the idea that isoproterenol stimulated cells from RSD mice may demonstrate attenuated pro-inflammatory signaling compared to unstimulated cells.

In addition to a possible isoproterenol-mediated attenuation in pro-inflammatory signaling among splenocytes from RSD mice, there were two other notable genes of interest that

demonstrated a difference in the magnitude of change in the RSD_I vs CON_R comparison vs. the RSD_R vs CON_R comparison. Specifically, the myeloid lineage gene *Cd14* demonstrated a .65-fold greater upregulation among isoproterenol stimulated CD11b+ splenocytes in the RSD_I vs CON_R comparison vs. unstimulated CD11b+ splenocytes in the RSD_R vs CON_R comparison. Given that the Cd14 protein is co-receptor for lipopolysaccharide (a bacterial component) and can help initiate innate immune response, it could be that isoproterenol serves as a signal to upregulate the primed and surveillance profile of CD11b+ splenocytes in RSD mice, while also downregulating pro-inflammatory signaling pathways (e.g., TNF). Among other notable genes, the glucocorticoid receptor signaling gene *Fkbp5* had a .28-fold greater upregulation in isoproterenol stimulated CD11b+ splenocytes in the RSD_I vs CON_R comparison vs. unstimulated CD11b+ splenocytes in the RSD_R vs CON_R comparison. *Fkbp5* encodes a protein involved in inhibition of GR translocation to the nucleus; thus, the implication is that isoproterenol stimulation of cells from RSD mice could shift these cells to a state of increased resistance to glucocorticoid signaling. Given that an increase in glucocorticoid resistance is typically thought of as *more* permissive to pro-inflammatory signaling, this cellular mechanism may be independent of a possible isoproterenol mediated attenuation in pro-inflammatory signaling.

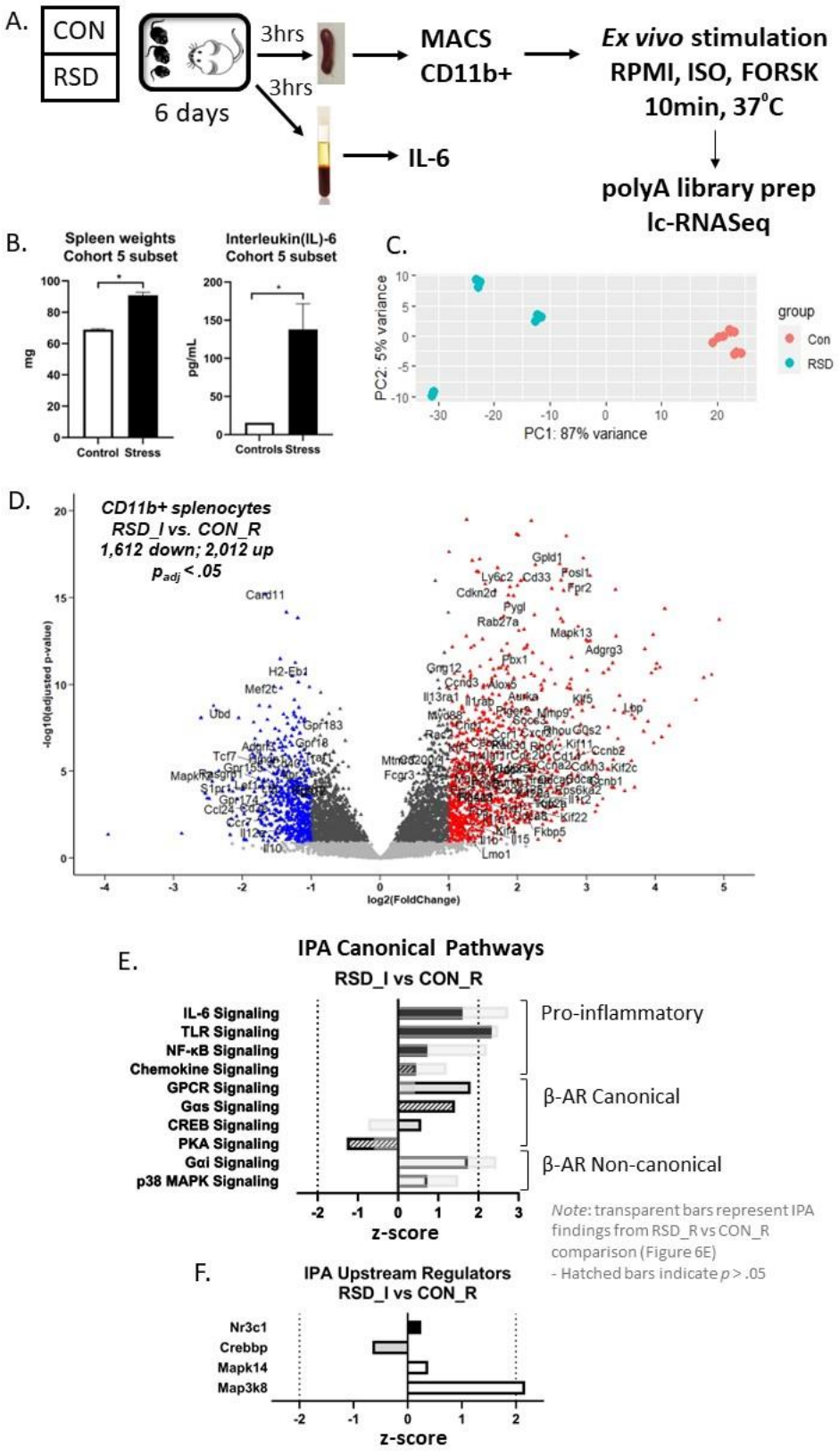


Figure 27. Stress + Stimulation Effect: Splenocytes

The experiment (described in A) resulted in a significant effect of Repeated Social Defeat (RSD) on splenomegaly (B), peripheral pro-inflammatory proteins (B), and differential gene transcription (C, D). Differentially regulated genes included those involved in pro-inflammatory signaling pathways (E, F), as well as β-adrenergic receptor (β-AR) canonical and non-canonical signaling pathways (E, F). Contrary to our hypothesis, stimulation of CD11b+ cells from Repeated Social Defeat (RSD) mice with isoproterenol may attenuate pro-inflammatory signaling in the short term (i.e., 10 min; E) and activate some canonical β-adrenergic signaling pathways (E) in the context of evidence for molecular scaffolding of non-canonical β-adrenergic signaling (E, F).

- **A.** Diagram of experiment. Male C56BL/6 mice (6-8 weeks old) underwent repeated social defeat (RSD) for 6 days or were left undisturbed in their cages in the control room (CON) until sacrifice. Mice were sacrificed within three hours of the last round of social defeat. After sacrifice, the spleen and peripheral blood was harvested from the mice for isolation of CD11b+ cells and plasma, respectively. CD11b+ splenocytes were isolated using Miltenyi Biotec CD11b+ Microbeads (Miltenyi Biotec Cat #130-049-60). Levels of IL-6 were measured using a quantitative sandwich enzyme immunoassay kit (BD OptEIA Mouse IL-6 ELISA Cat#555220; BD Biosciences, San Jose, CA), according to the manufacturer's directions. CD11b+ splenocytes were either left unstimulated (RPMI 1640 cell culture media alone) or were subject to stimulation by isoproterenol (ISO), a non-specific β-adrenergic agonist, or forskolin (FORSK), a non-specific activator of adenylate cyclase (AC) for 10 minutes at 37ºC. RNA was then isolated. A subset of these samples (N = 3 in each group CON/RSD) were sent for polyA library preparation (poly-A tail was used to amplify mRNA using oligo(dT) primers for mRNA library generation; the poly-A tail is a long chain of adenosine nucleotides added to 3' end RNA to prevent degradation and mark it for transport to ribosomes to be translated into protein) and lc-RNAseq (mRNA library generation for limiting cell (lc = low input RNA amounts: 10pg-1ng) was performed using the Clontech SMART-Seq HT kit (Takara Bio Inc., Mountain View, CA, Cat #634456) and the Nextera XT DNA Library Prep Kit (Illumina, Inc., San Diego, CA, Cat #FC-131-1096). RNA-Seq = Samples were then sequenced at a depth of 15-21million 2x100bp paired-end clusters on an Illumina NovaSeq6000 platform (Illumina, Inc., San Diego)). hrs = hours; IL = interleukin.
- **B.** Quantitative measures of stress in animals experiencing repeated social defeat (RSD/"Stress") include splenomegaly and elevated levels of peripheral pro-inflammatory proteins. The data comparing spleen weights in the subset of mice sent for sequencing is reproduced from Figure 2G (N = 3 mice in each CON/RSD group). The graph comparing interleukin(IL)-6 levels in the subset of mice sent for sequencing (N = 3 mice in each CON/RSD group) represents a subset of the data presented in Figure 2H. *$p < .05$
- **C.** Principle Component Analysis (PCA) plot of all genes for all treatment groups (RPMI, ISO, FORSK) and mice (CON (N = 3), RSD (N = 3) included in the analysis. Separation between groups (CON/RSD) accounted for greater variance (87%) than separation between treatment conditions (RPMI, ISO, FORSK; 5%).
- **D.** Volcano plot for all up and down-regulated genes for RSD_I = RSD_ISO group (N = 3 mice/samples) vs. CON_R = CON_RPMI group (N = 3 mice/samples) after CLEAR selection for robust/low-noise transcripts (Walker et al., 2020) and filtering out all genes with an average median count of ≤ 2 (across all samples). Differential gene expression was

estimated using the DESeq2 package in R (Love et al., 2014). Genes in dark gray, blue, and red are significantly up or down regulated using a *p*-adjusted value of .05 (N = 2,012 significantly upregulated genes; N =1,612 significantly downregulated genes). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg in DESeq2 (Love et al., 2014). Red genes represent a > 1 fold upregulation in RSD_I vs. CON_R samples; Blue genes represent a > 1 fold downregulation in RSD_I vs . CON_R samples.

(E, F). Qiagen Ingenuity Pathway (IPA) Analyses based on significantly up and down regulated genes using a p-adjusted value of .05, performed using the default settings in IPA with specific selection for species (mus musculus) and cell type (splenocytes).

E. IPA Canonical Pathway analysis for RSD_I = RSD_ISO (N = 3) vs. CON_R = CON_RPMI (N=3) demonstrates an attenuation in the upregulation in pro-inflammatory gene transcription pathways observed among unstimulated cells (i.e., RSD_R vs. CON_R comparison, reproduced on this figure from Figure 6E [in light gray]), as well as increased activation of β-adrenergic receptor (β-AR) canonical signaling pathways compared to unstimulated cells (i.e., RSD_R vs. CON_R comparison, reproduced on this figure from Figure 6E [in light gray]), and an attenuation in signaling via non-canonical β-AR pathways compared to unstimulated cells (i.e., RSD_R vs. CON_R comparison, reproduced on this figure from Figure 6E [in light gray]).
Interleukin(IL)-6 Signaling: Z-score = 1.604, *p* = .014454
Toll-like Receptor (TLR) Signaling: Z-score = 2.333, *p* = .001072
NF-κB Signaling: Z-score = .728, *p* = .048978
Chemokine Signaling: Z-score = .447, *p* = .460257
G-Protein Coupled Receptor (GPCR) Signaling: Z-score =1.806, *p* = .000003
$G\alpha_s$ Signaling: Z-score = 1.414, *p* = .225944
cAMP response element binding protein (CREB) Signaling: Z-score: -.75, *p* = .003388
Protein Kinase-A (PKA) Signaling: Z = 1.279, *p* = .287740
$G\alpha_i$ Signaling: Z-score = 1.732, *p* = .053703
p38 mitogen-activated protein kinase (MAPK) Signaling: Z-score = .728, *p* = .000355

F. IPA Upstream Regulator analysis for RSD_I = RSD_ISO (N = 3) vs. CON_R = CON_RPMI (N=3) found an overall upregulation in glucocorticoid receptor regulated signaling (NR3C1), a downregulation in cAMP response element binding (CREB) related gene transcription (CREBBP), and an upregulation in mitogen activated protein kinase related gene transcription (MAPK14, MAPK3K8).
NR3C1: ligand-dependent nuclear receptor, Activation Z-score = 0.251, *p* = 5.25E-04
Genes identified:
CXCL10, Cxcl9, IFIT1B, IL1B, IL7R, ISG15, MMP13, NFIL3, OASL, PTGR1
CREBBP: transcription regulator, Activation Z-score = -0.651, *p* = 4.93E-12
Genes identified:
ADORA2A, BANK1, CCND1, CCND3, CCR7, CD160, CKB, CNN3, CRISPLD2, EDARADD, ELANE, EVL, FOS, FOSL2, GFI1B, GIMAP4, GIMAP7, GPR18, GRAMD2B, HSD11B1, IFIT1B, IGF1R, IL18RAP, IL7R, JUP, KCNIP3, LTA, MAFG, MCF2L, MEF2C, MFHAS1, NOTCH1, OAS3, PIP5K1B, PPFIBP2, RGL1, RNASEL, RSAD2, RTP4, SDC1, SDC4, SELP, SLAMF1, SOCS3, ST6GAL1, TRAF1, TUBA8

MAPK14: kinase, Activation Z-score = 0.378, *p* = 1.12E-03
Genes identified: CD74, HLA-DQA1, IL1B, RGS1, SOCS3, SPP1, TXN
MAP3K8: kinase, Activation Z-score = 2.169, *p* = 3.91E-07
Genes identified:
CCR1, CCR5, Cd33, CIITA, COBLL1, FLNB, FSCN1, GAB1, GCA, GPR146, GPR160, GPR171, HP, IER3, IFNG, IGF1R, IL12A, IL1B, MAD2L1, P2RY1, SESN1, SNN, TDRKH, TSPAN33

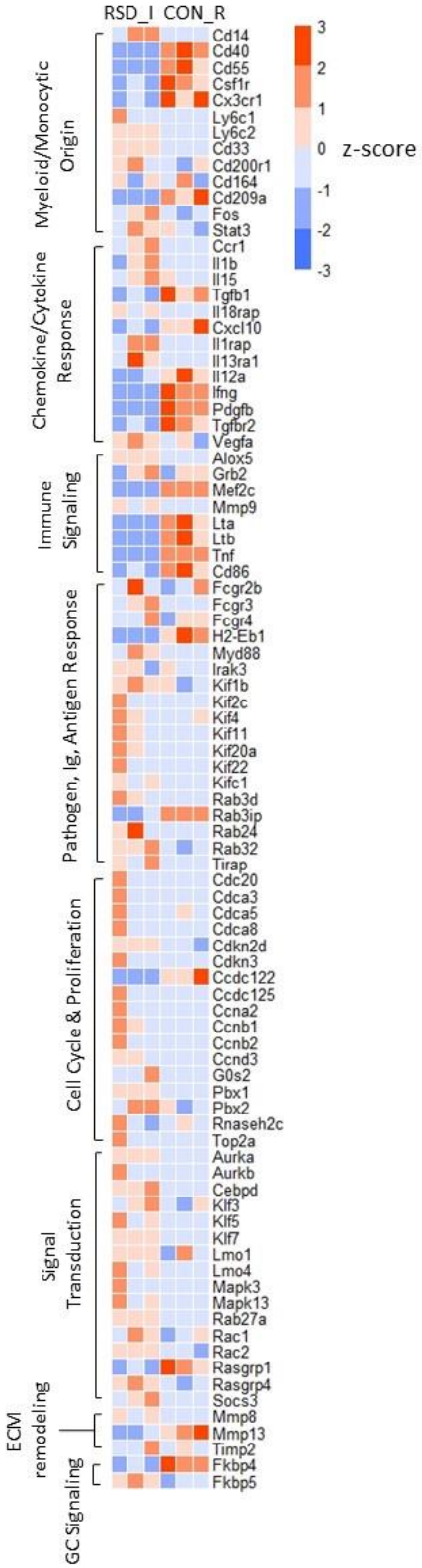


Figure 28. Stress + Stimulation Effect: Splenocytes - Genes previously identified in Repeated Social Defeat (RSD) literature

Heat map of individual genes previously identified in Repeated Social Defeat (RSD) literature (Cole et al., 2010; Powell et al., 2013; Niruala, Witcher, et al., 2018). Stimulation of CD11b+ cells from RSD mice with isoproterenol compared to unstimulated CD11b+ cells from control mice resulted in differential gene transcription in domains similar to unstimulated CD11b+ cells from RSD mice compared to unstimulated CD11b+ cells from control mice.

Notes: Individual gene z-scores depicted in the heat map (see Legend) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg in DESeq2 (Love et al., 2014). RSD_I = RSD_ISO group (N = 3 mice/samples) vs. CON_R = CON_RPMI group (N = 3 mice/samples); Each column represents an individual mouse. Ig = Immunoglobin; ECM = extracellular matrix; GC = glucocorticoid. ‡Indicates that this gene was differentially regulated in the same direction (i.e., upregulated or downregulated) in the RSD_R vs CON_R analysis.

Upregulated genes from the RSD_I vs CON_R comparison included those of myeloid origin (*Cd14*‡, *Ly6c1*‡, *Ly6c2*‡, *Ly6g*‡, *CD33*‡, *Cd200r1*‡, *Cd164*‡, *Fos*, and *Stat3*‡), those involved in cytokine and chemokine regulatory signaling (*Ccr1*‡, *IL1b*‡, *Il15*‡, *Il18rap*‡, *IL1rap*‡, *Il13ra1*‡, and *Vegfa*‡*)*, and genes involved in immune and inflammatory-related signaling (*Alox5*‡, *Grb2*, *Mmp9*†), including pathogen, Ig or antigen related signaling (*Fcgr2b*‡, *Fcgr3*‡, *Fcgr4*‡, *Myd88*‡, *Irak3*‡, *Kif1b*‡, *Kif2c*‡, *Kif4*‡, *Kif11*‡, *Kif20a*‡, *Kif22*‡, *Kifc1*‡, *Rab3d*‡, *Rab24*‡, *Rab32*‡, and *Tirap*‡). Upregulated genes also included those involved in cell cycling and proliferation (*Cdc20*†, *Cdca3*†, *Cdca8*†, *Cdkn2d*‡, *Cdkn3*‡, *Ccdc125*‡, *Ccna2*‡, *Ccnb1*‡, *Ccnb2*‡, *Ccnd3*‡, *G0s2*‡, *Pbx1*‡, *Pbx2*‡, *Rnaseh2c*‡, and *Top2a*‡), signal transduction (*Aurka*‡, *Aurkb*‡, *Cebpd*‡, *Klf3*, *Klf5*‡, *Klf7*‡, *Lmo1*, *Lmo4*‡, *Mapk3*‡, *Mapk13*‡, *Rab27a*‡, *Rac1*‡, *Rac2*‡, *Rasgrp4*‡, *Socs3*‡), and extracellular matrix remodeling (*Mmp8*‡ and *Timp2*). Among genes involved in glucocorticoid signaling, a gene important in inhibition of GR translocation to the nucleus was significantly upregulated (*Fkbp5*‡), which is consistent with the development of glucocorticoid resistance among these cells (Niraula et al., 2018). Notably, there was no significant difference in expression of the GR gene (*Nr3c1)*, which has previously served as a negative control for both gene expression and protein levels in RSD mice (Powell et al., 2013, Bailey 2009, Engler 2008; Quan 2003).

Downregulated genes also included those involved in myeloid and early monocytic lineage (*Cd40*‡, *Cd55*‡, *Csf1r*‡, *Cx3cr1*‡, *and Cd209a*‡), those involved in cytokine and chemokine regulatory signaling (*Tgfb1*‡, *Cxcl10*, *IL12a*‡, *Ifng*‡, *Pdgfb*‡, *and Tgfbr2*), genes involved in immune, pathogen, Ig, and antigen signaling (*Mef2c*‡, *Lta*‡, *Ltb*‡, *Tnf*, *Cd86*, *H2-Eb1*‡ (*MHCII protein*), and *Rab3ip*‡), a gene involved in cell cycling and proliferation (*Ccdc122*‡), and signal transduction (*Rasgrp1*‡). Other downregulated genes included a gene involved in matrix remodeling (*Mmp13)*.

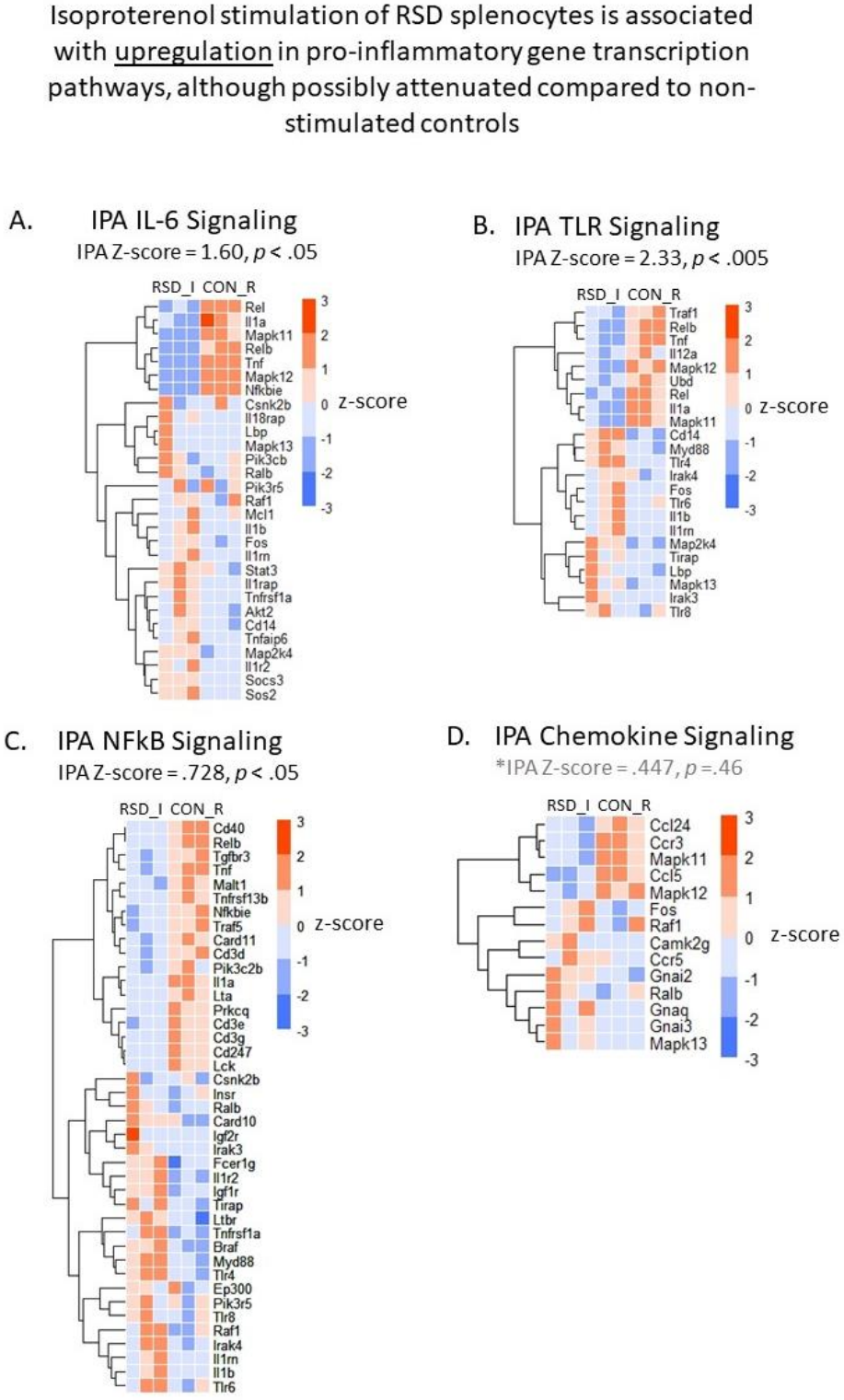


Figure 29. Stress + Stimulation Effect: Splenocytes – Individual genes identified in IPA Canonical Pathway analyses, pro-inflammatory pathways

Heat maps of individual genes identified in IPA Canonical Pathway analyses representing pro-inflammatory pathways (A-D) support the idea that isoproterenol stimulation of CD11b+ cells from Repeated Social Defeat (RSD) mice is associated with upregulation in pro-inflammatory gene transcription pathways, although possibly attenuated compared to non-stimulated controls (i.e., RSD_R vs CON_R analysis).

Notes: Individual gene z-scores depicted in the heat map (see legends) are based on raw counts data for each individual gene, normalized across all genes displayed in each group. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg in DESeq2 (Love et al., 2014). RSD_I = RSD_ISO group (N = 3 mice/samples) vs. CON_R = CON_RPMI group (N = 3 mice/samples). Each column represents an individual mouse. Cluster analysis is based on similarity in individual gene z-score distribution pattern within each group.

- **A.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in interleukin(IL)-6 signaling pathways. Activation score (Z = 1.60, $p < .05$) indicates an overall significant upregulation in IL-6 related genes among isoproterenol stimulated CD11b+ splenocytes from RSD mice compared to untreated CD11b+ splenocytes from control mice.
- **B.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in toll-like receptor (TLR) pathways. Activation score (Z = 2.33, $p < .005$) indicates an overall significant upregulation in TLR-related genes among isoproterenol stimulated CD11b+ splenocytes from RSD mice compared to untreated CD11b+ splenocytes from control mice.
- **C.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in nuclear factor – kappaBeta (NF-κB) signaling pathways. Activation score (Z = 0.728, $p < .05$) indicates an overall significant upregulation in NF-κB-related genes among isoproterenol stimulated CD11b+ splenocytes from RSD mice compared to untreated CD11b+ splenocytes from control mice.
- **D.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in chemokine-related signaling pathways. Activation score (Z = 0.447, $p = .46$) indicates a non-significant upregulation in chemokine signaling related genes among untreated CD11b+ splenocytes from RSD mice compared to untreated CD11b+ splenocytes from control mice.

5.4.5.2 Peripheral Blood

CD11b+ peripheral cells from RSD mice that were stimulated with isoproterenol compared to unstimulated CD11b+ peripheral cells from control mice (RSD_I vs CON_R comparison) resulted in differential expression of 659 mRNA gene transcripts (315 upregulated; 344 downregulated, $p_{adj}<.05$, Figure 30D). Up and downregulated genes among stimulated CD11b+ peripheral cells from the RSD_I vs CON_R comparison included many associated with a primed

and pro-inflammatory gene expression profile (Figure 30E, Figure 31A), although it was less clear compared to splenocytes whether pro-inflammatory signaling was attenuated among isoproterenol stimulated *peripheral* cells. For example, differentially regulated genes from isoproterenol stimulated CD11b+ peripheral cells in the RSD_I vs CON_R comparison resulted in a significant upregulation in IPA-identified IL-6 signaling pathways ($Z = 1.00$, $p < .05$; Figure 30E, Figure 31B). This observation is in contrast to a lack of significant findings from IPA for *unstimulated* CD11b+ peripheral cells in the RSD_R vs CON_R comparison (Section 5.4.1.2), which could be interpreted as an overall *up*regulation in pro-inflammatory signaling among isoproterenol stimulated peripheral cells from RSD mice (RSD_I vs CON_R comparison) compared to *unstimulated* peripheral cells from RSD mice (RSD_R vs CON_R comparison). Conversely, when up and downregulated genes were examined using Panther overrepresentation analysis, there was a significant enrichment in genes involved in inflammation mediated by chemokine and cytokine signaling (7.53-fold enrichment, $p = .015$; identified genes: *Pk2b, Fpr2, Rel, Cxcr4, Nfatc2, Ccl4, Itga4, Cx3cr1, Nfkbie, Ifngr1, Ccr5, Ccl3, Itgb2l, Stat3*), which was no longer significant after adjustment for multiple comparisons ($FDR = .267$). This observation is in contrast to a significant enrichment in genes involved in inflammation mediated by chemokine and cytokine signaling among *unstimulated* CD11b+ peripheral cells in the RSD_R vs CON_R comparison, which remained significant after multiple comparisons (7.75-fold, $p = .0002$, $FDR = .008$; identified genes: *Arpc3, Ccr3, Ptk2b, Fpr2, Ccrl2, Gna11, Ccl2, Grap2, Pak4, Cxcr4, Nfatc2, Fpr2, Itga4, Cx3cr1, Nfkbie, Ifngr1, Ccr5, Itgb2I*). These findings could be taken as evidence for a possible *attenuation* in pro-inflammatory signaling among stimulated peripheral cells from the RSD_I vs CON_R comparison vs. unstimulated peripheral cells from the RSD_R vs CON_R comparison.

In addition to differences in patterns of pro-inflammatory signaling among peripheral cells from RSD mice, there were several notable individual genes that demonstrated a difference in the magnitude of change in the RSD_I vs CON_R comparison vs. the RSD_R vs CON_R comparison. Specifically, and similar to stimulated splenocytes (Section 5.4.5.1), isoproterenol stimulated CD11b+ peripheral cells from RSD mice in the RSD_I vs CON_R comparison had a .50-fold greater upregulation in the myeloid lineage gene *Cd14* compared to unstimulated peripheral cells from RSD mice in the RSD_R vs CON_R comparison. As discussed in Section 5.4.5.1, it could be that isoproterenol serves as a signal for an upregulation in the primed phenotype displayed by these cells, independent from active modulation of pro-inflammatory signaling pathways. Also similar to stimulated splenocytes (Section 5.4.5.1), there was a .22-fold greater upregulation in the glucocorticoid receptor signaling gene *Fkbp5* among CD11b+ peripheral cells from the RSD_I vs CON_R comparison vs. the RSD_R vs CON_R comparison. As discussed in Section 5.4.5.1, this could suggest a shift to a cellular state of increased resistance to glucocorticoid signaling. Given that an increase in glucocorticoid resistance is typically thought of as *more* permissive to pro-inflammatory signaling, this cellular mechanism may be independent of any isoproterenol-mediated modulation in pro-inflammatory signaling pathways. Finally, and independent of observations among stimulated splenocytes, there was a .25-fold greater upregulation in a gene involved in extracellular matrix remodeling, *Timp2,* among stimulated CD11b+ peripheral cells in the RSD_I vs CON_R comparison vs. the RSD_R vs CON_R comparison. The protein encoded by the *Timp2* gene is unique in its ability to directly suppress the proliferation of endothelial cells (Oh et al., 2004); thus, a greater upregulation in this gene among stimulated peripheral CD11b+ cells from RSD mice vs unstimulated cells from RSD mice may indicate an upregulation in signaling directed at remodeling of endothelial cells (Yang, Zhang et al., 2014).

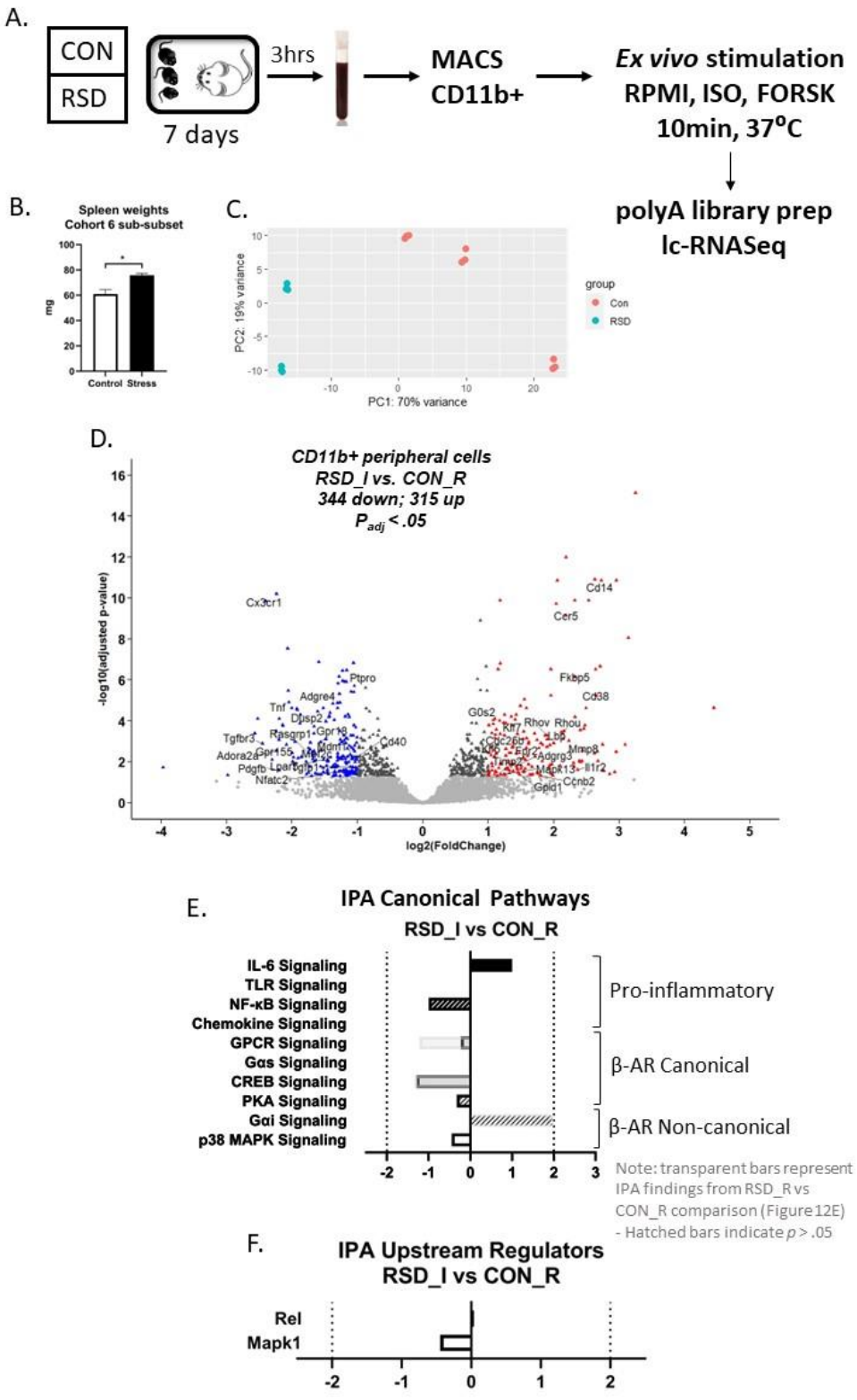


Figure 30. Stress + Stimulation Effect: Peripheral Cells

The experiment (described in A) resulted in a significant effect of Repeated Social Defeat (RSD) on splenomegaly (B) and differential gene transcription (C, D). Differentially regulated genes included those involved in pro-inflammatory signaling pathways (E, F), as well as β-adrenergic receptor (β-AR) canonical and non-canonical signaling pathways (E, F). Contrary to our hypothesis, stimulation of CD11b+ peripheral cells from Repeated Social Defeat (RSD) mice with isoproterenol may activate some canonical β-adrenergic signaling pathways (E) in the context of evidence for molecular scaffolding of non-canonical β-adrenergic signaling (E, F).

- **A.** Diagram of experiment. Male C56BL/6 mice (6-8 weeks old) underwent repeated social defeat (RSD) for 6 days or were left undisturbed in their cages in the control room (CON) until sacrifice. Mice were sacrificed within three hours (hrs) of the last round of social defeat. After sacrifice, the peripheral blood was harvested from the mice for isolation of CD11b+ cells. CD11b+ peripheral cells were isolated using Miltenyi Biotec CD11b+ Microbeads (Miltenyi Biotec Cat #130-049-60). CD11b+ peripheral cells were either left unstimulated (RPMI 1640 cell culture media alone) or were subject to stimulation by isoproterenol (ISO), a non-specific β-adrenergic agonist, or forskolin (FORSK), a non-specific activator of adenylate cyclase (AC) for 10 minutes at 37ºC. RNA was then isolated. A subset of these samples (N = 3 in each CON/RSD group) were sent for polyA library preparation (poly-A tail was used to amplify mRNA using oligo(dT) primers for mRNA library generation; the poly-A tail is a long chain of adenosine nucleotides added to 3' end RNA to prevent degradation and mark it for transport to ribosomes to be translated into protein) and lc-RNAseq (mRNA library generation for limiting cell (lc = low input RNA amounts: 10pg-1ng) was performed using the Clontech SMART-Seq HT kit (Takara Bio Inc., Mountain View, CA, Cat #634456) and the the Nextera XT DNA Library Prep Kit (Illumina, Inc., San Diego, CA, Cat #FC-131-1096). RNA-Seq = Samples were then sequenced at a depth of 15-21million 2x100bp paired-end clusters on an Illumina NovaSeq6000 platform (Illumina, Inc., San Diego)).
- **B.** A quantitative measure of stress in animals experiencing repeated social defeat (RSD/"Stress") includes splenomegaly. The sub-subset of data displayed here represents the mice for the samples used in the analyses (N = 2 RSD, N = 3 CON). Specifically, one RSD mouse that was sent for sequencing was removed from the analyses due to significant overlap with gene expression among control mice. The data comparing spleen weights in the subset of mice sent for sequencing can be seen in Figure 2J. *$p < .05$
- **C.** Principle Component Analysis (PCA) plot of all genes for all treatment groups (RPMI, ISO, FORSK) and mice (CON (N = 3), RSD (N = 2)) included in the analysis. Separation between groups (CON/RSD) accounted for greater variance (70%) than separation between treatment conditions (RPMI, ISO, FORSK; 19%).
- **D.** Volcano plot for all up and down-regulated genes for RSD_I = RSD_ISO group (N = 2 mice/samples) vs. CON_R = CON_RPMI group (N = 3 mice/samples) after CLEAR selection for robust/low-noise transcripts (Walker et al., 2020) and filtering out all genes with an average median count of ≤ 2 (across all samples). Differential gene expression was estimated using the DESeq2 package in R (Love et al., 2014). Genes in dark gray, blue, and red are significantly up or down-regulated using a *p*-adjusted value of .05 (N = 334 upregulated genes; N = 347 downregulated genes). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg (Love et al., 2014). Red genes represent a > 1-fold upregulation in RSD vs. CON samples; Blue genes represent a

> 1-fold downregulation in RSD vs. CON samples. Light gray genes were not significantly up or down-regulated.

(E, F) Qiagen Ingenuity Pathway (IPA) Analyses based on significantly up and down regulated genes using a p-adjusted value of .05, performed using the default settings in IPA with specific selection for species (mus musculus) and cell type (peripheral mononuclear cells & leukocytes).

E. IPA Canonical Pathway analysis for RSD_I = RSD_ISO (N = 2) vs. CON_R = CON_RPMI (N=3) demonstrates a downregulation in beta-adrenergic receptor (β-AR) canonical signaling pathways (although attenuated compared to unstimulated cells (i.e., RSD_R vs. CON_R comparison, reproduced on this figure from Figure 9E [in light gray])) and a downregulation in β-AR non-canonical signaling pathways.
Interleukin(IL)-6 Signaling: Z-score: 1.000, *p* = .030903
Toll-like Receptor (TLR) Signaling: n/a
NF-κB Signaling: Z-score: -1.000, *p* = .448745
Chemokine Signaling: n/a
G-Protein Coupled Receptor (GPCR) Signaling: Z-score = -.22942, *p* = .012303
$G\alpha_s$ Signaling: n/a
cAMP-response element binding protein (CREB) Signaling:
Z-score: -1.2909, *p* =.020893
Protein Kinase-A (PKA) Signaling: Z-score = -0.333, *p* = .155955
$G\alpha_i$ Signaling: n/a
p38 mitogen-activated protein kinase (MAPK) Signaling: Z-score: -.44721, *p* = .017378

F. IPA Upstream Regulator analysis for RSD_ISO (N = 2) vs. CON_RPMI (N=3) demonstrates a significant upregulation in activator protein (AP)-1 related gene transcription (REL) and a downregulation in non-canonical β-adrenergic receptor (β-AR) MAPK1 related gene transcription.
REL: transcription regulator; Activation Z-Score = 0.04, *p* = 5.78E-03
Genes identified: ATP11A, BCL2A1, BCL2L1, CD40, EGR2, GPT2, SH2B3, TNF
MAPK1: kinase, Activation Z-Score = -0.447, *p* = 6.48E-03
Genes identified: CCL3L3, EGR2, ENO3, NT5E, PDGFB, TNFRSF9

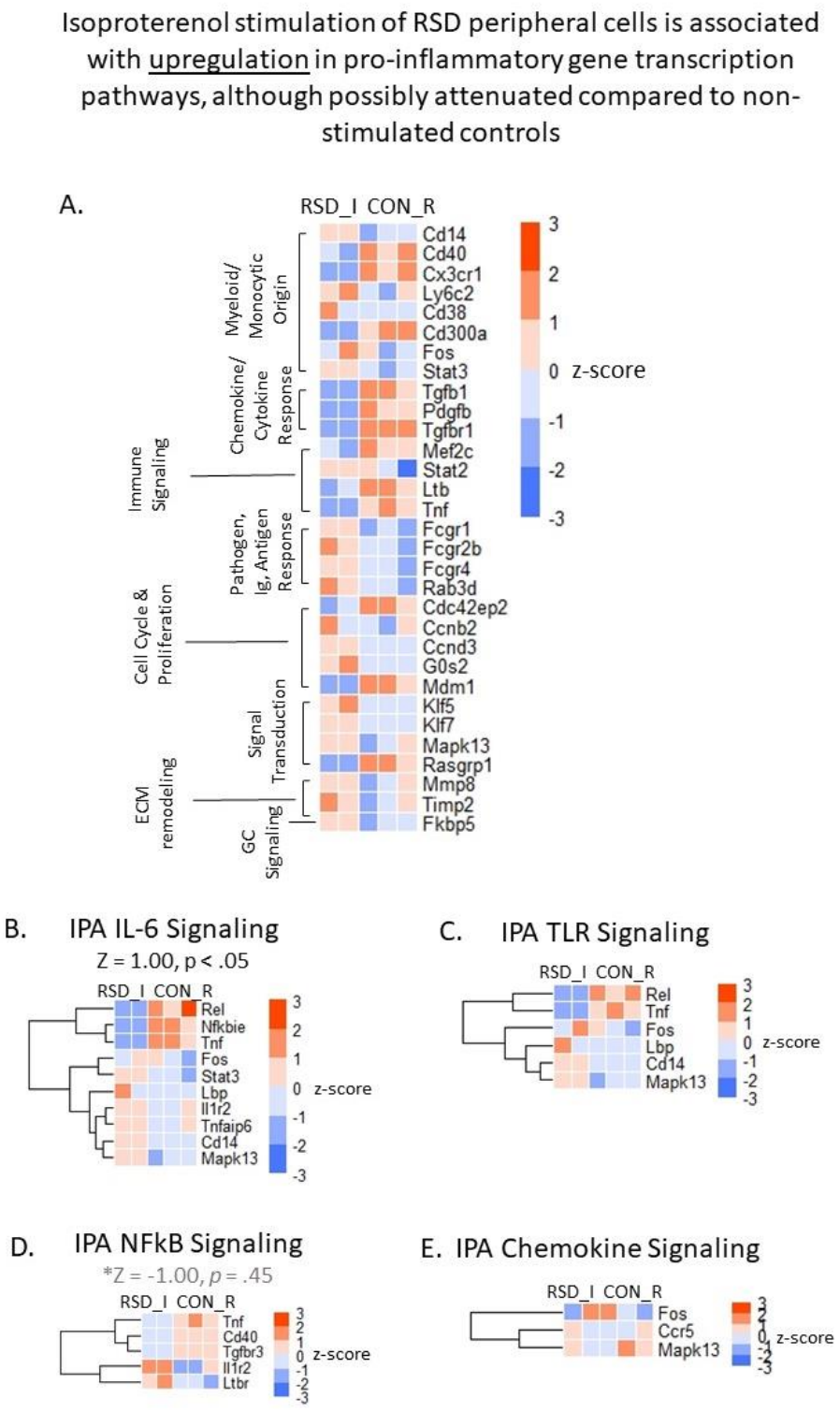


Figure 31. Stress + Stimulation Effect: Peripheral Cells - Genes previously identified in RSD literature & individual genes identified in IPA Canonical Pathway analyses, pro-inflammatory pathways

Heat map of individual genes previously identified in Repeated Social Defeat (RSD) literature (A; Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018). Stimulation of CD11b+ cells from RSD mice with isoproterenol compared to unstimulated CD11b+ cells from control mice resulted in differential gene transcription in domains similar to unstimulated CD11b+ cells from RSD mice compared to unstimulated CD11b+ cells from control mice (A vs Figure 9A). Heat maps of individual genes identified in IPA Canonical Pathway analyses representing pro-inflammatory pathways (B-E) support the idea that isoproterenol stimulation of CD11b+ cells from Repeated Social Defeat (RSD) mice is associated with upregulation in pro-inflammatory gene transcription pathways (B).

Notes: Individual gene z-scores depicted in the heat maps (see legends) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg (Love et al., 2014). RSD_I = RSD_ISO group (N = 2); CON_R = Control_RPMI group (N = 3); Each column represents an individual mouse.

A. Heat map of individual genes previously identified in RSD literature (Cole et al., 2010; Powell et al., 2013; Niraula, Witcher, et al., 2018) demonstrates that stimulation of CD11b+ cells from RSD mice with isoproterenol (RSD_I) compared to unstimulated CD11b+ cells from control mice (CON_R) resulted in differential gene transcription in domains similar to unstimulated CD11b+ cells from RSD mice compared to unstimulated CD11b+ cells from control mice (RSDI_R vs CON_R comparison). *Notes:* Ig = Immunoglobin; ECM = extracellular matrix; GC = glucocorticoid. ‡Indicates that this gene was differentially regulated in the same direction (i.e., upregulated or downregulated) in the RSD_R vs CON_R analysis.

- Upregulated genes in Cd11b+ peripheral cells from the RSD_I vs CON_R comparison included those of myeloid origin (*Cd14*‡, *Ly6c2*‡, *Ly6g*‡, *Cd38*‡, *Fos*, *Stat3*), those involved in immune, pathogen, Ig, or antigen related signaling (*Stat2*, *Fcgr1*‡, *Fcgr2b*‡, *Fcgr4*‡, *Rab3d*‡), genes involved in cell cycling and proliferation (*Ccnb2*, *Ccnd3*‡, *G0s2*), signal transduction (*Klf5*‡, *Klf7*‡, *Mapk13*), and extracellular matrix remodeling (*Mmp8*‡, *Timp2*‡). A gene important in inhibition of GR translocation to the nucleus was also significantly upregulated (*Fkbp5*‡), consistent with the development of glucocorticoid resistance. There was also no significant difference in expression of the GR gene (*Nr3c1*), which has previously served as a negative control for both gene expression and protein levels in RSD (Powell et al., 2013, Bailey 2009, Engler 2008; Quan 2003).
- Downregulated genes among isoproterenol stimulated peripheral CD11b+ cells from RSD mice compared to unstimulated peripheral CD11b+ cells from Control mice included those involved in myeloid and early monocytic lineage (*Cd40*‡, *Cx3cr1*‡, and *CD300a*‡), cytokine and chemokine regulatory signaling (*Tgfb1*, *Pdgfb*‡, and *Tgfbr1*‡), and immune and inflammatory-related signaling (*Mef2c*‡, *Ltb*, and *Tnf*). Other downregulated genes were involved in cell cycling and proliferation (*Cdc42ep2*, and *Mdm1*‡), and signal transduction (*Rasgrp1*‡). There was also a downregulation in a gene encoding a transcriptional activator of NFkB (*Relb*‡).

B. Heat map of individual genes identified in IPA Canonical Pathway analyses representing interleukin(IL)-6 pro-inflammatory signaling pathways. Cluster analysis is based on

similarity in individual gene z-score distribution pattern within each group. Activation score (Z = 1.00, $p < .05$) indicates an overall significant upregulation in IL-6 related genes among isoproterenol stimulated CD11b+ cells from RSD mice (RSD_I) compared to untreated CD11b+ cells from control mice (CON_R).

C. Heat map of individual genes identified in IPA Canonical Pathway analyses representing toll-like receptor (TLR) pro-inflammatory signaling pathways. Cluster analysis is based on similarity in individual gene z-score distribution pattern within each group. No IPA Z-score was calculated for this pathway, although a number of individual genes were identified.
D. Heat map of individual genes identified in IPA Canonical Pathway analyses representing nuclear factor – kappaBeta (NF-κB) pro-inflammatory signaling pathways. Cluster analysis is based on similarity in individual gene z-score distribution pattern within each group. Activation score (Z = -1.00, $p = .45$) indicates a non-significant downregulation in PKA-related genes among isoproterenol stimulated CD11b+ cells from RSD mice (RSD_I) compared to untreated CD11b+ cells from control mice (CON_R).
E. Heat map of individual genes identified in IPA Canonical Pathway analyses representing chemokine-related pro-inflammatory signaling pathways. No IPA Z-score was calculated for this pathway, although three individual genes were identified.

5.4.6 RSD_I vs CON_R: Contrary to our hypotheses, stimulation of RSD CD11b+ cells with isoproterenol may activate some canonical β-adrenergic signaling in the context of evidence for molecular scaffolding of non-canonical β-adrenergic signaling.

5.4.6.1 Spleen

A number of genes identified as differentially expressed in isoproterenol stimulated CD11b+ splenocytes from RSD mice compared to unstimulated splenocytes from Control mice (RSD_I vs CON_R comparison; $p_{adj} < .05$) were consistent with a pattern of activation of *canonical* β-adrenergic signaling in the context of evidence for molecular scaffolding of *non-canonical* β-adrenergic signaling (Figure 27E, Figure 32A). For example, IPA-identified a significant upregulation in CREB-mediated signaling pathways among isoproterenol stimulated CD11b+ splenocytes from the RSD_I vs CON_R comparison (Z = .58, $p < .005$; Figure 27E, Figure 33A), whereas CREB-mediated signaling pathways among *unstimulated* splenocytes in the RSD_R vs CON_R comparison were downregulated (Z = -.75, $p < .005$; Figure 6E, reproduced on Figure

27E using the light gray bars). An upregulation in CREB-mediated signaling pathways among stimulated CD11b+ splenocytes in the RSD_I vs CON_R comparison would be evidence for activation of canonical β-adrenergic signaling in the context of isoproterenol stimulation. Interestingly, IPA also identified a possible upregulation in $G\alpha_s$ mediated canonical GPCR signaling among stimulated CD11b+ splenocytes in the RSD_I vs CON_R comparison (Z = .41; Figure 27E, Figure 34B). Although this pathway was not statistically significant ($p > .05$), this observation is in contrast to a lack of identification of this pathway among *unstimulated* splenocytes from RSD mice in the RSD_R vs CON_R comparison (Figure 6E).

IPA-identified pathways consistent with molecular scaffolding for (and possibly some signaling through) *non-canonical* β-adrenergic pathways included activation of pathways utilizing $G\alpha_i$ in GPCR signaling (Z=1.73, $p < .05$; Figure 27E, Figure 34C), and an upregulation in p38 MAPK pathway utilization (Z=.73, $p < .005$; Figure 27E, Figure 33C; See Figure 1 for diagram of these pathways). Interestingly, the activation score for both of these pathways in *stimulated* splenocytes in the RSD_I vs CON_R comparison ($G\alpha_i$: Z=1.73; p38 MAPK: Z=.73) was quantitatively *less* than for *unstimulated* splenocytes in the RSD_R vs CON_R comparison ($G\alpha_i$: Z=2.45, $p < .005$, p38MAPK: Z=1.71, $p = .005$; Figure 6E, reproduced in the light gray bars in Figure 27E). This suggests a possible *reduction* in signaling through non-canonical β-adrenergic pathways in the context of isoproterenol stimulation, although the molecular scaffolding for non-canonical β-adrenergic signaling pathways is likely still present. Additional evidence in support of the presence of molecular scaffolding for non-canonical β-adrenergic signaling pathways was identified among individual genes of interest ($p_{adj} < .05$; Figure 32). Specifically, among stimulated splenocytes in the RSD_I vs CON_R comparison there was an upregulation in *Arrb2*, the gene encoding a scaffolding protein involved in non-canonical β-adrenergic receptor signaling (β-

arrestin-2; See Figure 1 for diagram of these pathways, Figure 32A for heat map, Figure 32E for exact gene counts). Notably, the magnitude of fold change for *Arrb2* was similar between stimulated splenocytes in the RSD_I vs CON_R comparison and unstimulated splenocytes in the RSD_R vs CON_R comparison (Figure 35E), suggesting that molecular scaffolding for non-canonical β-adrenergic signaling pathways was similar in both conditions.

Among other IPA-identified pathways, there was a significant upregulation in GPCR signaling among isoproterenol stimulated splenocytes in the RSD_I vs CON_R comparison (Z=1.81, $p < .0001$; Figure 27E, Figure 34A). Interestingly, the magnitude of the activation score for GPCR signaling was quantitatively *greater* in *stimulated* splenocytes from the RSD_I vs CON_R comparison (Z=1.81) compared to unstimulated splenocytes from the RSD_R vs CON_R comparison (Z=.43, $p < .005$; Figure 6E, also reproduced in Figure 27E in the light gray bars). Among *unstimulated* splenocytes from RSD mice in the RSD_R vs CON_R comparison, we inferred the upregulation in GPCR signaling as likely attributable to signaling through the non-canonical β-adrenergic pathway due to greater overall evidence for this pathway (See Section 5.4.2.1), in addition to the specific evidence for an upregulation in IPA-identified Ga_i-mediated GPCR signaling (Figure 6E, Figure 13C). Here, among isoproterenol *stimulated* splenocytes in the RSD_I vs CON_R comparison, the observed upregulation in GPCR signaling may be attributable to signaling through *both* canonical and non-canonical β-adrenergic receptor signaling pathways. Specifically, the evidence presented for the current RSD_I vs CON_R comparison is consistent with the activation of canonical β-adrenergic signaling in the context of evidence for molecular scaffolding for (and some signaling through) the non-canonical β-adrenergic signaling pathway.

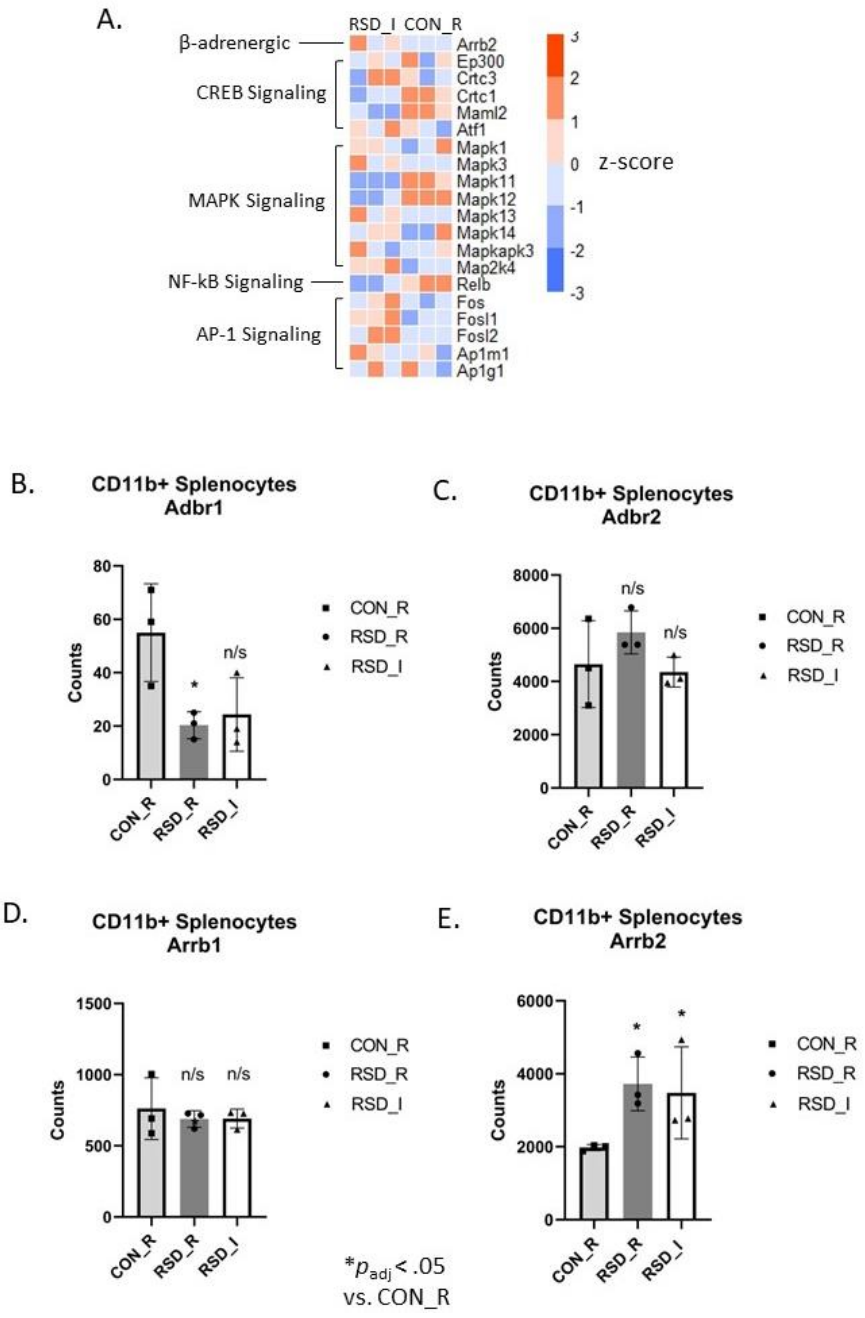


Figure 32. Figure 30. Stress + Stimulation Effect: Splenocytes – Individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching"

Heat map (A) and graphs of individual gene counts (B-E) support the idea that isoproterenol stimulation of CD11b+ splenocytes from Repeated Social Defeat (RSD) mice may promote gene transcription consistent with activation of canonical β-adrenergic receptor (β-AR) signaling (A, B) in the context of evidence for a molecular scaffolding involving signaling through non-canonical β-AR signaling pathways (A, E) compared to unstimulated CD11b+ splenocytes from control mice.

A. Heat map of individual genes identified a-priori as involved in β-AR "signal switching" support an upregulation in canonical β-AR signaling, although some non-canonical β-AR signaling pathways were also upregulated. Individual gene z-scores depicted in the heat map (see legend) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg in DESeq2 (Love et al., 2014). Each column represents an individual mouse. *Notes*: RSD_I = RSD_ISO group (N = 3 mice/samples) vs. CON_R = CON_RPMI group (N = 3 mice/samples). CREB Signaling = genes involved in cAMP response element binding protein (CREB) signaling; MAPK Signaling = genes involved in mitogen-activated protein kinase (MAPK) signaling; NF-κB Signaling = genes identified in nuclear factor – kappaBeta (NF-κB) pro-inflammatory signaling; AP-1 Signaling = genes involved in activator protein-1 (AP-1) pro-inflammatory signaling.

(B-E). Graphs of individual gene counts for particular genes of interest identified *a-priori* as involved in β-AR "signal switching." *Notes:* $*p_{adj} < .05$, n/s = not significant. CON_R = Control_RPMI group, RSD_R = RSD_RPMI group, RSD_I = RSD_ISO group. Individual points (squares, circles, triangles) in each column represent raw gene counts from an individual mouse (one point = one sample). CD11b+ = cell surface protein marker indicating mouse monocytes and granulocytes.

B. Among unstimulated CD11b+ splenocytes from RSD mice (RSD_R) there was significantly lower expression of Adrb1, the gene encoding the β1-adrenergic receptor, compared to untreated CD11b+ splenocytes from control mice (CON_R). While the difference in gene expression for Adrb1 between isoproterenol stimulated CD11b+ splenocytes from RSD mice (RSD_I) and unstimulated CD11b+ splenocytes from control mice (CON_R) was non-significant, the gene counts for Adrb1 were quantitatively lower among RSD_I mice compared to CON_R. This adds support to the idea that isoproterenol stimulation may upregulate canonical β-AR signaling pathways.

C. There was no significant difference in gene expression of Adrb2, the gene encoding the β2-adrenergic receptor, between untreated CD11b+ splenocytes from RSD mice (RSD_R) and untreated CD11b+ cells from control mice (CON_R), nor between isoproterenol treated CD11b+ splenocytes from RSD mice (RSD_I) and untreated CD11b+ cells from control mice (CON_R).

D. There was no significant difference in gene expression of Arrb1, the gene encoding the β-arrestin-1 protein, between untreated CD11b+ splenocytes from RSD mice (RSD_R) and untreated CD11b+ cells from control mice (CON_R), nor between isoproterenol treated CD11b+ splenocytes from RSD mice (RSD_I) and untreated CD11b+ cells from control

mice (CON_R). β-arrestin-1 is a key signaling protein involved in canonical β-adrenergic receptor signaling.

E. RSD resulted in a significantly greater expression of Arrb2, the gene encoding the β-arrestin-2 protein, among both untreated CD11b+ splenocytes from RSD mice (RSD_R) and isoproterenol stimulated CD11b+ splenocytes from RSD mice (RSD_I) compared to untreated CD11b+ splenocytes from control mice (CON_R). β-arrestin-2 is a key signaling protein involved in non-canonical β-adrenergic receptor signaling. This data adds support to the idea that isoproterenol stimulation may not alter the molecular scaffolding in place for a shift to non-canonical β-AR signaling pathways.

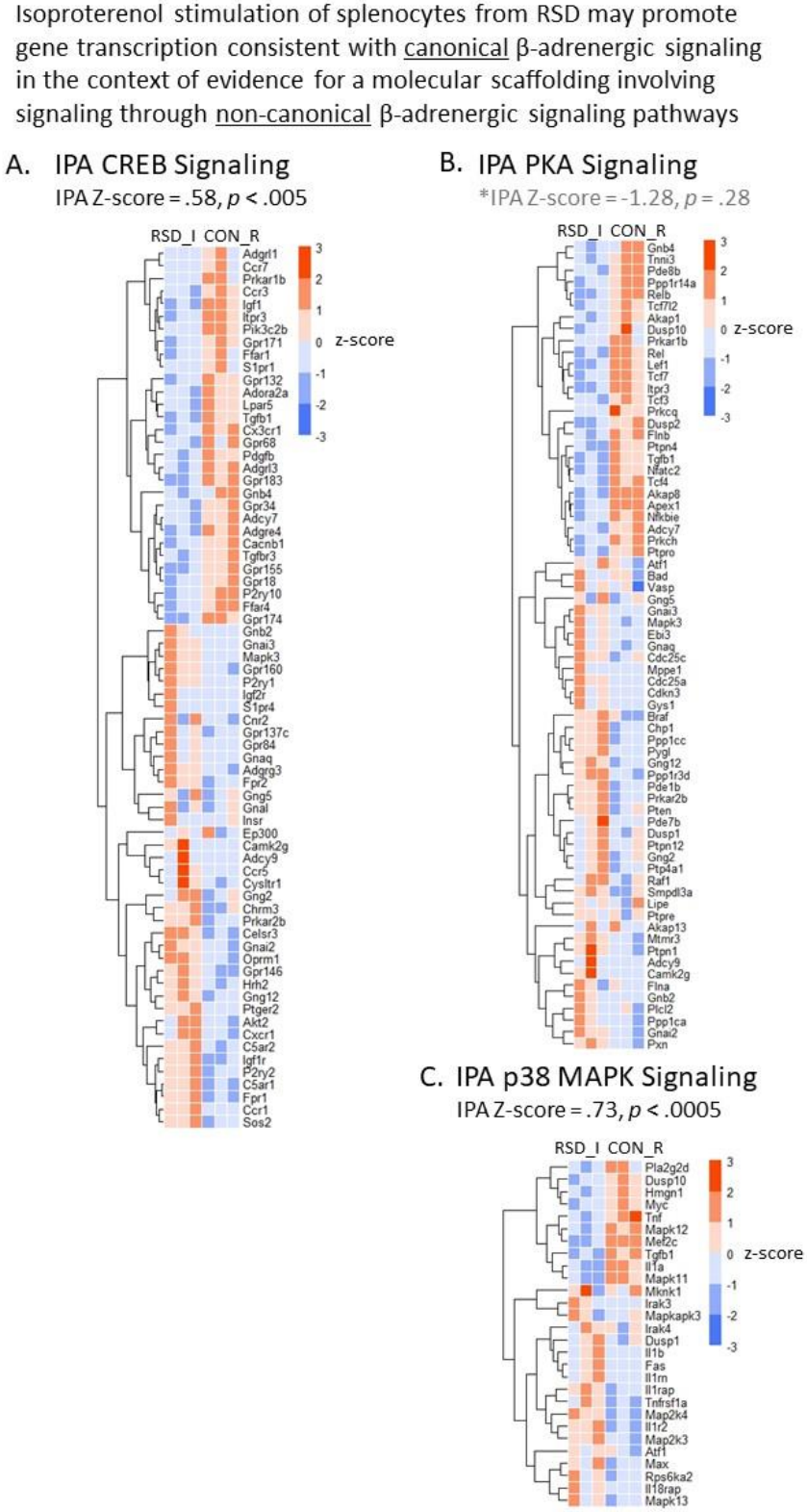


Figure 33. Stress + Stimulation Effect: Splenocytes – Individual genes identified in IPA Canonical Pathway analyses, protein kinase-A (PKA), cAMP response element binding (CREB), and mitogen-activated protein kinase (MAPK) pathways

Heat maps of individual genes identified in IPA Canonical Pathway analyses (A-C) support the idea that isoproterenol stimulation of CD11b+ splenocytes from Repeated Social Defeat (RSD) mice may promote gene transcription consistent with canonical β-adrenergic signaling pathways (A) in the context of evidence for molecular scaffolding for non-canonical β-adrenergic signaling pathways (C).

Notes: Individual gene z-scores depicted in the heat map (see legends) are based on raw counts data for each individual gene, normalized across all genes displayed in each group. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg in DESeq2 (Love et al., 2014). RSD_I = RSD_ISO group (N = 3 mice/samples); CON_R = Control_RPMI group (N = 3 mice/samples). Each column represents an individual mouse. Cluster analysis is based on similarity in individual gene z-score distribution pattern within each group.

A. Heat map of individual genes identified in IPA Canonical Pathway analyses involved in cAMP response element binding (CREB) protein signaling pathways. Activation score (Z = .58, $p < .005$) indicates an overall significant upregulation in CREB-related genes among isoproterenol stimulated CD11b+ splenocytes from RSD mice compared to untreated CD11b+ splenocytes from control mice.

B. Heat map of individual genes identified in IPA Canonical Pathway analyses involved in protein kinase-A (PKA) signaling pathways. Activation score (Z = -1.28, $p = .28$) indicates a non-significant downregulation in PKA-related genes among isoproterenol stimulated CD11b+ splenocytes from RSD mice compared to untreated CD11b+ splenocytes from control mice.

C. Heat map of individual genes identified in IPA Canonical Pathway analyses involved in mitogen-activated protein kinase (MAPK) signaling pathways. Activation score (Z = .73, $p < .0005$) indicates an overall significant upregulation in p38 MAPK-related genes among isoproterenol stimulated CD11b+ splenocytes from RSD mice compared to untreated CD11b+ splenocytes from control mice.

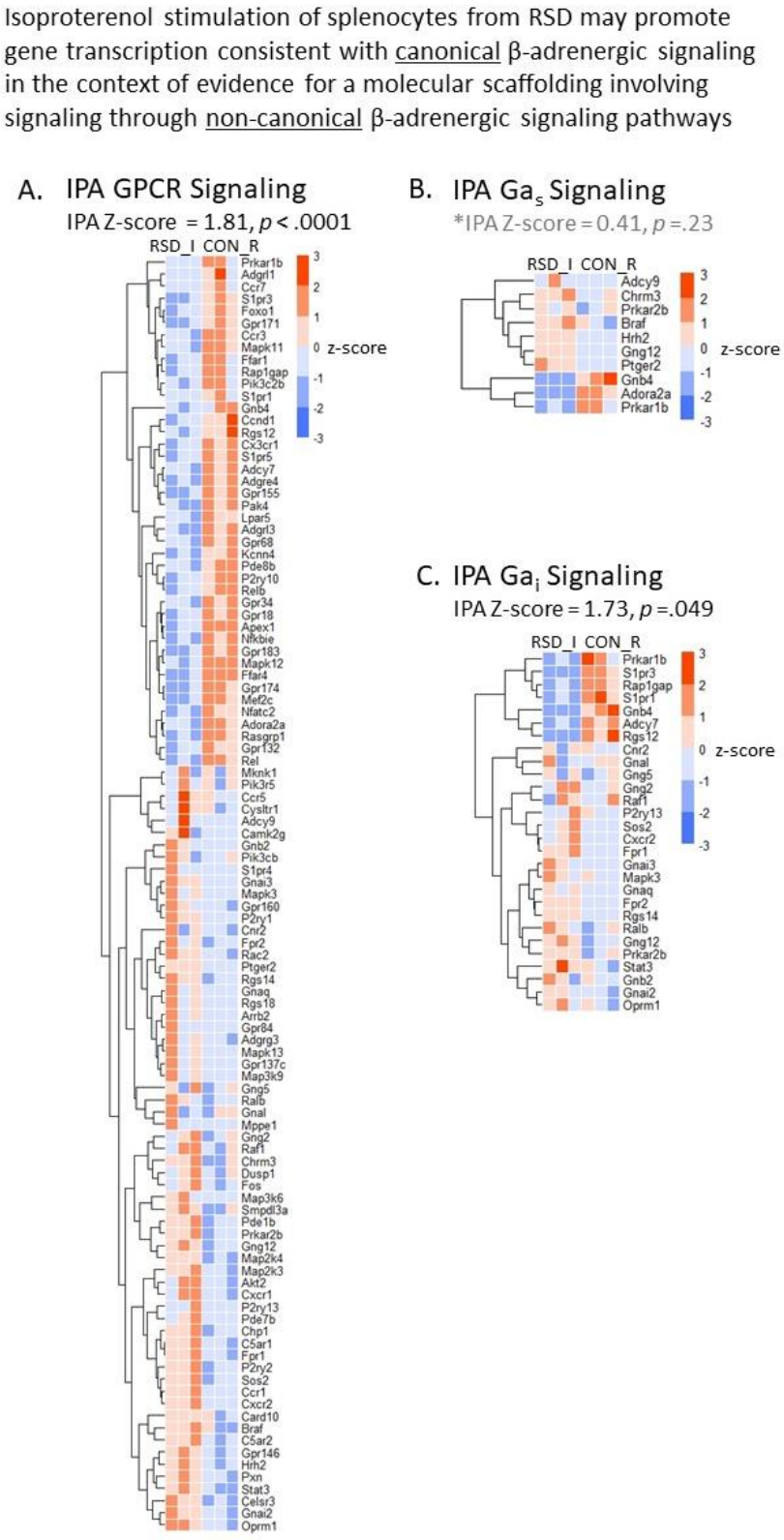


Figure 34. Stress Effect + Stimulation: Splenocytes – Individual genes identified in IPA Canonical Pathway analyses, G-protein coupled receptor (GPCR) signaling pathways

Isoproterenol stimulation of CD11b+ splenocytes from Repeated Social Defeat (RSD) mice may promote gene transcription consistent with canonical β-adrenergic signaling (A, B) in the context of evidence for a molecular scaffolding involving signaling through non-canonical β-adrenergic signaling pathways (C).

Notes: Individual gene z-scores depicted in the heat map (see legends) are based on raw counts data for each individual gene, normalized across all genes displayed in each group. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg in DESeq2 (Love et al., 2014). RSD_I = RSD_ISO group (N = 3 mice/samples) vs. CON_R = CON_RPMI group (N = 3 mice/samples). Each column represents an individual mouse. Cluster analysis is based on similarity in individual gene z-score distribution pattern within each group.

- **A.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in G-protein coupled receptor (GPCR) signaling pathways. The activation score (Z = 1.81, $p < .0001$) indicates an overall significant upregulation in GPCR-related genes among isoproterenol stimulated CD11b+ splenocytes from RSD mice compared to untreated CD11b+ splenocytes from control mice.
- **B.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in G-protein-alpha-s ($G\alpha_s$) GPCR signaling pathways. G-protein alpha (Gα) is a subunit of the G-protein complex that dissociates from the GPCR after ligand binding. Subunit (s) indicates the subtype of G-protein alpha; Ga_s signaling is involved in canonical β-adrenergic receptor signaling. Activation score (Z = 0.41, $p = .23$) indicates a non-significant upregulation in PKA-related genes among isoproterenol stimulated CD11b+ splenocytes from RSD mice compared to untreated CD11b+ splenocytes from control mice.
- **C.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in G-protein-alpha-i ($G\alpha_i$) GPCR signaling pathways. G-protein alpha (Gα) is a subunit of the G-protein complex that dissociates from the GPCR after ligand binding. Subunit (i) indicates the subtype of G-protein alpha; Ga_i signaling is involved in non-canonical β-adrenergic receptor signaling. The activation score (Z = 1.73, $p = .049$) indicates an overall significant upregulation in Ga_i GPCR-related genes among untreated CD11b+ splenocytes from RSD mice compared to untreated CD11b+ splenocytes from control mice.

5.4.6.2 Peripheral Blood

A number of genes identified as differentially expressed in isoproterenol stimulated CD11b+ peripheral cells from RSD mice compared to unstimulated CD11b+ peripheral cells from control mice (RSD_I vs CON_R comparison; $p_{adj} < .05$) could be consistent with a pattern of activation of canonical β-adrenergic signaling pathways in the context of evidence for molecular scaffolding of non-canonical β-adrenergic signaling pathways.

Among pathways consistent with a possible activation of canonical β-adrenergic signaling, the downregulation in IPA-identified GPCR signaling observed among stimulated peripheral cells in the RSD_I vs CON_R comparison (Z= -.229, $p < .05$; Figure 30E and 36B) was *attenuated* compared to unstimulated peripheral cells in the RSD_R vs CON_R comparison (Z= -1.51, $p < .005$; Figure 9E, reproduced in light gray bars in Figure 30E). This observation could be taken as evidence for an overall shift toward greater GPCR-mediated signaling, as might occur in the context of an activation of canonical β-adrenergic signaling, or at least a shift in cellular pressure away from non-canonical GPCR-independent signaling. Consistent with this interpretation, there was also a slight attenuation in the downregulation of IPA-identified CREB-mediated signaling among stimulated peripheral cells in the RSD_I vs CON_R comparison (Z= -1.29, $p < .05$; Figure 30E, Figure 36A) compared to unstimulated peripheral cells in the RSD_R vs CON_R comparison (Z = -1.34, $p < .005$; Figure 9E, reproduced in light gray bars in Figure 30E). An attenuation in the downregulation of CREB-mediated signaling could also possibly be taken as evidence for an overall shift toward CREB-mediated gene transcription, or a shift in cellular pressure away from non-canonical GPCR-independent signaling. Individual genes from the RSD_I vs CON_R comparison ($p < .05$[7]) that were consistent with this conjecture included the upregulation of two genes involved in CREB-related gene transcription (*Crtc3, Crem*; Figure 35A). Of these, there was a .11 greater upregulation (i.e., log fold change) in *Crem* among stimulated peripheral cells in the RSD_I vs CON_R comparison compared to unstimulated peripheral cells in the RSD_R vs CON_R comparison. *Crem* is a CREB-related gene encoding a protein that binds specifically to

[7] Given that individual genes involved in β-adrenergic pathways of interest were identified a-priori, and that we examined whether there was significant differential regulation among *unstimulated* cells using a non-adjusted $p < .05$ (RSD_R vs CON_R comparison), we used this criterion to explore differential regulation among isoproterenol *stimulated* CD11b+ peripheral cells from RSD mice vs. unstimulated CD11b+ peripheral cells from Control mice (RSD_I vs CON_R comparison).

the cAMP response element (CRE) to mediate gene transcription (Masquilier et al., 1993); thus, a greater upregulation in *Crem* among *stimulated* peripheral cells in the RSD_I vs CON_R comparison compared to unstimulated peripheral cells from the RSD_R vs CON_R comparison would be consistent with an upregulation in CREB-mediated gene transcription as a consequence of isoproterenol stimulation. Other observations among individual genes of interest[6] that were consistent with signaling through canonical β-adrenergic receptor pathways included the downregulation of a β-adrenergic receptor gene, *Adrb2* (Figure 35A, Figure 35C). Downregulation of this gene is interpreted here in the context of activation of canonical β-adrenergic signaling pathways because we also observed a significant downregulation in the *Adrb2* gene among CD11b+ splenocytes from control mice stimulated with isoproterenol (Section 5.4.3.2; Figure 18B).

Evidence among peripheral CD11b+ peripheral cells consistent with molecular scaffolding for (and possibly some signaling through) *non-canonical* β-adrenergic pathways was sparse or mixed at best. Among IPA-identified pathways, there was an overall downregulation in p38MAPK signaling observed among stimulated peripheral cells in the RSD_I vs CON_R comparison (Z= -.447, $p < .05$; Figure 30E, Figure 36E). At a minimum, this suggests the possible presence of molecular scaffolding for non-canonical β-adrenergic signaling pathways. In comparison to a lack of identification of signaling through this pathway among unstimulated peripheral cells in the RSD_R vs CON_R comparison (Figure 9E, reproduced in light gray bars in Figure 30E), the observation of a downregulation in p38MAPK signaling suggests a possible *reduction* in signaling through non-canonical β-adrenergic pathways in the context of isoproterenol stimulation. This would be in line with observations among stimulated CD11b+ splenocytes from RSD mice (Section *5.4.6.1*). Somewhat stronger evidence was identified among IPA-identified upstream

regulators. Specifically, there was a significant downregulation in genes regulated by *Mapk1* among stimulated peripheral cells in the RSD_I vs CON_R comparison (Z = -.447, $p < .01$; Figure 30F), whereas there was a significant activation of genes regulated by *Map3k8* among unstimulated peripheral cells in the RSD_R vs CON_R comparison (Z = .218, $p < .05$; Figure 9F). This observation adds support to the idea of a possible reduction in signaling through p38 MAPK non-canonical β-adrenergic pathways among peripheral cells in the context of isoproterenol stimulation.

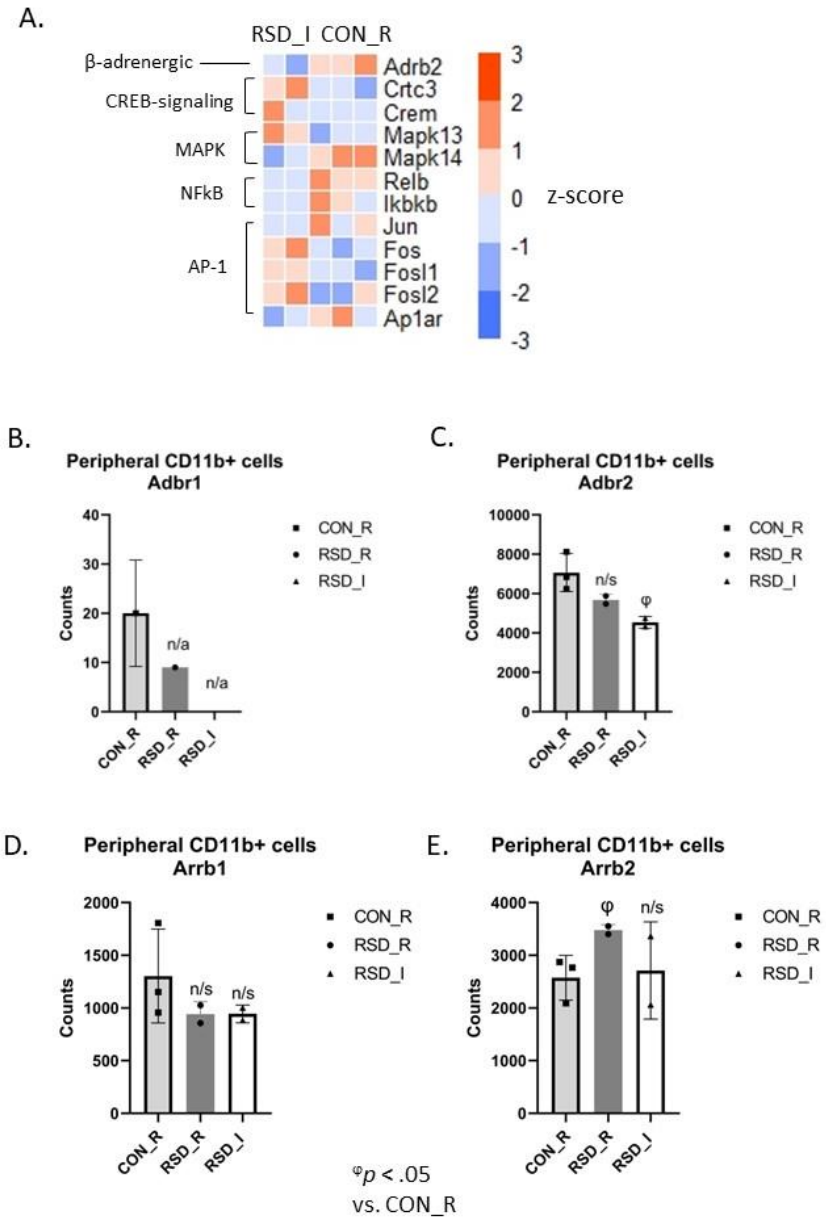


Figure 35. Stress + Stimulation Effect: Peripheral Cells – Individual genes identified *a-priori* as involved in β-adrenergic receptor (β-AR) "signal switching"

Heat map (A) and graphs of individual gene counts (B-E) support the idea that isoproterenol stimulation of CD11b+ peripheral cells from Repeated Social Defeat (RSD) mice may promote gene transcription consistent with activation of canonical β-adrenergic receptor (β-AR) signaling (A, B, C).

A. Heat map of individual genes identified *a-priori* as involved in β-AR "signal switching" support the idea that isoproterenol stimulation of CD11b+ peripheral cells from Repeated Social Defeat (RSD) mice may promote gene transcription consistent with activation of canonical β-adrenergic receptor (β-AR) signaling. Individual gene z-scores depicted in the heat map (see legend) are based on raw counts data for each individual gene, normalized across all genes displayed. All genes displayed were significantly differentially regulated using $p < .05$ (DESeq2), not adjusted for multiple comparisons. Each column represents an individual mouse. RSD_I = RSD_ISO group (N = 2 mice/samples); CON_R = CON_RPMI group (N = 3 mice/samples). CREB Signaling = genes involved in cAMP response element binding protein (CREB) signaling; MAPK Signaling = genes involved in mitogen-activated protein kinase (MAPK) signaling; NF-κB Signaling = genes identified in nuclear factor – kappaBeta (NF-κB) pro-inflammatory signaling; AP-1 Signaling = genes involved in activator protein-1 (AP-1) pro-inflammatory signaling.

(B-E). Graphs of individual gene counts for particular genes of interest identified a-priori as involved in β-AR "signal switching." *Notes:* CON_R = Control_RPMI group, RSD_R = RSD_RPMI group, RSD_I = RSD_ISO group. All cells were CD11b+, representing mouse monocytes and granulocytes. $*p_{adj} < .05$, $^{\Phi} p < .05$ (not adjusted for multiple comparisons), n/s = not significant, n/a = statistical comparison was not possible due to N = 1 sample in the RSD group. Individual points (squares, circles, triangles) in each column represent raw gene counts from an individual mouse (one point = one sample).

B. Although differential gene expression analysis was unable to be performed due to missing data in the RSD group, the available counts were consistent with a lower expression of Adrb1 in both untreated CD11b+ peripheral cells from RSD mice (RSD_R) vs. untreated CD11b+ peripheral cells from control mice (CON_R) and isoproterenol stimulated CD11b+ peripheral cells from RSD mice (RSD_I) vs. untreated CD11b+ peripheral cells from control mice (CON_R). This may be consistent with activation of canonical β-adrenergic signaling pathways.

C. Although there was no significant difference in gene expression of Adrb2, the gene encoding the β2-adrenergic receptor, among untreated CD11b+ peripheral cells from RSD mice (RSD_R) compared to untreated CD11b+ peripheral cells from control mice (CON_R), gene expression of Adrb2 was significantly lower in isoproterenol stimulated CD11b+ peripheral cells from RSD mice (RSD_I) compared to untreated CD11b+ peripheral cells from control mice (CON_R) using a p-value < .05 NOT adjusted for multiple comparisons ($^{\Phi}$). This is consistent with activation of canonical β-adrenergic signaling pathways because we also observed a significant downregulation in the Adrb2 gene among CD11b+ splenocytes from control mice stimulated with isoproterenol (CON_I vs CON_R in CD11b+ splenocytes; Figure 18B).

D. There was no significant difference in gene expression of Arrb1, the gene encoding the β-arrestin-1 protein, among untreated CD11b+ peripheral cells from RSD mice (RSD_R) compared to untreated CD11b+ peripheral cells from control mice (CON_R), nor between isoproterenol stimulated CD11b+ peripheral cells from RSD mice (RSD_I) compared to untreated CD11b+ peripheral cells from control mice (CON_R). β-arrestin-1 is a key signaling protein involved in canonical β-adrenergic receptor signaling.

E. RSD resulted in a significantly greater expression of Arrb2, the gene encoding the β-arrestin-2 protein, among untreated CD11b+ peripheral cells (RSD_R) using a p-value < .05 NOT adjusted for multiple comparisons ($^{\Phi}$), compared to untreated CD11b+ peripheral cells from control mice (CON_R), but not between isoproterenol stimulated CD11b+ peripheral cells (RSD_I) compared to untreated CD11b+ peripheral cells from control mice (CON_R). β-arrestin-2 is a key signaling protein involved in non-canonical β-adrenergic receptor signaling.

Isoproterenol stimulation of peripheral cells from RSD may promote gene transcription consistent with canonical β-adrenergic signaling in the context of evidence for a molecular scaffolding involving signaling through non-canonical β-adrenergic signaling pathways

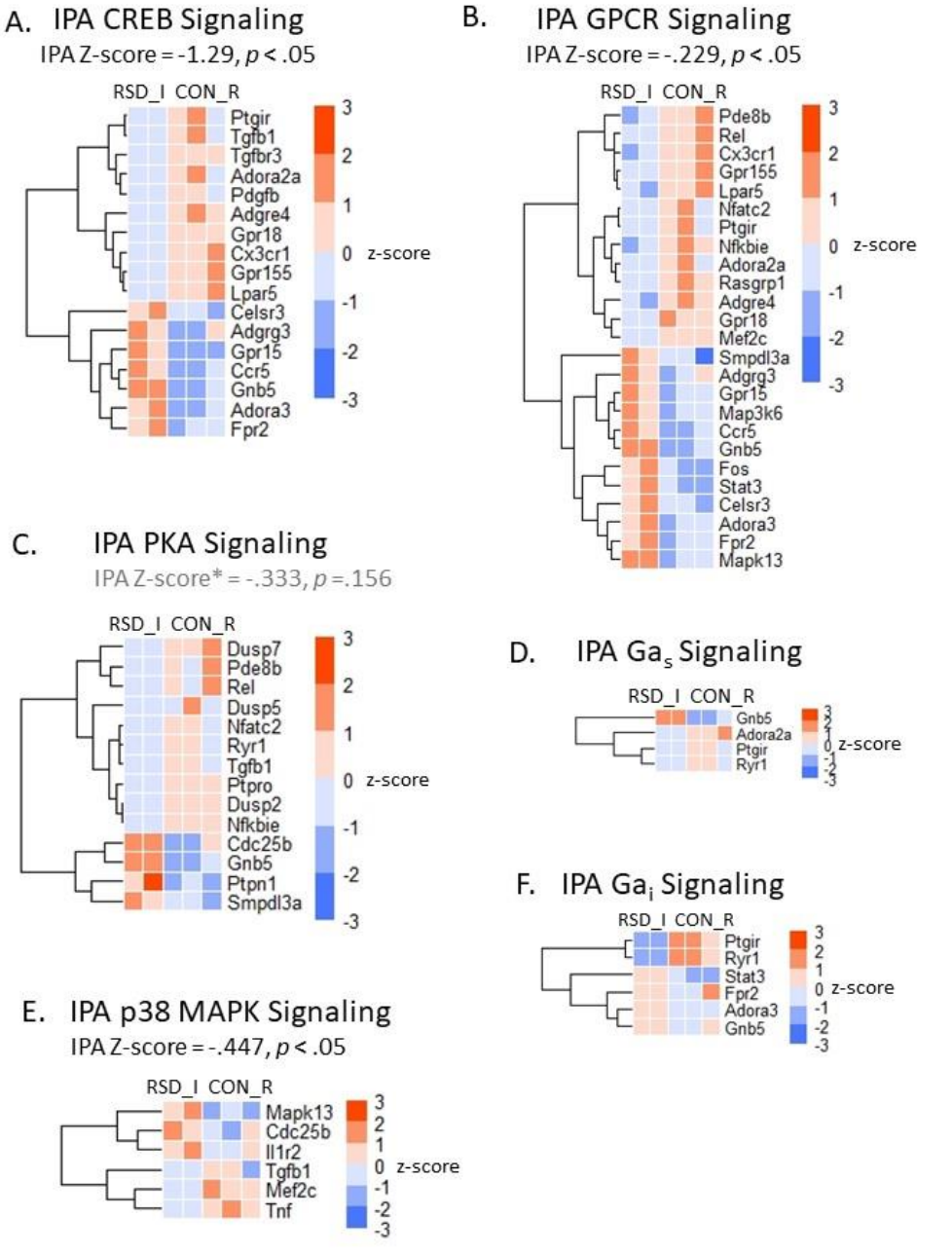


Figure 36. Stress + Stimulation Effect: Peripheral Cells – Individual genes identified in IPA Canonical Pathway analyses, protein kinase-A (PKA), cAMP response element binding (CREB) protein, mitogen-activated protein kinase (MAPK), and G-protein coupled receptor

Heat maps of individual genes identified in IPA Canonical Pathway analyses (A-F) suggest that isoproterenol stimulation of CD11b+ peripheral cells from Repeated Social Defeat (RSD) mice may promote gene transcription consistent with a shift toward canonical β-adrenergic signaling (A, B, C) in the context of molecular scaffolding for signaling through non-canonical β-adrenergic signaling pathways (E).

Notes: Individual gene z-scores depicted in the heat maps (see legends) are based on raw counts data for each individual gene, normalized across all genes displayed in each group. All genes displayed were significantly differentially regulated using $p_{adj} < .05$ (DESeq2). Adjustment for multiple comparisons was performed using the procedure of Benjamini and Hochberg (Love et al., 2014). RSD = RSD_RPMI group (N = 2 mice/samples); CON = Control_RPMI group (N = 3 mice/samples). Each column represents an individual mouse. Cluster analysis is based on similarity in individual gene z-score distribution pattern within each group.

- **A.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in cAMP response element binding (CREB) protein signaling pathways. The activation score ($Z = -1.29$, $p < .05$) indicates an overall significant downregulation in CREB-related genes among isoproterenol stimulated CD11b+ peripheral cells from RSD mice (RSD_I) compared to untreated CD11b+ cells from control mice (CON_R). This represents a slight attenuation in downregulation compared to unstimulated cells (i.e., RSD_R vs. CON_R comparison; Figure 9E, Figure 15A).
- **B.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in G-protein coupled receptor (GPCR) signaling pathways. The activation score ($Z = -.229$, $p < .05$) indicates an overall significant downregulation in GPCR-related signaling genes among isoproterenol stimulated CD11b+ cells from RSD mice (RSD_I) compared to untreated CD11b+ cells from control mice (CON_R). This represents an attenuation in downregulation compared to unstimulated cells (i.e., RSD_R vs. CON_R comparison; Figure 9E, Figure 15B).
- **C.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in protein kinase-A (PKA) signaling pathways. The activation score ($Z = -.333$, $p = .156$) indicates a non-significant downregulation in PKA-related genes among isoproterenol stimulated CD11b+ peripheral cells from RSD mice (RSD_I) compared to untreated CD11b+ cells from control mice (CON_R). IPA Z-score was calculated for this pathway for unstimulated cells (i.e., RSD_R vs. CON_R comparison; Figure 9E, Figure 15C).
- **D.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in G-protein-alpha-s ($G\alpha_s$) GPCR signaling pathways. G-protein alpha ($G\alpha$) is a subunit of the G-protein complex that dissociates from the GPCR after ligand binding. Subunit (s) indicates the subtype of G-protein alpha; Ga_s signaling is involved in canonical β-adrenergic receptor signaling. No IPA Z-score was calculated for this pathway, although a number of individual genes were identified. Similarly, no IPA Z-score was calculated for this pathway for unstimulated cells (i.e., RSD_R vs. CON_R comparison; Figure 9E, Figure 15D).
- **E.** Heat map of individual genes identified in IPA Canonical Pathway analyses involved in mitogen-activated protein kinase (MAPK) signaling pathways. The activation score ($Z = -.447$, $p < .05$) indicates an overall significant downregulation in p38 MAPK-related signaling genes among isoproterenol stimulated CD11b+ peripheral cells from RSD mice

(RSD_I) compared to untreated CD11b+ cells from control mice (CON_R). No IPA Z-score was calculated for this pathway for unstimulated cells (i.e., RSD_R vs. CON_R comparison; Figure 9E, Figure 15E).

F. Heat map of individual genes identified in IPA Canonical Pathway analyses involved in G-protein-alpha-i ($G\alpha_i$) GPCR signaling pathways. G-protein alpha ($G\alpha$) is a subunit of the G-protein complex that dissociates from the GPCR after ligand binding. Subunit (i) indicates the subtype of G-protein alpha; Ga_i signaling is involved in non-canonical β-adrenergic receptor signaling. No IPA Z-score was calculated for this pathway, although a number of individual genes were identified. There was an overall non-significant upregulation in Ga_i GPCR-related genes ($Z = 2.00$, $p = .09$) for unstimulated cells (i.e., RSD_R vs. CON_R comparison; Figure 9E, Figure 15F).

6.0 Discussion

Chronic social stress, in both mice and humans, associates with an upregulation in peripheral pro-inflammatory markers as well as an increase in peripheral pro-inflammatory monocytes that have cell surface markers indicative of early myeloid lineage (high expression of Ly6c in mice, low expression of CD16 in humans; Yang, Zhang et al., 2014). In a well-established murine model of chronic social stress, Sheridan and colleagues (Avitsur et al., 2002) show that RSD associates with behavioral and physiological consequences that have parallels in human models of chronic social stress (Walsh et al., 2021). This includes the development of anxiety-like behavior, social isolation, a decrease in grooming behavior, and physiological alterations in peripheral pro-inflammatory processes including an elevation in peripheral pro-inflammatory proteins (e.g., IL-6, TNF-α, and IL-1β) and an increase in immunologically primed and pro-inflammatory cells in peripheral circulation compared to controls. The RSD model also demonstrates alterations in stress hormones such as an increase in circulating levels of corticosterone, reflecting activation of the HPA-axis, and an increase in circulating levels of catecholamines, reflecting activation of the sympathetic nervous system. A growing body of literature supports the development of cellular glucocorticoid resistance in response to chronic activation of the HPA-axis in both the RSD model and among humans undergoing chronic social stress (see Walsh et al., 2021 for a review), which contributes to an increase in peripheral pro-inflammatory processes (Niraula, Wang, et al., 2018). However, alterations in HPA-axis signaling do not fully account for the chronic stress-related upregulation in peripheral inflammation in either human or murine models of chronic social stress (Walsh et al., 2021). Thus, recent attention has turned to the possible contribution of sympathetic nervous system signaling. In this regard, initial

evidence among humans and mice suggests that chronic social stress associates with prolonged downregulation in β-adrenergic receptor surface expression together with upregulation in peripheral pro-inflammatory phenotypes (see Walsh et al., 2021 for a review). One cellular model that may explain how decreased β-adrenergic receptor signaling may promote peripheral pro-inflammatory phenotypes is a molecular "switch" from canonical or typical β-adrenergic signaling through the β-adrenergic receptor (β-AR) and CREB-mediated gene transcription, to non-canonical β-AR signaling via pro-inflammatory MAPK signal transduction. More specifically, canonical β-AR signaling via CREB-mediated gene transcription can be anti-inflammatory (i.e., suppression of TNF-α) or pro-inflammatory (i.e., promotion of IL-6) depending on context (Elenkov et al., 2000), while non-canonical β-AR signaling via MAPK pathways is primarily pro-inflammatory (Bellinger and Lorton, 2018; Cargnello & Roux, 2011). In addition, non-canonical β-AR signaling has been shown to occur within intracellular compartments (i.e., endosomes) and in some cases independently from the β-AR itself (Carmona-Rosas et al., 2018; DeFea, 2008). While evidence for this molecular "switch" for β-AR signaling from canonical to non-canonical intracellular signaling pathways has been observed in murine models of pathogenic inflammation (e.g., rheumatoid arthritis; Lorton et al., 2013) as well as stably transfected cell lines (Shenoy et al., 2006), it has not been explored among models of chronic social stress. Thus, in this project we aimed to characterize β-AR-regulated intracellular signaling pathways within Ly6c+ peripheral monocytes from RSD mice compared to control mice. Generally, we hypothesized that we would find evidence consistent with β-AR "signal switching" among peripheral CD11b+ Ly6c+ cells from RSD mice. More specifically, we hypothesized that among peripheral CD11b+ Ly6c+ cells from RSD mice we would see diminished intracellular cAMP accumulation on stimulation with isoproterenol, greater intracellular β-arrestin-2 protein utilization vs β-arrestin-1, and an alteration

in differential gene transcription consistent with an upregulation in p38MAPK/ERK1/2 signaling and a downregulation in CREB-mediated gene transcription compared to control mice. This evidence would indicate a shift from canonical signaling through the β-AR and PKA-CREB pathways, to the use of non-canonical β-AR signaling pathways, including β-arrestin-2 mediated and/or β-AR independent signaling via pro-inflammatory MAPK signal transduction (Bellinger & Lorton, 2018; Carmona-Rosas et al., 2018; DeFea, 2008). As evidence that this shift in intracellular signaling may promote previously established pro-inflammatory phenotypes, we hypothesized that isoproterenol stimulation of peripheral CD11b+ Ly6c+ cells from RSD mice would exacerbate any differences in gene transcription observed among unstimulated cells, including greater upregulation in pro-inflammatory gene transcription and greater utilization of non-canonical β-AR signaling pathways among RSD mice compared to controls. Due to technical difficulties in obtaining enough CD11b+ Ly6c+ cells for our assays, we performed our experiments on CD11b+ cells from spleen and peripheral blood. Previous work by Sheridan and colleagues has shown that CD11b+ cells are a mixture of CD11b+ Ly6c+ monocytes and CD11b+ Ly6c- Ly6g+ granulocytes (2/3 granulocytes > 1/3 monocytes; Powell et al., 2013; Niraula, Witcher et al., 2018). We chose to examine CD11b+ cells isolated from both the spleen and peripheral blood given the greater ease in obtaining splenocytes, and prior work demonstrating that the spleen serves as an extramedullary source of primed and pro-inflammatory CD11b+ Ly6c+ monocytes in the RSD model (McKim et al., 2018). Prior work has shown that these primed and pro-inflammatory CD11b+ Ly6c+ monocytes are maintained in the spleen and can be released into peripheral circulation for up to 24 days following social threat (McKim et al., 2018; Wohleb et al., 2013, 2014). Notably, these primed cells can also traffic to the brain and play a role in anxiety behavior, social withdrawal, and alterations in memory as a consequence of RSD (Niraula, Witcher et al., 2018, DiSabato et al.,

2021). Thus, we applied our hypotheses for CD11b+ Ly6c+ monocytes from the periphery to CD11b+ cells from both the peripheral blood and spleen of RSD mice.

Findings from the current study provided partial support for our hypotheses. In line with our hypotheses, we found initial evidence indicating a downregulation in β-AR canonical signaling pathways together with an upregulation in non-canonical β-AR signaling through MAPK among CD11b+ cells isolated from both the spleen and peripheral blood of RSD mice compared to controls. Downregulation of the canonical signaling pathway was observed as a reduction in intracellular cAMP accumulation on stimulation with isoproterenol across both CD11b+ splenocytes and peripheral cells from RSD mice compared to control mice. Among CD11b+ splenocytes from RSD mice, we also observed a downregulation in gene transcription involved in β-AR canonical signaling (i.e., PKA and CREB signaling) together with an upregulation in gene transcription pathways involved in non-canonical β-AR signaling (i.e., p38MAPK signaling and G-protein-α-I coupled GPCR signaling) compared to control mice. Among CD11b+ peripheral cells from RSD mice, we similarly observed a downregulation in CREB signaling compared to controls. Consistent with a switch to non-canonical β-AR signaling, we found evidence for a downregulation in the gene encoding the β1-adrenergic receptor among CD11b+ splenocytes from RSD mice compared to controls and an upregulation in the gene encoding the β-arrestin-2 scaffolding protein in both CD11b+ splenocytes and peripheral cells from RSD mice compared to controls. Also, in line with our hypotheses and consistent with prior work (Powell et al, 2013; Niruala, Witcher, et al., 2018), we observed an upregulation in pro-inflammatory gene transcription among unstimulated CD11b+ splenocytes and peripheral cells from RSD mice compared to controls. To examine the effects of *ex vivo* isoproterenol stimulation on CD11b+ cells from RSD mice, we compared both stimulated and unstimulated CD11b+ cells from RSD mice to

unstimulated CD11b+ cells from control mice, which served as a baseline for gene expression. In this way, we were able to examine differences in differential gene transcription due to isoproterenol stimulation alone in CD11b+ cells from RSD mice. Using this strategy, we found that contrary to our hypotheses, there was an attenuation (rather than the expected exaggeration) in the upregulation of pro-inflammatory gene transcription among stimulated vs unstimulated CD11b+ splenocytes from RSD mice. Also contrary to our hypotheses, among isoproterenol stimulated vs unstimulated CD11b+ splenocytes from RSD mice, we observed evidence supporting an upregulation in canonical β-AR signaling (i.e., CREB-mediated gene transcription) together with an attenuation in non-canonical β-AR signaling (i.e., p38MAPK and G-protein-α-I coupled GPCR signaling). These findings suggest that canonical β-AR signaling may still be possible in the context of evidence for molecular scaffolding for (and likely some signaling through) non-canonical β-adrenergic signaling pathways. In addition, these findings suggest that *ex vivo* isoproterenol stimulation of CD11b+ splenocytes from RSD mice may attenuate pro-inflammatory signaling, at least at 10uM of agonist for 10 minutes. Interestingly, this latter finding is consistent with extant literature in the RSD model demonstrating an attenuation in *ex vivo* LPS-stimulated levels of TNF-α when incubated with norepinephrine compared to LPS stimulation without norepinephrine (Avitsur et al., 2005). Among CD11b+ cells isolated from the peripheral blood of RSD mice, findings were broadly consistent with those reported in splenocytes, although we were generally underpowered to make any definitive conclusions based on differential gene transcription in peripheral cells (n = 2 RSD mice in the final analyses). Thus, all together, we found that evidence for an upregulation in pro-inflammatory gene transcription among CD11b+ splenocytes and peripheral cells from RSD mice compared to controls may be related to hypothesized differences in intracellular β-AR signaling patterns, including a downregulation in

β-AR canonical signaling and an upregulation in β-AR non-canonical signaling in cells from RSD mice. The results were less clear when examining the cellular response to *ex vivo* isoproterenol stimulation, although initial evidence suggested that *ex vivo* stimulation with a β-AR agonist may promote β-AR canonical signaling and downregulate β-AR non-canonical signaling.

6.1 Findings parallel extant RSD literature

Our results for differential gene expression patterns in CD11b+ splenocytes and peripheral cells from RSD mice were similar to those previously reported in the RSD literature, including an upregulation in pro-inflammatory signaling, an upregulation in myeloid function, and a downregulation in glucocorticoid signaling (Powell et al., 2013; Niraula, Witcher et al., 2018). Specifically, we observed an RSD-related upregulation in patterns of pro-inflammatory gene transcription in CD11b+ splenocytes that is consistent with prior reports (Powell et al., 2013) including upregulation in IL-6 signaling, upregulation in toll-like receptor signaling (important in pathogen pattern recognition and downstream activation of immune response), and upregulation in pro-inflammatory signaling mediated by the transcription factor NFkB. Also consistent with prior literature (Powell et al., 2013; Niraula, Witcher et al., 2018), we observed an enrichment in pro-inflammatory gene transcription mediated by chemokine and cytokine signaling, and an upregulation in markers of myeloid lineage and myeloid lineage effector functions (e.g., cytokines and their receptors, prostaglandin synthesis, reactive oxygen species-related molecules, pathogen pattern recognition receptors and associated signaling molecules, Ig receptors, and mediators of ECM remodeling) among both CD11b+ splenocytes and peripheral cells from RSD mice compared to controls. Among cell surface markers indicative of myeloid lineage, we observed an

upregulation in the *Ly6c* and *Ly6g* genes, which encode cell surface markers for monocytes and granulocytes respectively. Notably, we observed a relative ratio of *Ly6c* to *Ly6g* differential gene expression within both CD11b+ splenocytes and peripheral cells that was similar to the ratio of Lyc6+ to Ly6g+ cells previously reported in Flow cytometry experiments for the RSD model (Powell et al., 2013; Niraula, Witcher et al., 2018). In addition, in both CD11b+ splenocytes and peripheral cells from RSD mice there was a significant upregulation in the *Fkbp5* gene, which encodes a protein that inhibits the glucocorticoid receptor from translocating to the nucleus and thus contributes to known resistance of these cells to glucocorticoids (Avitsur, Stark et al., 2001, 2002; Quan et al., 2003; Pirkl & Buchner, 2001; Sinars et al., 2003; Stark, Avitsur et al., 2001, 2002; Niraula, Witcher et al., 2018). Notably, there was no significant difference in expression of the GR gene (*Nr3c1*) in either CD11b+ splenocytes or peripheral cells, which has previously served as a negative control for both gene expression and protein levels in RSD mice (Powell et al., 2013, Bailey 2009, Engler 2008; Quan 2003). Thus, across differential gene expression patterns in CD11b+ splenocytes and peripheral cells from RSD mice compared to Controls we found evidence for cellular markers and processes that are consistent with those previously reported in RSD mice, including an upregulation in pro-inflammatory gene transcription, an upregulation in markers of myeloid lineage and myeloid lineage effector functions, and the development of glucocorticoid resistance.

6.2 Novel findings suggest a downregulation in canonical β-AR signaling and use of non-canonical β-AR signaling pathways among RSD mice

For the first-time in RSD mice, we show evidence consistent with a proposed molecular "switch" from canonical β-AR signaling pathways through cAMP, protein kinase-A (PKA), and CREB-mediated gene transcription, to a non-canonical β-AR signaling pathway through β-arrestin-2 and pro-inflammatory MAPK signal transduction. First, we present evidence among CD11b+ splenocytes from RSD mice that there was a significant downregulation in the gene encoding the β1-adrenergic receptor (*Adrb1*). Although differential gene expression was unable to be performed among CD11b+ peripheral cells due to missing data, the available counts showed a similar pattern to that observed in splenocytes. Downregulation in β-AR surface expression is consistent with the known internalization and degradation of the β-AR in the context of prolonged sympathetic nervous system signaling (Bellinger & Lorton, 2018; Elenkov et al., 2000) and has been previously reported among human models of chronic stress (Dimsdale et al., 1994; Mausbach et al., 2007; Mills et al., 2004). Among murine models of chronic stress, studies of repeated restraint stress have reported no difference in density of β-ARs on splenocytes and lymphocytes from peripheral blood after 5-7 days of stress exposure (Kubera et al., 1992; Li et al., 2018); however, restraint stress lacks the behavioral and physiological parallels to human chronic stress that are recapitulated in RSD. Here, we report initial evidence that RSD is associated with downregulation of the gene encoding the β1-AR, consistent with a downregulation in canonical β-AR signaling. This appears specific to the β1-adrenergic receptor, as the gene encoding the β2-adrenergic receptor (*Adrb2*) was not significantly different between RSD mice and controls.

Other evidence consistent with an RSD-related downregulation in canonical β-adrenergic signaling included significantly lower cAMP accumulation on stimulation with isoproterenol

among both splenocytes and CD11b+ peripheral cells from RSD mice. The production of cAMP is directly related to activation of the β-AR, given that the dissociation of the G-protein-α from the β-AR results in activation of adenylate cyclase (AC), which then catalyzes the conversion of ATP to cAMP within the cell (Figure 1). Thus, reduced cAMP on stimulation of the β-AR in cells from RSD mice is consistent with a downregulation of the β-AR on the cell surface and/or a switch away from intracellular signaling through this pathway. This downregulation in cAMP on stimulation of the β-AR has been previously reported among peripheral blood mononuclear cells from humans experiencing the chronic social stress of caregiving (Mausbach et al., 2007; Mills et al., 2004), as well as among splenocytes in a murine model of rheumatoid arthritis (Lorton et al., 2013). However, this is the first-time reduced cAMP on stimulation with a β-adrenergic agonist has been reported in the murine model of RSD. Thus, we present novel evidence for a downregulation in differential gene transcription of the gene encoding the β1-AR among CD11b+ splenocytes, together with reduced cAMP on stimulation of the β-AR in splenocytes and CD11b+ peripheral cells from RSD mice compared to controls.

Strengthening the case for decreased canonical β-AR signaling pathways among CD11b+ cells, we report a downregulation in PKA-mediated signaling pathways among CD11b+ splenocytes and a downregulation in CREB-mediated gene transcription among both CD11b+ splenocytes and peripheral cells from RSD mice compared to controls. Within the canonical β-AR signaling pathway, cAMP stimulates PKA, which activates CREB-mediated gene transcription through phosphorylation of CRE. Interestingly, PKA is also involved in phosphorylation of the β-AR itself, which marks the β-AR for internalization, reduces its affinity for G-protein-α-S (indicative of canonical signaling), and increases its affinity for G-protein-α-I, which is indicative of a "switch" to non-canonical signaling pathways. Thus, to our knowledge, we provide the first

evidence for chronic stress-induced downregulation in PKA-mediated signaling. We also observed an RSD-related downregulation in CREB-mediated signaling pathways among both CD11b+ splenocytes and peripheral cells from RSD mice. This is inconsistent with previously reported upregulation in CREB-related genes among CD11b+ splenocytes in response to RSD (e.g., *Creb3*: Powell et al., 2013), but is consistent with a downregulation in CREB-related gene transcription previously reported among CD11b+ peripheral cells (Powell et al, 2013); however, another paper found no difference in expression of CREB-related genes among peripheral CD11b+Lyc6+ cells (Niraula, Witcher et al., 2018). Differences across study samples may be attributable to variability in methods used to evaluate gene transcription (TELiS versus individual genes: Powell et al, 2013; Nanostring: Niraula, Witcher et al., 2018) and/or differences in cell subtypes. A strength of the current study was consistent analysis across cell subtypes, with sequencing together in the same Illumina sequencing batch. Thus, among evidence consistent with a downregulation in canonical β-AR signaling, we report novel evidence of a downregulation in PKA-mediated signaling pathways among CD11b+ splenocytes, and a downregulation in CREB-mediated gene transcription among both CD11b+ splenocytes and peripheral cells, together with evidence supporting downregulation in the gene encoding the β1-AR among CD11b+ splenocytes and reduced cAMP on stimulation of the β-AR in splenocytes and CD11b+ peripheral cells from RSD mice compared to controls.

In addition to evidence for a downregulation in canonical β-AR signaling, we also report novel evidence consistent with a proposed molecular "switch" to non-canonical β-AR signaling through β-arrestin-2 and pro-inflammatory MAPK-mediated gene transcription. Specifically, we found evidence for an upregulation in the gene encoding the scaffolding protein β-arrestin-2 (*Arrb2*), but not β-arrestin-1 (*Arrb1*), among CD11b+ splenocytes from RSD mice compared to

controls. While we demonstrated the presence of β-arrestin-1 and β-arrestin-2 proteins among splenocytes using Western Blot, we did not attempt to detect these proteins in our samples of interest due to low overall protein yield. Among CD11b+ peripheral cells, we also observed an upregulation in the gene encoding β-arrestin-2 (*Arrb2*), but not β-arrestin-1 (*Arrb1*), when differential gene expression was examined using a *p*-value that was uncorrected for multiple comparisons. Prior studies have shown that both the β1-AR and the β2-AR can utilize β-arrestin proteins 1 and 2 to scaffold the binding of a MAPK signaling complex within endosomes after stimulation and subsequent internalization of the β-adrenergic receptor (DeFea, 2008; Martin et al., 2004; Steinberg, 2018). Indeed, this appears to occur in a time-dependent manner in which early after β-AR internalization (0-5 min), the β-AR may continue to use G-protein-α-S and cAMP-PKA signaling while also scaffolding β-arrestin-1 or 2 and other MAPK signaling proteins (Carmona-Rosas et al., 2018; DeFea, 2008). At later time points after β-AR internalization (5-60min), the β-AR couples with G-protein-α-I and/or β-arrestin-1 or 2 to mediate downstream MAPK signaling (Carmona-Rosas et al., 2018; DeFea, 2008). Our findings in RSD mice suggest that compared to CD11b+ splenocytes from control mice, there may be an upregulation in the use of the scaffolding protein β-arrestin-2 within CD11b+ splenocytes after 6 days of social stress. In addition to an upregulation in β-arrestin-2, we also report evidence for significant activation of gene transcription pathways utilizing G-protein-α-I ($G\alpha_i$) in GPCR signaling among CD11b+ splenocytes from RSD mice. While this pathway was also identified as activated among CD11b+ peripheral cells, it was not statistically significant. Thus, together with evidence for an upregulation in the gene encoding the β-arrestin-2 protein, activation of gene transcription pathways utilizing Ga_i in GPCR signaling within CD11b+ splenocytes from RSD mice is consistent with an association of the β-AR with $G\alpha_i$ and β-arrestin-2 to mediate downstream

MAPK signaling. Indeed, among CD11b+ splenocytes from RSD mice, we found evidence for significant activation of gene transcription pathways involved in p38 MAPK signaling. While a broad upregulation of this pathway was not found in CD11b+ peripheral cells from RSD mice, there were some individual genes involved in MAPK signaling that were upregulated when differential gene transcription was examined using a non-adjusted *p*-value ($p < .05$; *Mapk13*, *Cdc25b*). Our findings are in contrast to one prior study that reported downregulation in MAPK signaling in response to RSD (Niraula, Witcher et al., 2018); however, this study was not designed to look at this pathway, and reported downregulation in the *Mapk8* gene only among CD11b+Ly6c+ peripheral cells from RSD mice compared to control mice (Niraula, Witcher et al., 2018). Thus, our evidence for an upregulation in p38 MAPK signaling detected using comprehensive bioinformatic pathway analysis among CD11b+ splenocytes from RSD mice provides stronger evidence than prior findings. In sum, together with evidence for a downregulation in canonical β-AR signaling, we report novel evidence suggesting the use of non-canonical β-AR signaling among CD11b+ splenocytes from RSD mice, including the potential scaffolding of β-arrestin-2 and $G\alpha_i$, and an upregulation in pro-inflammatory MAPK-mediated gene transcription.

6.3 *Ex vivo* isoproterenol stimulation of cells from RSD mice results in a possible attenuation in pro-inflammatory gene transcription, upregulation in canonical β-AR signaling, and attenuation in non-canonical β-AR signaling.

While we initially hypothesized that isoproterenol stimulation of CD11b+ cells from RSD mice would exacerbate any differences in gene transcription observed among unstimulated cells

(i.e., greater upregulation in pro-inflammatory gene transcription and greater utilization of non-canonical signaling pathways among RSD mice), our findings did not support this hypothesis. In order to examine differences in differential gene transcription due to isoproterenol stimulation alone in cells from RSD mice, we compared both isoproterenol stimulated and unstimulated CD11b+ cells from RSD mice to unstimulated CD11b+ cells from control mice as a baseline for gene expression. Using this strategy, we found that, contrary to our hypotheses, isoproterenol stimulation of CD11b+ cells from RSD mice may attenuate pro-inflammatory gene transcription, upregulate canonical β-AR signaling, and attenuate non-canonical β-AR signaling compared to CD11b+ cells from RSD mice that are not stimulated. More specifically, among pro-inflammatory pathways, we found an attenuation in gene transcription pathways involved in IL-6 signaling, toll-like receptor signaling, and NFkB pro-inflammatory signaling among stimulated CD11b+ splenocytes from RSD mice compared to unstimulated cells. Interestingly, there was specific evidence for downregulation in the gene encoding the pro-inflammatory cytokine TNF-α (*Tnf*) among both stimulated CD11b+ splenocytes and peripheral cells from RSD mice, which was NOT observed among unstimulated cells from RSD mice. This observation is consistent with previously reported findings in which CD11b+ splenocytes from RSD and control mice were stimulated *ex vivo* with norepinephrine and LPS and lower levels of TNF-α were produced compared to LPS stimulation in the absence of norepinephrine (Avitsur et al., 2005). However, our findings are novel in that we report a downregulation in *Tnf* in the absence of an immune stimulant. Among other observations due to *ex vivo* isoproterenol stimulation, we found evidence consistent with an upregulation in canonical β-AR signaling. Specifically, there was a significant upregulation in CREB-mediated gene transcription among stimulated CD11b+ splenocytes from RSD mice compared to a significant downregulation in CREB-mediated gene transcription among

unstimulated CD11b+ splenocytes from RSD mice. Among stimulated CD11b+ peripheral cells from RSD mice, we observed a slight attenuation in the downregulation of CREB-mediated gene transcription observed in unstimulated CD11b+ peripheral cells from RSD mice. These findings together provide evidence consistent with an upregulation in canonical β-AR signaling on stimulation with 10uM isoproterenol. In addition to evidence consistent with an upregulation in canonical β-AR signaling, we observed evidence for a possible attenuation in non-canonical β-AR signaling. In particular, among stimulated CD11b+ splenocytes from RSD mice there was an attenuation in the activation of gene transcription pathways involved in Ga_i GPCR and p38MAPK signaling compared to unstimulated CD11b+ splenocytes from RSD mice. Interestingly, the magnitude of fold change for the gene encoding the β-arrestin-2 protein was similar between stimulated CD11b+ splenocytes from RSD mice and unstimulated cells, suggesting that the molecular scaffolding that couples Ga_i GPCR and p38MAPK signaling within the cell was not different after 10 minutes of isoproterenol stimulation. Thus, it may be that the molecular scaffolding for non-canonical β-adrenergic receptor signaling is unchanged by isoproterenol stimulation for 10 min (i.e., β-arrestin-2 scaffolds the association of the β-AR, Ga_i, and downstream MAPK signaling proteins in the endosome), while relative signaling through the endosomal complex of β-AR, Ga_i, and MAPK signaling proteins may decrease. Thus, although evidence is preliminary, we found that among isoproterenol stimulated CD11b+ splenocytes from RSD mice, there was an attenuation in pro-inflammatory gene transcription, an upregulation in canonical CREB-mediated gene transcription, and a possible attenuation in the upregulation of Ga_i GPCR and p38MAPK related gene transcription compared to unstimulated cells, with no difference in expression of the β-arrestin-2 gene. Together, this evidence suggests that stimulation of CD11b+ splenocytes from RSD mice with 10uM isoproterenol for 10 minutes may attenuate

pro-inflammatory gene transcription, upregulate canonical β-AR signaling, and attenuate signaling through non-canonical β-AR pathways.

6.4 Novel evidence supports an increase in an acute phase protein among RSD mice

In exploratory analyses, we found evidence for an upregulation in a marker of acute phase activation, serum-amyloid protein-P (SAP), in the peripheral circulation of RSD mice. Specifically, we extend existing evidence showing increases in peripheral levels of IL-6 in response to RSD (e.g., Stark et al, 2002; Hanke et al., 2012; Niruala, Wang, et al., 2018) to demonstrate increased levels of SAP. Elevation in an acute phase marker of inflammation in the model of RSD is indicative of longer-term inflammatory processes than elevation in IL-6. This is of specific interest in the RSD model because peripheral levels of IL-6 are only moderately correlated with spleen weight, another indicator of the stress effect, and IL-6 is an immediate early release inflammatory response gene that can be activated rapidly in response to acute stress (Gruys, et al., 2005; Janeway et al., 2017; Naugler & Karin, 2008). In particular, the process of removing mice from their housing in order to sacrifice them can be acutely stressful and may contribute to elevated levels measured in the plasma of these mice. In addition, recent work has demonstrated that many of the physiological consequences of RSD are independent of IL-6, including stress-related increases in percentages of CD11b+ cells in the periphery and bone marrow as well as an upregulation in pro-inflammatory gene transcription among these cells (Niraula, Witcher et al., 2018). Interestingly, our study found that levels of SAP were highly correlated with measured levels of IL-6 ($r = .92$), and both IL-6 and SAP demonstrated moderate correlations with spleen weight (IL-6: $r = .64$; SAP: $r = .51$). In making translational comparisons with humans, there is a

low to moderate correlation (r =.3-.5) between peripheral levels of IL-6 and CRP, the human homologue of SAP (Il'yasova et al., 2005; Walsh et al., 2019). Despite this, we demonstrate novel evidence for a social-stress related elevation in SAP, an acute phase protein, which may be of interest because it provides a more reliable marker of chronic inflammation than other more transient proinflammatory mediators.

6.5 Limitations

Although we present a number of novel findings, they are not without limitations. Most saliently, across all results presented, there was a low number of experimental replicates. That is, even among experiments with N = 3 technical replicates and N = 6 biological replicates (i.e., mice), each experiment presented was only performed in one cohort of mice. Thus, the results presented here should be considered preliminary and replicated to confirm findings. In addition, among experiments using differential gene expression, we report findings using the minimum number of replicates (N=1 technical replicate, N=3 biological replicates) in each of the RSD and control groups, and a subthreshold number of mice (N=2) in the RSD group for peripheral cells. For peripheral cells, this resulted in an underpowered analysis and thus results for differential gene transcription among peripheral cells should be interpreted with caution. Furthermore, when we examined the treatment effects for both splenocytes and peripheral cells (i.e., isoproterenol and forskolin stimulation), there was greater variation in differential gene transcription between biological replicates (mice) than between treatment conditions. This low variation in differential gene transcription between treatment conditions may indicate that a 10-minute stimulation was not long enough to detect downstream differences in gene transcription. Indeed, the 10-minute

stimulation time frame was chosen to match the cAMP assay, which measures protein accumulation upstream of a number of intracellular processes prior to alterations in gene transcription. This low variation in differential gene transcription also contributed to lack of statistical power to reliably differentiate signaling pathways in and between forskolin and isoproterenol stimulated cells, even when examining *p*-values unadjusted for multiple comparisons. Although we were able to discern some differences in differential gene transcription between isoproterenol stimulated CD11b+ splenocytes from RSD mice compared to unstimulated CD11b+ splenocytes from RSD mice, we would have greater confidence in these findings if we were able to confirm the positive and negative stimulation controls for our treatment effect. Another major limitation to the presented findings includes a lack of *ex vivo* or *in vivo* pharmacological blockade or knock out model(s), which would help establish a causal relationship between chronic social stress and the intracellular and gene-transcription processes of interest. In this regard, we initially planned to perform *ex vivo* assays in which we would pharmacologically block intracellular signaling via the β-AR canonical pathway (using the AC inhibitor SQ22536, Sigma #S153) or the β-AR non-canonical pathway (using the ERK1/2 antagonist U0126; Sigma #662005), and measure the effect on both upstream signaling molecules (e.g., cAMP) and downstream differential gene transcription. However, due to time and resource constraints, we were unable to perform these assays; thus, future work should include these pharmacological blockade studies to help establish causality. Another limitation of this study is the assumption that observed alterations in β-AR expression and intracellular signaling patterns occur in the bone marrow during differentiation and development of the myeloid cell. Our current methods focus on CD11b+ cells harvested immediately after the termination of the last cycle of RSD, during which catecholamines have been shown to be elevated in the peripheral circulation of RSD mice (i.e., for

up to three hours after the end of the last RSD cycle; Hanke et al., 2012). Thus, it is possible that the current findings, including downregulation in β1-AR gene transcription and/or a shift away from intracellular β-AR canonical signaling toward β-AR non-canonical signaling, may be related to elevated levels of peripheral catecholamines rather than differential release of specific pre-programmed cell subtypes from the bone marrow. Thus, future experiments should examine whether observed differences in β-AR intracellular signaling patterns are developmentally programmed in the bone marrow or are a product of RSD-related activation of the sympathetic nervous system by examining time points greater than three hours to several days after the end of the last cycle of RSD. A final limitation is the inclusion of only male mice in these experiments, thus limiting the generalizability of the findings to chronic social stress in males and not females. Thus, future experiments should focus on replicating the results in female mice subjected to RSD (e.g., Yin et al., 2019).

6.6 Conclusions / Implications / Future Directions

This project aimed to expand our understanding of peripheral CD11b+ cells from RSD mice, a murine model of chronic social stress that has behavioral and physiological parallels to human chronic social stress. We aimed to gain greater understanding of intracellular β-AR signaling pathways in order to contribute to knowledge of how chronic social stress may promote pro-inflammatory states, and thus risk for CVD and other diseases with inflammatory pathophysiology. We present evidence for an upregulation in pro-inflammatory gene transcription among CD11b+ cells from stressed mice compared to controls, together with novel evidence for a downregulation in β1-AR, but not β2-AR, gene transcription and a decrease in cAMP

accumulation on stimulation with isoproterenol. These findings suggest a desensitization of peripheral monocytes and granulocytes to adrenergic signaling in response to chronic social stress, and parallel observations in humans undergoing chronic social stress that involve an interpersonal component (e.g., caregiving for a loved one who cannot effectively care for themselves). To explain how a downregulation in cellular sensitivity to adrenergic signaling may relate to an upregulation in pro-inflammatory signaling, we present novel evidence for alterations in intracellular β-AR signaling patterns. Specifically, we found a downregulation in canonical or typical β-AR signaling pathways through cAMP, PKA, and CREB-mediated gene transcription, together with an upregulation in non-canonical, or alternative, β-AR signaling pathways utilizing β-arrestin-2 to scaffold downstream MAPK signaling proteins. These findings may explain an upregulation in pro-inflammatory signaling together with decreased cellular sensitivity to adrenergic signaling in that signaling via canonical β-AR pathways is dependent on surface expression of the β-AR and can result in pro-inflammatory or anti-inflammatory signaling based on context, while an upregulation in non-canonical β-AR signaling is independent of β-AR surface expression and primarily promotes pro-inflammatory signaling. Thus, a downregulation in β-AR surface expression may decrease acute sensitivity to prolonged or repeated sympathetic nervous system signaling in the context of chronic stress, while β-AR intracellular scaffolding that can promote a primed and pro-inflammatory immune response with less energy input by the cell (i.e., in response to SNS signaling) may be adaptive in the context of chronic stress when energy resources are low. A broad pro-inflammatory phenotype in the periphery may also serve an important physiological function in providing a negative feedback signal at the level of the brain to decrease SNS-related signaling, and to promote social withdrawal and general “sickness behavior” that preserves overall energy resources.

We also present preliminary evidence that *ex vivo* adrenergic stimulation of CD11b+ cells from stressed mice results in activation of canonical β-AR signaling, including CREB-mediated gene transcription, and a downregulation in some pro-inflammatory gene transcription such as the gene encoding the cytokine Tnf-α. We also present evidence that molecular scaffolding for the non-canonical β-AR pathway may be unchanged after *ex vivo* adrenergic stimulation, although active signaling through non-canonical β-AR signaling pathways is somewhat attenuated. These findings may suggest a difference in response to acute vs chronic SNS signaling as may occur in response to acute vs chronic social threat. However, prior work in the RSD model suggests that plasma norepinephrine levels immediately after the 6th cycle of RSD are ~300 times lower than the concentration we used in our stimulation assay (Hanke et al., 2012). Thus, it may be that our findings from *ex vivo* adrenergic stimulation reflect a cellular response to supraphysiological concentration of β-adrenergic agonist. Therefore, future work should investigate the parameters around which differential use of β-adrenergic signaling pathways may occur, such as the timing and/or the dependence on agonist concentration.

Understanding sympathetic nervous system-related intracellular signaling pathways underlying the pro-inflammatory phenotype of peripheral CD11b+ cells in a murine model of chronic social stress is important. Indeed, greater knowledge of these pathways aids in revealing new targets for study of psychosocial processes that may contribute to inflammatory disease risk, as well as the development of targeted interventions to prevent risk for CVD and other diseases with inflammatory pathophysiology known to accompany chronic social stress. Identifying modifiable targets early on in the signaling process may provide aims for future investigations that can decrease the impact of exposure to chronic social stress on risk for chronic diseases of aging. Thus, future work should focus on replicating and refining our results, both in models of chronic

social stress in mice and in translating these findings to an understanding of pathways of relevance to health in humans.

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