The Sympathetic Nervous System Mediates Disease Progression in *C. difficile* Infection

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Abstract

Clostridioides difficile infection (CDI) is a leading cause of hospital-acquired infections in the United States, known for triggering severe disease by hyperactivation of the host response. In this study, we determine the impact of the sympathetic nervous system (SNS) on CDI disease severity. Mouse models of CDI are administered inhibitors of SNS activity prior to CDI. Chemical sympathectomy or pharmacological inhibition of norepinephrine synthesis greatly reduces mortality and disease severity in the CDI model. Pharmacological blockade or genetic ablation of the alpha 2 adrenergic receptor ameliorates intestinal inflammation, disease severity, and mortality rate. These results underscore the role of the SNS and the alpha 2 adrenergic receptor in CDI pathogenesis and suggest that targeting neural systems could be a promising approach to therapy in severe disease.

Keywords: Clostridioides difficile; adrenergic receptors; colitis; norepinephrine; sympathetic nervous system.

Chapter 1 - A Very Brief History of Stress and Infection

The sympathetic nervous system (SNS) is popularly recognized as the body's system governing the "flight or fight" response to stressful stimuli. A less well popularized but emerging function of the SNS is its role in coordinating defenses against environmental threats. Stress responses have been repeatedly tied to non-infectious and infectious diseases contexts in clinical patients and models. Recently, a variety of neuroimmune interactions have been uncovered highlighting the extensive reach and variety of responses coordinated by neuronal systems. This chapter will briefly touch on the earliest signs that stress systems modulate infection risk and disease outcome.

The connection between psychological stress and susceptibility to infection has been well appreciated for a long time. It was recognized as early as the turn of the 20th century that stress was a general modulator of disease and could be experimentally linked to infectious susceptibility. Long before rigorous studies in humans worked this idea, studies in animal models pioneered the connection between stress and infection outcome. A number of studies in rodents and rabbits demonstrated that with viral, bacterial, and parasitic infections, prior subjection to stress in the form of forced exercise, social stress, cold exposure, crowding, and restraint generally led to an increased incidence of mortality in the animal model.[1] Even then, there were noticeable nuances in that conclusion as stress seemed to protect against some infectious agents and some studies showing the result depends on the type of stressor and features of the host (e.g. sex). Although the link between stress and infection hasn't been a straight path, the goal here is to highlight some of the major steps along the way.

The first *human* studies in the 1950 and 1960s building the connection between stress and infection were retrospective ones, associating the incidence of traumatic life events with that of infection based on self-report. A seminal example are the collections of studies by the group of Thomas Holmes which found an association in tuberculosis infection risk in individuals experiencing hardships such as death of a family member and alcoholism.[2] This was set upon a backdrop of other studies showing an the association between life experiences and general illness.[3] The earliest of studies quickly started to realize the connection between stress and infection in different infectious contexts and began incorporating the observed effects of stress on the immune system (mostly immunosuppressive) in the paradigm.[4,5] A compendium of following studies progressed from this point through more controlled, predominantly prospective studies where subjects were exposed to various viruses.[1,6] The conclusion from many of these studies were that individuals with more reported stressful experiences were more likely to react more severely to infectious challenges. Through time, this association became more generalized including different types of infectious challenge (e.g. response to vaccine) and more aspects of the psychosocial spectrum contributing to disease susceptibility.[7] To the latter, studies

demonstrate that personality differences and "power motivation", an individual's drive to gain power, correlate both with higher levels of norepinephrine/epinephrine and vulnerability to infection.[8] Ensuing studies demonstrated that both acute stressors and chronic stress alter immune cell number and function, connecting these circumstances with changes in leukocyte proliferative capacity, cytotoxicity, antibody production, etc. Many of these investigations highlighted the immunosuppressive effects of stress and form the foundation of many current studies determining the relationship between molecular correlates of stress and changes in immune cell behavior. By the 1980s and 90s, firmer associations between psychosocial factors and infections were made with larger scale human studies and animal studies.[9] There were new studies demonstrating potential direct connections between neural components and immune effectors such as innervation of lymphoid structures and a functional study showing that chemical sympathectomy modulates the level of circulating antibodies.[21,22] Investigators at the time pointed out that the there was still much work to do in establishing the "psychoimmunologic nexus" but these studies formed a foundation for the hypothesis that increased susceptibility of infection in stressed individuals was due to an influence of stress mediators on immunologic function.[6]

Later studies in the 1990s and early 2000s stressed more rigorous means of determining stress-immune relationships but found that the conclusions of these studies were less simple. A number of studies provided varying conclusions on the effects of psychological stress on the level of circulating antibodies[7,10]. Additionally, studies at the time showed other facets of vulnerability to disease after infection related to stress such as the reactivation of latent viruses latency and wound healing.[1,11] While the problem of the relationship between stress and vulnerability to infection proved increasingly complex, human and animal studies at this point better clarified the molecular components at play. More investigations connected the action of stress hormones and catecholamines with susceptibility to infection.[12–18]

For example, rodent models with surgical removal of the pituitary gland lose their resistance to *S. typhimurium* and succumb to infection at much higher rates.[19] Growth hormone was sufficient to restore resistance by, as the results of in vitro killing assays suggest, enhancing the killing capacity of peritoneal macrophages. Likewise, defective corticosteroid production in rodent models worsened inflammatory disease in rodent models of streptococcal cell wall peptidoglycan polysaccharide-induced arthritis. [20]. In opposite fashion, activation of the HPA axis by restraint stress increased the susceptibility of mice to *Mycobacterium avium* infection.[14] In this case, glucocorticoid signaling hampered the ability of splenic macrophages to handle *M. avium* growth. These studies furthered the hypothesis that the hypothalamic-pituitary adrenal (HPA) axis and the sympathetic nervous system, through hormones and neurotransmitters, respectively, drive stress-related changes in vulnerability to infection predominantly (but not solely) through modulation of the immune system.

A functional advance to studies was the use of 6-hydroxydopamine (6-OHDA), a neurotoxin capable of chemical sympathectomy in animal models. 6-OHDA was used in a number of studies starting in the 1980s to isolate the role of the sympathetic nervous system from that of other stress systems on immunity and infection susceptibility. [23,24] One such study was one that demonstrated sympathetic innervation to lymph nodes but also functionally determined using 6-OHDA that lymph node norepinephrine is necessary for the primary IgM response to antigen (a mouse strain specific effect). Another example is a series of papers published by Hermann and colleagues in 1993 and 1994 where they used 6-OHDA to distinguish the role of the SNS from other stress components.[13,17] The authors found that DBA/2 mice were less susceptible to influenza-induced mortality after restraint stress whereas 2 other strains of mice were not (see Chapter 2 of the dissertation for more examples of context-specific effects of adrenergic systems).[17] Further, they discovered that glucocorticoids levels could not explain the strain-specific difference in mortality which suggested that the sympathetic nervous system could mediate the effect.[25] Using 6-OHDA, they demonstrated that SNS activity contributes to their observed phenotype.[13]

Another example highlights the added nuance the importance of both host factors and infectious context to the relationship between stress mediators and infection. Silverstein and Johnson building upon recent animal models of the time demonstrated that glucocorticoid deprivation by adrenalectomy increased TNF alpha in CF-1 mice in response to challenge with *S. aureus* or *E. coli*.[26] Interestingly, the lethal dose of bacteria was affected by glucocorticoid deprivation or supplementation in the case of *S. aureus* challenge but not that of *E. coli.* Further, neither lethal dose was affected by dexamethasone administration in C3H/HeJ mice. Both characteristics of bacteria and host affect the role of glucocorticoid action. Other factors can additionally affect this relationship. In this case, the authors found that antibiotic administration not only made CF-1 mice lethal dose sensitive to dexamethasone administration for both *S. aureus* and *E. coli,* but it also did the same for the C3H/HeJ which was once insensitive in either case.

With nearly every pathogen challenging society, the association between stress and susceptibility has been made. The findings up to the late 1990s set the stage for studies on the associations of psychological stress and HIV infection. Psychological factors such as social and inhibition and life stressors were associated with higher infection rates and dampened immune responses to HIV. [27–30] In the 2010s and 2020s the number of publications addressing stress and infection have grown substantially (much too many to review here) and the field, naturally, has been divided into more divided and specialized paths of inquiry. The best studied means by which the stress and the sympathetic nervous system influences infection and disease progression is through modulation of the immune system (of much focus in Chapter 2) but there are many facets of stress that have come to light over the years. Notable examples are of how stress affects infection in immune-independent ways. One of the earliest reported non-immune defenses mediated by stress was that of vesical urothelial shedding in mice against *E.*

coli.[31,32] Another is the tendency of psychological stress to reactivate latent viruses such as herpes simplex virus.[33] Related, there is a rich literature of "microbial endocrinology" how microbes (both pathogenic and non-pathogenic) stress hormones and catecholamines.[34,35] While these effects have large implications on infection outcome, they have been dutifully described elsewhere. The scope of this dissertation is to explore the subset of sympatho-immune interactions that have been demonstrated to affect disease, especially considering how those interactions are studied (Chapter 2) and how they could relate to *C. difficile* infection (Chapter 3).

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Chapter 2 -Analyzing the Effects of Adrenergic Signaling on Recruited Immunity within Context

(Adapted from area paper 5/20/21)

Introduction

Rationale

The complexity of the immune system cannot be understated and with the added layer of integration with other organ systems, it can seem especially daunting. In spite of this, we have been able to leverage model systems to more and more reliably alter the immune system in ways we can predict. Our lab has realized the potential of fine-tuning the immune system in resolving infectious diseases and understand that this could be generalized to other disease contexts. Direct manipulation of immune cells and molecules can allow us to orchestrate immune responses but an understanding of the broader modes of regulating the immune response can help us increase our predictive power and flexibility in immunotherapeutic intervention. Nervous system regulation of the immune system has been a fast-moving field, and it is becoming increasingly apparent that the two systems are highly intertwined. Here, we will seek patterns in how neurotransmitters orchestrate immunity and scrutinize the way we've come to these understandings.

This review will take an "immunocentric" view of neuroimmunology and has the principal goal to invite immunologists of a variety of fields to consider the contributions of neuroimmune interactions in their work. I will use the adrenergic signaling platform throughout as an example of neuroimmune study. Adrenergic signaling in immune cells has been well studied, involves a large variety of cellular subsets and can be contextualized in stress, which is known to alter the progression of a variety of diseases and has well-defined circuits. The immune cells considered here will be monocytes, neutrophils, and natural killer (NK) cells as recruited immune cells work in a variety of contexts whereas resident cells might be organ specific. To maintain scope eosinophils have been left out but the existing three cellular subsets are sufficient to highlight the important facets of studying the contextual variables that influence the interaction between adrenergic signaling and immune cell behavior.

An important feature of this review is that it will rely heavily on *in vivo* data. Not only does this allow for a more concentrated scope, but it will also allow us to build an understanding in the context of physiological conditions. This poses a limit on the types of questions we can ask, but *in vivo* methods today can cover much of what *in vitro* methods offer. An ambitious goal should be to learn from

current *in vivo* data how we might predict the efficacy of translation from models to humans and between different disease contexts. For this review, *in vivo* data will be gleaned from multiple *in vivo* models and clinical data ranging over a variety of disease contexts with the aim to decode the language between adrenergic signals and immune cell responses.

Methodology

In PubMed the following search terms were used: "NK cell adrenergic" "monocyte adrenergic" "neutrophil adrenergic" with search years set from 1978 to 2021. Data included in this review are primarily from *in vivo* experiments from all studies found in search. Experiments were included if they specifically target adrenergic receptors within an *in vivo* context. NK cells, monocytes, and neutrophils were chosen as a focus as there are a diversity of *in vivo* studies and models to draw from (contrast that to those of eosinophils and basophils) and the focus on infiltrating immune cells is to limit the scope and explore both tissue resident and remote adrenergic mechanisms.

The approach taken here is a synthesis of *in vivo* data from all accessible sources on PubMed concerning adrenergic receptors and a change in the state of inflammation. That means there is wide variability in the model systems, methodology in measurement, dosing, etc. in the literature considered, but that variability is central to the goal of this review. When it comes to different species, inflammatory stimuli, gender, and other variables, are there pervasive patterns in how immune cells respond to adrenergic signals? For example, will an NK cell in the lung respond to beta 2 adrenergic receptor stimulation the same way as an NK cell in the intestine, or an NK cell in the lung of a different animal, or an NK cell in a lung responding to an allergic stimulus? Finally, the studying of one model system would make it more difficult to define cause-effect relationships between adrenergic signaling and immune cell effects. If adrenergic signaling protects against tissue damage in an immune-cell independent manner, the orchestration and recruitment of immune cells could still be affected. Gleaning data from multiple model systems will give more confidence in the patterns one might find between adrenergic signals and immune cell activity. The synthesis of a broad range of fields and model systems will allow us to reveal how generalizable adrenergic signaling across different contexts.

There are many advantages in taking this approach to find patterns among the literature. First, sampling of in vivo data from different papers is unbiased. While, the clustering of different studies is based on one perspective, this review gleans information from studies large and small, and many times the data presented for one cell type will come from a paper primarily focused on a different cell type but collecting data for both cell types. Second, the sampling of all in vivo data available allows for strength of confidence in numbers. Experiments with

agonists, antagonists, and genetic models all giving the same idea will bolster the reproducibility of a finding. In all fields there are exceptions due to differences in experimental methods, random chance, etc. but if there are 18 papers pointing toward the same direction and 2 papers dissenting, the former set of data is more likely to represent data that can be reproduced and leveraged. With that said, those 2 papers might have conflicting conclusions for an important reason such as the use of female subjects, so including all of these studies is crucial for a complete analysis. Following, a limitation of this approach is that, if there are not many studies within a disease context or for a cell subtype, it is difficult to determine patterns within the data. Third, while it might seem useful to simply probe different cell subsets for their adrenergic receptor expression, this would not be practical. As will be exemplified in the studies presented, receptor expression is dynamic, and the functional adrenergic receptor will change based on the cell type and environmental context. Instead, focusing on the effects of experimental interventions targeting adrenergic receptors in separate contexts will reveal the "functional relevance" of each receptor subtype within different models and disease contexts. Last, the breadth of studies sample will aid in determining whether gender, species, inflammatory context, organ, or other variables affect the functional relevance of an adrenergic receptor within a specific disease context. The main goal of this review is to compare studies to identify 1) the variables that make an adrenergic receptor subtype targetable within a disease context and 2) the effects of targeting an adrenergic receptor subtype on orchestrating the cellular response for different cell subsets. This analysis might help researchers and clinicians predict the effects (or even side-effects) of therapeutic interventions and tests targeting adrenergic receptors with patients and model systems of disparate disease contexts.

Note: The field uses a large variety of pharmacological compounds. Also be aware that due to the large disparity in the amount of information for each cellular subset, the organization for each cellular section is different and customized to fit the patterns found from the data in each field.

Adrenergic Signaling and Natural Killer (NK) Cells

Natural killer (NK) cells are important mediators of type 1 immunity and play an important role in the defense against cancer progression and viral infection. [1–3] Researchers beginning to study NK cells in the context of adrenergic signaling tend to be interested in the effect of stress responses on NK cell recruitment and cytotoxic activity (NKCA). They can do this directly with rodent models or human volunteers, or they can isolate the effects of adrenergic signals by injecting adrenaline, noradrenaline, or isoproterenol into rodents or humans. Recently, there has been an increasing trend in testing the function of adrenergic receptors in the context of cancer and viral infection models. Pharmacological studies dominate this field with many of the first studies using nonselective

adrenergic receptor blockers, but the field is developing a clearer picture of how NK cells respond to adrenergic stimuli with the recent introduction and use of genetic models and more selective pharmacological agents. Although the connection between adrenergic signals and NK cell function is very much in a state of development, the field repeatedly implicates the beta 2 adrenergic receptor in altering NK cell action. This section walks through what the field has to offer today.

Adrenergic Signaling Effects on NK Cells in Various Models

Stress Models Demonstrate Beta-Receptor Dependent Positive Regulation of NK cell Number and Function

Separate **stress models** in mice (social disruption, sleep deprivation), rats (social stress), and humans (mental stress, parachute jumping, psychosocial stress) demonstrate that stress increases NK cell number.[4–9] There is one exception in which Kanemi et al find that NK cell number in the lung and blood are decreased in a model of restraint stress within mice. Ostensibly, this study is the only of the stress model studies using exclusively females, others using male rodents and volunteers.[10] The strong effect of gender on the function and expression of the beta 2 adrenergic will be explored in the discussion section below but this provides a promising explanation for the distinct results in this study compared to the others. All but one of these studies utilize propranolol to attempt to reverse the effect of stress and every attempt is successful (even in the case of Kanemi et al). The one study not using propranolol, utilizing labetalol a nonselective antagonist of beta and alpha adrenoreceptors, consistently abrogates stress induced increases in NK cell cytotoxicity and number.[4]

Of these studies, four of them (representing mouse, rat, and human) test NK cell function or activation marker expression. Consistent with one another Benschop et al (two studies), De Lorenzo et al (2015) and Bachen et al (1995) show that NKCA is increased with stress and Tarr et al (2012) that inhibitory receptors NKG2a+ and Ly49A+ are decreased in stressed rats.[4–6,8,9] Additionally, both of these effects are reversible by propranolol. However, Tarr et al (2012) unexpectedly found that NKCA was increased with propranolol treatment above the level of increase with social disruption alone. The effect of beta adrenoreceptors on NKCA in the context of stress merits further study.

Overall, stress studies using beta blockers consistently impute beta adrenergic receptors as positive regulators of NK cell number across stress models and species with potential dependence on gender. Four of five studies imply that beta adrenergic receptors are also positive regulators of NKCA. Studying beta adrenoreceptors in the context of stress should benefit from studies with selective agonists and antagonists and genetic models to identify the specific receptor responsible for these phenotypes and, perhaps, to make clearer the relationship between NKCA and beta adrenoceptor function.

Exercise Studies Also Implicate Beta Receptors in Positive Regulation of NK cell Action

Exercise studies show that exercise tends to increase NK cell function and number in a manner dependent on beta adrenergic receptors. In both humans and mice, exercise increases the number of circulating NK cells but this increase can be abrogated by non-selective beta receptor antagonists such as nadolol and propranolol.[11–13] Additionally, in a mouse model with a tumor, propranolol ablates exercise-induced increases of NK cell number within the tumor and decreases in number within the spleen, suggesting that beta adrenoreceptors are important for NK cell egress from the spleen.[13] NKCA and the expression of maturation/ activation markers NKG2C and CD57 are also increased with exercise and blunted by nonselective beta blockers.[11,12] One study is slightly contradictory in that it finds NK cell activity is decreased with exercise, but it also finds that propranolol and atenolol have no effect, suggesting that this phenotype may not beta adrenoceptor mediated.[14]

As with stress models, exercise models tend to show an increase of NK cell activity and number dependent on beta adrenergic receptors. As most of these studies are in human, it is difficult to generalize these findings across species but in the studies presented there seems to be a clear positive correlation between beta adrenoreceptor function and NK cell number and activity within the context of exercise.

Infusion Studies at Homeostasis Additionally Implicate the Beta 2 Receptor as Particularly Important and Highlight Its Nuanced Effects on IFNγ Production

An infusion study is one where a rodent or human is directly administered (intraperitoneally or osmotic pump for mice, intravenously for humans) adrenaline, noradrenaline, isoproterenol. This mimics the effect of stress but isolates the effect of adrenergic signals from other stress-induced mediators (e.g. glucocorticoids). Consistently with stress studies, the **infusion of adrenaline** into humans or mice tend to increase the number of NK cells in circulation and the cytolytic activity of these cells but there seem to be caveats with this finding. Some studies do show simply that adrenaline infusion increases NK cell number and function.[15–19] Ben-Eliyahu and colleagues, however, show a decrease of NKCA with adrenaline but the killing target for these cells is different from that of other infusion studies: tumors.[20] Whether the tumor microenvironment can alter the effect of adrenergic stimuli on NK cell activity is discussed below. Another commonality between these studies is that they tend to sample from NK cells in blood. Watanabe et al found that when they infused mice with adrenaline that NKCA was increased in cells isolated from the liver but not from spleen.[21] This would be consistent with the finding of Schedlowski et al who found that in splenectomized patients adrenaline still increases NK cell number and NKCA,

albeit to a lesser extent.[22] Demographic and disease state factors also play a role in the effect of adrenergic influence on NKCA. NK cells in female arthritic patients have a dampened increase in number with adrenaline yet an increased NKCA compared to healthy volunteers.[23] Finally, a caveat that touches the core motivation of this review, is the finding that effect of adrenaline can depend on the presence of other modulators. Although the study is singular, Bedoui and colleagues demonstrated that neuropeptide Y (NPY) modulation of NK cell number depends on the amount of adrenaline administered.[24] Mice administered a low level of adrenaline had increased numbers of NK cells when administered NPY and, contrarily, mice administered high doses of adrenaline had reductions in the number of NK cells when administered NPY. Altogether, it seems clear that number and NKCA of blood NK cells is augmented in healthy humans with adrenaline infusion but that NK cell tissue site, combinatorial effects with other modulators, and disease milieu should be considered when trying to predict the effect of adrenaline on NK cells.

Noradrenaline infusion studies do not create a clear picture of the role of noradrenaline in NK cell number and cytotoxic activity. A couple of studies show that noradrenaline has no effect on NK cell number.[22,25] Kappel and colleagues show in one study that noradrenaline increases NKCA in conditions where they are unstimulated, IL-2 stimulated, or IFNα stimulated, but in a second study shows no change under IFNα stimulation.[17,18] In the same two studies they also show an increase in NK cell number with noradrenaline fusion in one study but no change in cell number with the second study. The reason for these differences is unclear although they do use largely different concentrations of injection between studies and explain that noradrenaline-induced adrenaline could influence the outcome of these studies. Finally, a study shows that morphine induced reductions in NKCA are exacerbated with the co-administration of noradrenaline.[26] This study stands out in the same way as others as its effects are deviant from other studies and it uses exclusively female patients. It seems that if noradrenaline does cause an effect in NK cells through direct or off-target effects, it would increase number and increase NKCA, but the relative inconsistencies here might also indicate that adrenaline is the more relevant, potent stimulator of NK cells *in vivo*.

Lastly, **isoproterenol**, a nonselective beta receptor agonist, has been used in some infusion studies to shed light on the effect of beta-adrenergic receptors on NK cell number and function. Isoproterenol increases NK cell number in tumorized mice but not in mice deficient in the beta 1 and beta 2 adrenergic receptors.[25,27] Although isoproterenol increases NK cell number, the proportion of IFNγ+ NK cells is reduced.[27]

Collectively, these infusion studies tell a similar story to that of stress studies: NK cell number is increased by beta receptor agonism. Further, with these infusion studies we start to see more cases where adrenergic stimulation results in decreased NK cell function in terms of IFNγ cytokine production but increases in

NKCA. Thus, in both contexts of stress and isolated infusion, there must be underlying factors that alter the effect of adrenergic stimuli on NKCA. It is not very common for these studies to have adrenergic receptor antagonists co-administered, so it is difficult to pin down the specific receptors responsible for the observed phenotypes. One study did show that adrenaline-induced increases in NK cell number and NKCA are prevented by propranolol but not bisoprolol, suggesting that beta 2 adrenergic receptors are most likely responsible for the change.[22] Studies described in ensuing sections continually implicate this receptor, and these infusion studies set the foundation for more targeted inquiry of beta adrenergic receptors in NK cells.

Selective Agents and Models Implicate Beta 2 Adrenergic Receptors in Controlling NK Cell Activity but Demonstrate Mechanistic Uncertainty and Potential Gender Effects

The above studies hint that beta 2 adrenergic receptors are functionally the most important for driving changes in NK cell function and number, but more selective pharmacological agents and genetic models help solidify this reasoning.

Selective pharmacological studies implicate beta 2 adrenergic receptor in the control of NK cell number and function. Injection of metaproterenol, a selective beta 2 adrenergic agonist, increases NK cells in mice but decreases NKCA, both phenotypes by non-selective beta blockers nadolol or propranolol.[28] Another study shows within a murine hemorrhage model that metoprolol, a beta 1 receptor antagonist, and propranolol decrease NK cell number.[29] This runs consistently with other studies demonstrating that beta receptors are positive regulators of NK cell number but contrary to the finding that bisoprolol, another beta 1 receptor antagonist had no effect on NK cell number. As metoprolol is less specific for beta 1 adrenergic than bisoprolol, it is conceivable that the effect is mediated through beta 2 adrenergic receptors, but it also could be that beta 1 adrenergic receptors are more relevant in the context of the hemorrhage model.[30] Models with genetic ablation of the beta 1 adrenergic receptors will shed more light on this question.

Genetic models have only tested the function of the beta 2 receptor (barring one study using B3ar siRNA discussed later) in altering NK cell function and find opposing conclusions. The most selective study connecting beta 2 adrenergic receptor function and NK cell activity utilizes a Cre-lox system that selectively ablates the receptor under the NKp46 promoter (Diaz-Salazar et al. 2020).[31] The study found firsts that during murine cytomegalovirus (MCMV) infection NK cells lie in close apposition to adrenergic neurons and that they upregulate transcriptional expression of the *Adrb2* gene. They further found that the NK cell-specific *Adrb2* KO decreases the number of NK cells in the lung, blood, liver and spleen and that the remaining NK cells express fewer markers of maturation and exhibit dampened killing capacity. Consistently, when transferring NK cells into NK cell deficient mice (Ly49H deficient) they found that the expansion of *Adrb2*

KO NK cells lagged significantly behind WT cells. This difference between *Adrb2* KO and WT NK cell pool expansion could be abrogated if the host was sympathectomized and depleted of catecholamines, confirming that catecholamine stimulation of the Adrb2 receptor in NK cells is essential in the maintenance (rather than development) of their number. Finally, they confirmed that this reduced number is not due to increased apoptosis but rather diminished proliferation. This study supports a model in which the beta 2 adrenergic receptor in NK cells increases NK cell number and function, consistent with many but not all of the findings in stress response and infusion studies.

Diaz-Salazar et al, by selectively ablating the beta 2 adrenergic receptor in NK cells, put forth a model in which this receptor acts intrinsically on NK cell function, but another study proposes a cell extrinsic effect of the beta 2 adrenergic receptor. Wieduwild et al found that in a murine model of MCMV infection in which the *Adrb2* gene is ablated in Ncr1+ cells, covering all immune cells, or in LysM+ cells, covering myeloid cells, there is no effect on NK cell number, cytokine production, or survival in the model.[32] Instead, NK cells transferred to *Adrb2*-deficient hosts have augmented IFNγ production and improve survival in the model. Another contrary finding to Diaz-Salazar and others is that they demonstrate that global *Adrb2* ablation does not affect NK cell number in blood, spleen, or liver, but it does increase NK cell IFNγ production. Lastly, the study demonstrates that 6-OHDA induced sympathectomy does not affect IFNγ circulation or survival in the model, not proving but strongly implying that the adrenal gland is the source of adrenergic stimulation. This does not necessarily conflict with the findings of Diaz-Salazar et al who found that 6-OHDA coadministered with metyrosine affects NK cell function as metyrosine could act to deplete catecholamines in the adrenal gland. In sum, Wieduwild et al supports a model in which the beta 2 adrenergic receptors in non-NK cells negatively regulate NK cell cytokine production but not number.

What might account for these opposite effects in two MCMV models of infection? While sex cannot always explain these differences, it is notable that Diaz-Salazar et al uses exclusively female mice. Kanemi et al (2005), discussed before in stress studies, ran contrary to all other studies in that it found beta-receptor dependent decreases in NK cell number in response to stress and also used exclusively female subjects. While Diaz-Salazar et al doesn't find differences in NK cell number as Kanemi et al (2005) does, they do find that the beta 2 receptor acts as a negative regulator in the capacity of NK cells to act. This might suggest that the adrenergic "code" regulating NK cells in females is model-specific in mechanism but that it functions to downregulate NK cell function in either case.

Contextual Details and Variables Controlling the Relationship Between Adrenergic Signaling and NK Cell Behavior

The prior studies show trends of beta receptor function in controlling NK cell activity and, as with any field, present exceptions where external factors might play a role. An understanding of the system in which adrenergic signals reside will allow us to create a clearer picture of how mechanistically NK cells respond to these signals and, following, how external forces might work on the environment to change the function of the system. Here is a brief discussion of what the field has found in trying to define the source of adrenergic stimulation and in how beta-adrenergic receptor sensitivity is changed with the environmental context.

Defining the source: The Relative Roles of Sympathetic Neurons vs the Adrenal Gland in Arranging NK Cells

What organ is the most important source of adrenergic stimulation for NK cells between the sympathectomy nervous system and the adrenal gland? This is difficult to answer with the current data as there are not many studies (I have only found one) using adrenalectomy models compared to the sympathectomy study. The one study shows that adrenalectomy slightly decreases NK cell number induced by a social stressor but that propranolol completely ablates it.[7] While adrenaline infusion studies consistently show an effect on NK cells, they could artificially push the system through the same receptors utilized by endogenous norepinephrine. The study by Wieduwild et al (2020) implicated the adrenal gland by highlighting the absence of an effect of sympathectomy. This could highlight a difference in the relative contribution of the adrenal gland in males in females as the sympathetic nervous system plays less of a role in this study using females compared to other studies.

Sympathectomy studies are more common and show larger effects. One study shows that splenic stimulation decreases splenic NK cell number, reversible by nadolol .[33] Another shows that NK cell recruitment to the lung in an influenza model is decreased with sympathectomy.[34] A third study shows that sympathectomy plus depletion of catecholamines (with 6-hydroxydopamine and metyrosine) inhibits the expansion of the NK cell population.[31] These findings would fit a model in which adrenergic stimulation allows for proliferation of NK cells within the spleen and egress from the spleen to other organs. One study that deviates from this idea is that of Brenner et al showing that sympathectomy does not change NK cell trafficking to the lung in a tumor model but this might be a feature of the model.[35] The next section will discuss why the tumor microenvironment might alter the effect of adrenergic signals in NK cell function.

It seems that timing affects the regulation of beta receptor impact on NK cell function. All of the aforementioned studies concerning the effect of beta receptors on IFNγ production in NK cells show an inverse relationship between the two. Consistently, Logan et al (2011) find that splenic sympathectomy in male rats increases IFNγ in NK cells but with an important caveat: they were able to see

this effect when splenic norepinephrine levels are lowest in a circadian cycle but not when sympathectomizing mice at the point when norepinephrine levels are highest.[36] In addition to sympathetic nervous system effects being sensitive to timing within the circadian cycle, there is also evidence that beta receptor sensitivity changes through development. Reder et al found that sympathectomizing mice aged 4 weeks increases NK cytotoxicity but sympathectomizing mice at 6 weeks or 10 weeks results in decreased NK cytotoxicity.[37] Many of the studies covered in this review utilize adult mice which might explain why we see a positive correlation between NKCA and adrenergic signaling. It is important to keep the developmental stage of subjects in mind here and in other cases where we attempt to put together a signaling code.

Environmental Context Drives the Expression of Beta-Adrenergic Receptors in Stress and Cancer Models: Emerging Role for the Beta 3 Adrenergic Receptor

There are a few studies showing that environmental context regulates expression and sensitivity of NK cells to beta adrenergic receptor stimuli. NK cell expression of beta 2 adrenergic receptors is increased after mental stress tasks in human.[38] It seems that receptor stimulation can have an effect on beta 2 receptor sensitivity as well. Jetschmann et al (1997) found that adrenaline infusion decreases beta 2 (and alpha 1) adrenergic receptors in NK cells, possibly creating a negative feedback loop on the receptor's stimulation.[15]

Recently, the role for the beta 3 adrenergic receptor in NK cells has come to light, especially in the context of models of cancer. The beta 3 adrenergic receptor is increased in NK cells within tumors compared to NK cells outside of it. SR59230a (SR) and beta 3 adrenergic receptor-specific siRNAs but not propranolol increase perforin+ and CD107A+ NK cells within the melanoma model suggesting that beta 3 adrenergic receptor stimulation downregulates NK cell infiltration into tumors and cytotoxic activity.[39] Calvani and colleagues explain this mechanism as they show in a separate study that beta 3 adrenergic receptor is upregulated in placental NK cells of pregnant mice and that SR increases the number of CD107a+ NK cells within the placenta(Calvani et al. 2020).[40] These authors propose that the hypoxic environment in both models of cancer and pregnancy underlie beta 3 adrenergic receptor mediated immunosuppression of NK cells. Consistently, adrenaline infusion in one model of cancer and beta receptor blockade in another decrease NKCA and increase NK cell infiltration into metastases, respectively.[20,41] Although the studies of Calvani and others provide good support of a model mediated by the beta 3 adrenergic receptor, there are studies which put its generalizability and mechanism into question. Wrobel et al (2016), who found that beta blockade in the melanoma increases NK cell infiltration into metastases, did so using propranolol which decidedly had little effect in the studies of Calvani et al.[41] In a different model of cancer, a B cell lymphoma model, isoproterenol, stimulating beta receptors (which has low affinity for beta 3 adrenergic receptor), acts to increase NK cell number in blood.[27] These studies put into question not only the role of beta 2

adrenergic receptors in cancer models but to what extent we might generalize about the effects of beta receptor function in NK cells between different types of cancer. Still, the studies in total considering cancer models demonstrate that the environmental context of cancer (and potentially pregnancy) can switch beta receptor agonist and antagonist effects on NK cell function in a way opposite to how we might have predicted in the context of stress, exercise, and healthy baseline subjects.

Summary of the Adrenergic Code of NK Cells

In summary, a synthesis of the adrenergic data would suggest that NK cells are positively regulated by beta 2 adrenergic receptors in number and killing capacity, but that there are external factors that can change this trend. With regards to the differences across species, there are no clear instances where rodent data follow one trend and human data follow another. Similarly, across different models of stress, infusion, and inflammation there seems to be a consistent effect on number. The two instances where we see no change or decreases in NK cell number are in studies using female rodents where other studies use male rodents and human volunteers. While there is a clear effect of beta 2 adrenergic on NKCA, the direction of its effect is variable. There is a general trend that beta receptors increase NKCA but there are some unexplained exceptions. Notably, a chunk of these exceptions lies within tumor models where the beta 3 adrenergic receptor is supposed to have a dominant effect. A curious thing from this synthesis is that IFNγ production has an inverse relationship to beta receptor function, even in the case of female subjects. At face value, mobilized cytotoxic NK cells seem less productive of their major cytokine. As more studies emerge using NK cells in models of inflammation such as viral infection, it would not only become clearer how different inflammatory contexts alter the adrenergic code in NK cell function, but also tell us whether recruited NK cells act effectively under beta adrenergic stimulation. Finally, there are a few recent studies that highlight the role of patient health status, circadian rhythm, and development in altering the NK cell adrenergic code. To create consensus in the field it would be advantageous to take heed of and present these factors in publication, even when it is not practical to control these variables. Ostensibly absent from these studies are those of adrenergic signaling and NK cells are those concerning alpha adrenergic receptors. There was only one study found in search and it shows that dexmedetomidine has no effect on NK cell number.[42] Collectively, NK cell recruitment, cytolytic activity, and cytokine production is primarily controlled by beta adrenergic receptors and especially the beta 2 adrenergic receptor which frequently augments NK cell recruitment and NKCA. The potential roles of gender effects, beta 3 adrenergic receptors, extrinsic effects, patient health status, and development all are emerging in the literature as overlays on this signaling code that can alter the mechanism or effect of adrenergic stimulation on NK cell behavior.

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Adrenergic Signaling and Neutrophil Activity

Introduction to Studying the Effects of Adrenergic Signaling in Neutrophils: Arcaroli et al Demonstrate Important Variables within the Adrenergic Code

Neutrophils are the most abundant circulating immune cell and take part in multiple inflammatory diseases. Following, strategies to alter the recruitment and function of neutrophils has been a great focus for researchers of a large variety of fields (Nemeth et al 2020).[43] The literature concerning the effect of adrenergic signaling on neutrophils is quite rich compared to that of NK cells and monocytes. As a result, this section is structured differently to reflect the greatest divide in the data: inverse relationships and promoting relationships between adrenergic stimulation and neutrophil activity. Separating analysis this way allows for better handling of the large number of studies as there is good representation of studies describing both kinds of relationships between adrenergic signaling and neutrophil activity. Fortunately, there seems to be clear reasons for why a study might fall into one category or the other. This section will explore these reasons but first the section will begin with an example of how neutrophil studies show important nuances in adrenergic signaling that highlight the environmental context and receptor subtypes as essential variables of study.

The central motivation of this review is the realization that the effect of an adrenergic agonist or antagonist will depend on characteristics of an inflammatory model, yet which variables are most important in predicting the effectiveness of these interventions within a particular context are ill-defined. Arcaroli et al (2002) serves as an exemplary study as it clearly demonstrates how the most relevant adrenergic receptor in modulating neutrophil recruitment can be different for distinct inflammatory contexts.[44] Further, it portrays this phenomenon is not restricted to a comparison as to whether beta or alpha receptors are more responsive within context but also, for instance, whether alpha 1 or alpha 2 adrenergic receptors might be the more important within a model system.

They use either a systemic LPS model and or cardiac puncture hemorrhage (HEM) model and monitor the influx of lung neutrophils by proxy of lung myeloperoxidase (MPO). Propranolol has no effect on MPO in the HEM model but decreases MPO in LPS endotoxemia, suggesting that beta adrenergic stimulation in the latter model would be important in the recruitment of neutrophils but not in the HEM model. The nonselective alpha-adrenergic blocker, phentolamine has a different effect; it decreases MPO in HEM but increases it in LPS endotoxemia. A simple comparison of propranolol and phentolamine effects in the LPS model demonstrates opposite effects of nonselective blockade of alpha vs beta adrenergic receptors on neutrophil

infiltration. The introduction of selective alpha agonists creates nuance in this picture. Consistent with the results for phentolamine, phenylephrine, a selective alpha 1 adrenergic agonist, increases MPO in the HEM model but, inconsistently, it also increases MPO in the LPS model. The latter inconsistency would suggest a contribution of the alpha 2 adrenergic receptor in the effect of phentolamine. UK 14304, the selective alpha 2 adrenergic receptor agonist, has no effect MPO in HEM but decreases MPO In the LPS model. If we compare the effects of these alpha-adrenergic antagonist and agonists, we see the following:

1) The effect of phentolamine suggests a positive effect of alpha-adrenergic stimulation on neutrophil infiltration in the HEM model but a negative one in the LPS model.

2) These effects are a combination of the effects of alpha 1 and alpha 2 adrenergic receptor functions. The positive effect in the HEM model is recapitulated by phenylephrine suggesting a dominant effect of the alpha 1 adrenergic receptor in the HEM model. The negative effect in the LPS model is recapitulated by UK 14304 suggesting a dominant effect of the alpha 2 adrenergic receptor in the LPS model.

3) Finally, the effect of phenylephrine in the LPS model demonstrates that although the alpha 2 adrenergic receptor has a dominant effect, that the alpha 1 adrenergic receptor can still be targeted to orchestrate the neutrophil response. In contrast, the lack of effect of UK 14304 in the HEM model tells us that sometimes the non-dominant receptor is not targetable.

This study also goes against the idea that beta adrenergic receptors are anti-inflammatory and that alpha adrenergic receptors are pro-inflammatory. Propranolol by blocking beta receptors downregulates the neutrophil response suggesting a negative relationship between beta adrenergic stimulation and neutrophil recruitment. Unfortunately, this study does not dissect the relative contribution of beta 1, 2, and 3 adrenergic receptors with selective pharmacological agents, but either beta 1 or beta 2 adrenergic receptors mediate a pro-inflammatory effect in the context of LPS endotoxemia. Add to this, alpha 1 and alpha 2 adrenergic receptors have opposite effects on MPO infiltration. Thus, subsets of beta- and alpha-adrenergic receptors act in different ways on neutrophil responses.

This study forwards a model in which beta-adrenergic receptors and alpha 1 adrenergic receptor have a positive effect on neutrophil recruitment while the alpha 2 adrenergic receptor has a negative effect on neutrophil recruitment. This model further would suggest that the HEM model is dominated by alpha 1 adrenergic receptors while the LPS model is dominated by alpha 2 and beta-adrenergic receptors. As such, in these inflammatory conditions, the therapeutic target would be different for these separate inflammatory models. Extended, this could mean that for patients with distinct inflammatory diseases that the most

appropriate intervention should change. The goal of this section is to identify which variables within an inflammatory context best predict which adrenergic receptor is the most pertinent and how that adrenergic receptor acts to orchestrate the neutrophil response.

Adrenergic Signaling Effects on Neutrophils in Various Models:

Beta Adrenergic Stimulation at Homeostasis Tends to Stimulate Neutrophil Recruitment

Neutrophil studies unlike those of NK cells utilize rodent models of inflammation more readily than infusion studies at baseline, but these baseline studies tend to show that adrenergic signals promote neutrophil responses at homeostasis. Adrenaline infusion in male volunteers and rats alike increase the circulation of blood neutrophils.[16,45] The rat study found that this adrenaline infusion effect is partially dependent on the spleen. Isoproterenol infusion increases the number of neutrophils to the heart but this might be site-specific as isoproterenol induces myocardial infarction (Filho et al. 2011).[46] Still, these studies might indicate that both adrenaline and isoproterenol promote neutrophilia in homeostatic conditions.

Similarly, some noradrenaline infusion studies find that the intervention in humans increases the number of circulating neutrophils.[17,47] The route of administration of noradrenaline could play a role as oral norepinephrine has no effect on blood neutrophil number in rats.[48] In contrast, Nicholls et al (2018) find that mice superfused with fMLP, a strong chemotactic factor directing neutrophils, have neutrophils with decreased adhesion and transmigration in vivo with the coadministration of noradrenaline.[49] This difference could be due to the added stimulus of fMLP. Consistent with this idea, CLP mice receiving *ex vivo* stimulated neutrophils with noradrenaline or neutrophils from burn mice have higher mortality, which the authors presume to be due to decreased neutrophil function.[50] This demonstrates that additional signals in the inflammatory milieu can switch the effects of noradrenaline on neutrophil activity from positive to negative. These studies suggest that adrenaline, isoproterenol, and noradrenaline promote neutrophil circulation at baseline, but that inflammation can alter the effect of adrenergic signals on the neutrophil response. The ensuing sections demonstrate inflammatory contexts where adrenergic signals might be induced or sensitized by inflammatory stimuli compared to baseline.

Studies at baseline through other experimental methods could support the idea that adrenergic receptors stimulate neutrophil activity at homeostasis. A study showing that mice transferred bone marrow cells deficient in both the beta 1 and beta 2 receptors further suggests that this might be cell intrinsic as the number of neutrophils in circulation is decreased in this chimeric model. Bartley et al (2018)

show that global KO in these receptors increases the number of "young" or immature (CD184+, CD62Lhi) neutrophils raising the possibility that adrenergic signals at baseline are important for neutrophil development.[51] There is one study, however, that opposes the idea that adrenergic signals act positively on neutrophil activity at baseline. Skurikhin et al (2016) found that reserpine, a compound which inhibits monoamine neurotransmitter release from, increases neutrophils within serum. Further, they show that mononuclear cells from reserpine-treated donors augment the number of neutrophils in the recipient.[52] Reserpine, however, can act to alter dopaminergic and serotonergic pathways, so it is difficult to single out noradrenergic signaling in this case. Altogether, infusion studies and non-infusion studies alike suggest that adrenergic signaling at baseline is a stimulator of the neutrophil response.

BETA ADRENERGIC RECEPTOR STUDIES

I. Inverse Relationships Between Neutrophil Activity and Beta Receptor Activity

When considering how different adrenergic receptors affect neutrophil responses, it has been useful to separate the results of experiments as describing "inverse" or "promoting" relationships between adrenergic activity and neutrophil activity. Promoting relationships describe where agonist or genetic overexpression of adrenergic receptors results in an increased neutrophil response (which can manifest as recruitment, egress, ROS production, etc.) or where antagonist or genetic ablation result in a dampened neutrophil response. On the other hand, inverse relationships describe where the agonist or genetic overexpression of adrenergic receptors results in decreased neutrophil response or where antagonist or genetic ablation results in an augmented neutrophil response. By separating studies this way, certain patterns arise in which either a specific receptor works similarly across different experimental contexts or where a specific experimental context consistently implicates a specific receptor. This section will discuss these patterns and their evidence.

Broadly, the majority of studies concerning neutrophil activity and adrenergic signaling describe an inverse relationship between the two in the context of inflammatory models. The majority of these effects are mediated via beta adrenergic receptors. Beta 2 adrenergic receptors, especially within context of vagal nerve stimulation and lung inflammation, tend to underlie these inverse relationships. In contrast, in separate models' beta 1 adrenergic receptors tend to promote the neutrophil response and stress models particularly have a beta 1 adrenergic receptor dominant effect on neutrophil recruitment. Beta 1- and beta 2-adrenergic receptors have opposing roles in controlling the neutrophil response but seem to work within diverse contexts.

Adrenergic Stimulation Downstream of the Vagus Nerve Results in Spleen-Dependent Downregulation of Neutrophil Recruitment

A subset of studies suggests that adrenergic stimulation of the spleen downstream of vagus nerve stimulation downregulates neutrophil recruitment. A couple show that propranolol and splenectomy have similar effects in increasing neutrophil recruitment in inflammatory models.[53,54] These studies implicate the role of cholinergic receptors within this model by the use of mecamylamine. Although the effects of the vagus nerve are outside of the scope of this review, one model describes a downregulation of inflammation via the vagus nerve, splenic nerve, and T cells that act to dampen cytokine production in splenic macrophages.[55–57] In agreement with this model, vagus nerve stimulation decreases arthritic severity and neutrophil infiltration but not under the condition of propranolol treatment. Some studies might indicate that beta adrenergic signals act to modulate neutrophil egress from the spleen. One study shows that propranolol decreases splenic KC, providing a means by which adrenergic signals can affect neutrophil trafficking from spleen. [58] In a second, renal denervation and peripheral blockade of beta 2 adrenergic receptors via ICI 118,551 increase splenic neutrophil number and decrease recruitment to damaged heart tissue.[59,60] One of these groups, Grisanti et al (2019), forward a cell-intrinsic effect of the beta 2 adrenergic receptor by demonstrating that beta 2 adrenergic receptor KO cells transferred to a wild type host have increased splenic neutrophils and decreased infiltrates to the heart. They additionally show that reintroduction of a beta 2 adrenergic receptor transgene into these transferred cells reverses these effects. Together these studies show that beta 2 adrenergic receptors can mitigate neutrophil egress from spleen likely downstream from vagus nerve stimulation.

Beta 2 Adrenergic Receptors Downregulate Lung Neutrophilia

Another subset of studies tests the effect of beta 2 adrenergic agonist within the context of asthma or lung injury. Many of them demonstrate that in patients, mice, and guinea pigs that formoterol, salmeterol, olodaterol, and salbutamol decrease neutrophil recruitment to the lung in diverse models of lung damage and inflammation.[61–69] Consistently, nonselective agonists dobutamine and dopexamine decrease neutrophils in bronchial lavage fluid with LPS induced acute lung injury (ALI).[70] Conversely ICI 118, 551, a selective blocker of the beta 2 adrenergic receptor, increases neutrophil influx and worsens ALI. [71] The effect of the beta 2 adrenergic receptor seems not to be restricted to the type of inflammatory insult within the lung. The aforementioned studies represent both type 1 (LPS induced lung injury) and type 2 (asthma) dominant modes of inflammation within the lung. Additionally, a study demonstrated that in 3 separate models of airway inflammation that mice deficient in the beta 2 adrenergic receptor have an increased neutrophil response.[72] The effects in these models 1) extrinsic crude (NTHi), 2) extrinsic defined(PAM2/ODN), and 3) intrinsic (IL-17) demonstrate that these effects are generalizable across different types of neutrophilic stimuli. The same study also proposes that this effect could be mediated extrinsically. They show that nadolol and genetic ablation of the beta 2 adrenergic receptor induce airway neutrophil, the latter of which can be reversed by introduction of a beta 2 adrenergic transgene specifically in epithelia.

Both the epithelial transgenically introduced beta 2 adrenergic receptor and formoterol are sufficient to reduce neutrophil infiltration into the airway. The consistent effect in the lung could arise from a strong extrinsic effect conferred by the lung epithelia. Finally, mice treated with PAM2, the extrinsic defined neutrophilic stimulus, have the same neutrophil response whether they proficient or deficient in the enzyme Phenylethanolamine N-methyltransferase (PNMT), indicating that norepinephrine rather than epinephrine would mediate the effects seen in the lung. Despite the semblance of a clear picture here, there are studies that are not completely consistent. One study that could run contrary to these patterns is one showing that carvedilol decreases MPO in lung tissue in a paraquat induced lung injury.[73] The simple explanation here is that although carvedilol is characterized as a beta blocker, it also inhibits the alpha 1 adrenergic receptor (Yoshikawa et al 1996).[74] A couple of studies suggest that while formoterol can dampen neutrophilic inflammation, it cannot do so alone but in tandem with other compounds such as fluticasone .[75,76] Finally, there are mechanistic details that may either be inconsistent or model-dependent. Salbutamol increases L -selectin in ex vivo stimulated neutrophils from ARDS patients, suggesting a cell intrinsic effect of the beta 2 adrenergic receptor. [77] Körner et al (2019) also implicate sympathetic nerves showing that 6-OHDA induced sympathectomy increases neutrophil recruitment but propose that beta adrenergic receptors act within sympathetic neurons to mediate release of a molecule, repulsive guidance molecule A (RGMA), which should indirectly regulate neutrophil recruitment through the modulation of macrophage cytokine production.[69] Overall, these studies suggest that beta 2 adrenergic receptors downregulate neutrophilia in diverse contexts of lung inflammation but whether the mechanism is shared between these contexts is up for debate.

Studies Outside of the Spleen and Lung are not as Well-Defined in Implicating Beta Adrenergic Receptors in Affecting Neutrophil Activity

The remaining studies implicating a negative effect or no effect of adrenergic stimuli on neutrophil use propranolol. One study finds that propranolol decreases MPO and elastase within skin lesions in a mouse model of pressure ulcers.[78] Another finds that neutrophils of mice housed with a sick partner (Ehrlich tumor-bearing) have decreased oxidative bursts and phagocytosis and that this is prevented if the sick partner is treated with propranolol .[79] The one model not finding an effect of beta adrenergic signaling in altering neutrophil activity, found that inhaled propranolol had no effect on neutrophil recruitment induced by intranasal LPS.[80] The route of administration or the studies use of female mice could play a role here. While these studies describing an inverse relationship outside of the lung or spleen are few in number, they do exist and future experiments in similar models will help to determine why beta receptors downregulate inflammation in these contexts.

II. Promotion of Neutrophil Activity Through Beta Adrenergic Receptors

Stress Models Implicate Beta 1 Adrenergic Receptors as a Promoter of Neutrophil Recruitment

A pocket of the dissenting studies, ones that show a promoting effect of beta-adrenergic receptors on the neutrophil response, involve stress models. Blockade of beta receptors by propranolol or atenolol within acute stress models abrogates the increase in number of circulating neutrophils .[7,81] While the selective beta 2 adrenergic blocker ICI 118, 551 has no effect on acute cold restraint stress induced neutrophilia, mice genetically deficient in the beta 1 adrenergic receptor do not have an increase in circulating neutrophils with the stressor.[81] In line with these findings, a study using an LPS endotoxemia model demonstrates that propranolol or superior cervical ganglionectomy increases the number of neutrophils within bone marrow and limits egress to the lung.[82] At the surface, it might seem that the beta 1 adrenergic receptor acts to inhibit egress from the bone marrow but the details are not clear. Zieziulewicz et al (2013) found that the number of granulocytes within bone marrow is decreased in wild type mice experiencing acute cold restraint stress but not in beta 1 adrenergic receptor deficient under the same conditions.[81] Consistently Liu et al (2009) find that propranolol reverses hemorrhagic shock mediated reduction in bone marrow neutrophils.[83] In contrast, Jin et al find that their mice undergoing chronic psychological stress have increased bone marrow neutrophils and this effect is abrogated by propranolol.[84] They still find that stress increases the number of neutrophils in blood but do not test whether this effect is mediated by adrenergic receptors. The difference in effect of stress on bone marrow neutrophil number could be a consequence of Jin et al using exclusively female mice (a sex effect) or of their chronic model of stress which could behave differently compared to an acute model. Interestingly, Jin et al also found that infusion of epinephrine or isoproterenol did not have an effect on neutrophils in bone marrow suggesting that stress combines the effects of adrenergic signals and other signals to mediate neutrophil egress from bone marrow. These stress-induced adrenergic signals most likely come from innervation of the bone marrow. Xue et al (2018) found that restraint stress increases the recruitment of neutrophils to a corneal abrasion wound and that neutrophil recruitment could be prevented by nonselective beta blocker timolol or ablation of sympathetic nerves coming from the superior cervical ganglion.[85] Notice here that although these studies ablate noradrenergic neurons like those described involving the spleen, that these studies have opposite effects and could implicate separate neuronal circuits. Although the beta 1 adrenergic receptor in the context of stress consistently upregulates neutrophil recruitment in the context of stress, the effects on other neutrophil functions have not been well explored. Although one might assume that all other functions are upregulated, Shilov and Orlova (2000) in an immobilization stress model found that propranolol increases the phagocytic activity of neutrophils in vivo.[86] Studies here suggest that secondary signals in the context of stress drive beta 1 adrenergic receptor mediated promotion of neutrophil recruitment.

Beta 1 Adrenergic Receptor Dominant Inflammatory Models Increase Neutrophil Activity with Adrenergic Stimulation

As stress models implicate the beta 1 adrenergic receptor, some inflammatory models do the same. Metoprolol decreases infarct size within both patients and myocardial infarction mouse models, decreasing the infiltrating neutrophils in the latter.[87] Metoprolol also decreases the number of recruited neutrophils in a thioglycolate induced peritonitis mouse model in WT mice but not in those deficient for the beta 1 adrenergic receptor. Bone marrow transfer of beta 1 adrenergic receptor deficient cells demonstrates a cell intrinsic effect as the host genotype has no effect. Garcia-Prieto et al further characterize this cell intrinsic effect by showing local TNFa injection induced velocity, distance traveled and directionality is decreased by metoprolol and that neutrophil-platelet interactions (important for the infarct phenotype) are diminished with the drug.[87] This finding is supported by another seeing that TNFa and clenbuterol co-administration, but neither alone, increases neutrophil infiltration into the rat brain.[88] TNFa could sensitize the action of clenbuetrol and, broadly, other adrenergic signals in these TNFa administration experiments and with endogenous TNFa within inflammatory contexts. Clenbuterol, here might implicate, the beta 2 adrenergic but it is likely that it could have acted through the beta 1 adrenergic receptor.[89] Supporting this idea, metoprolol decreases levels of MPO within spinal cord tissue.[90] Still, the potential role of the beta 2 adrenergic receptor in upregulating neutrophil recruitment is supported as well. In a skin wound model, epinephrine or salbutamol can increase the number of neutrophils at the wound site and that wound size can be decreased with ICI 118, 551 or neutrophil depletion.[91] Thus, beta adrenergic receptor promotion of neutrophilia in various models of inflammation is mediated by the beta 1 adrenergic receptor but the influence of beta 2 adrenergic receptors remains a possibility.

There Remain Some Contexts in Which the Beta 1 Adrenergic Receptor Might Play a Role but Has Yet to be Tested

Some experiments utilizing non-selective adrenergic receptor pharmacology may implicate the beta 1 adrenergic receptor in positive regulation of neutrophils, but lack of target specificity precludes a firm conclusion. Wong et al (2018) finds that carvedilol decreases monosodium urate (MSU), an inflammasome activating crystal typically used in peritonitis models, induced neutrophil influx into the peritoneum.[92] This could happen through the beta 1 adrenergic receptor but carvedilol is nonselective across beta and alpha adrenergic receptors. Wrobel et al (2016) find that neutrophil influx into tumors decreases with propranolol.[41] Although this also could be mediated via the beta 1 adrenergic receptor (perhaps sensitized by hypoxia) there is also a strong possibility that propranolol causes a direct effect on the tumor. Finally, in thermal injury rats, propranolol decreases the number of neutrophils recruited to the burn site.[93] This seems to contradict the proposal of Nicholls et al (2017) who proposed that neutrophils have decreased function in response to adrenergic stimulation, but it is not clear in either case

which specific receptor is having an effect. Models here exhibiting a promoting relationship between neutrophil and beta-adrenergic receptor activity would most likely be through stimulation of the beta 1 adrenergic receptor but without selective pharmacological targeting or genetic manipulation, the receptor responsible remains uncertain.

III. Contextual Variables Controlling the Effect of Beta Receptor Activity on Neutrophil Function

Beta Adrenergic Effects Could be Sourced from the Adrenal Gland

The role of the adrenal gland is not entirely clear in this paradigm. One study demonstrates in an ozone induced lung damage model that adrenalectomy decreases the number of neutrophils in BAL fluid under the condition that clenbuterol and dexamethasone is administered [94] The same group demonstrated that propranolol administration also decreases neutrophils in BAL in this model.[95] In a non-stress model, Ferraz de Paula et al (2014) found that MDMA-treated mice have decreased oxidative burst and phagocytic activity and, that propranolol but not ICI 118, 551 or 6-OHDA can reverse effects, suggesting a beta 1 adrenergic receptor, adrenal gland-derived effect.[96] In comparison, a different study shows that adrenalectomy has no effect on social stress induced increase in neutrophils but that phentolamine abrogates this increase.[7] This could mean that while usually the effect of the adrenal gland is mediated through beta 1 adrenergic receptors, if the effect alpha receptors dominate, the adrenal gland will play a smaller role. If true, it would be important to know which factors create an alpha dominant versus a beta dominant adrenergic receptor environment.

Human Variation in the Effects of Beta-Adrenergic Receptors on Neutrophils Can be Explained by Sex Effects and Patient Health Variables

Gender effects, as with NK cells, are seen with the effect of the beta 2 adrenergic receptor on neutrophil function. In an air pouch model, LPS induces neutrophil recruitment in females but not males.[97] Males deficient in *Adrb2* have restored neutrophil recruitment suggesting that in males the beta 2 adrenergic receptor acts to inhibit the infiltration of neutrophils. In contrast, audiogenic stress increases neutrophil influx into rat air pouches in males but not in females.[98] Adrenal denervation decreases neutrophils in the air pouch model while isoproterenol increases them in males. The effect of adrenal denervation is recapitulated by propranolol in males. Gonadectomized males have a blunted neutrophil response with stress while there are no changes for females. Lastly, female mice, experience an increase in neutrophils with propranolol in response to LPS but not as much when exposed to both LPS and stress. The added stress stimulus complicates the effect of adrenergic signals on neutrophil function (discussed in subsequent sections) and interacts with the gender effect. Although these studies seem to suggest that neutrophils in males are more responsive to beta adrenergic stimulation than females, one study found that, at baseline, human neutrophils of

females express higher levels of the beta 2 adrenergic receptor than those of males.[99] Future studies will need to determine how this difference might change with inflammatory stimulus or stress. There are not very many other factors between humans that stand out to alter the sensitivity of adrenergic signals but there is one study showing that cystic fibrosis patients have neutrophils with lower beta 2 adrenergic receptor expression than healthy controls and obligate cystic fibrosis heterozygotes.[100] While the consequence of this has not been formally tested, other studies within the lung detailed below would suggest that this would increase the number of neutrophils within airways. Finally, diet might play a role in sensitizing adrenergic receptors as ICI 118, 551 treatment decreases neutrophil number and MPO in obese Zucker rats but not lean Zucker rats undergoing severe trauma of the hind limbs.[101] Collectively, the gender effect constitutes a major contribution of variation in beta receptor activity on the neutrophil response and future studies connecting models to patients will help to identify more factors that change the function of beta adrenergic receptors with respect to neutrophilia.

Beta Adrenergic Receptor in Neutrophils Summary

Both beta 1 adrenergic receptor and beta 2 adrenergic receptors play a part in neutrophilic inflammation but could play opposing roles. Generally, beta 1 adrenergic receptors tend to have a negative effect on neutrophil activity while beta 2 adrenergic receptors promote it. Stress models tend to implicate the beta 1 adrenergic receptor. Consistent with this, the beta 1 adrenergic receptor has been shown to be strongly expressed within neutrophils upon stress.[81] Some of the aforementioned studies suggest that adrenergic signals work in tandem with others in the context of stress and perhaps these other signals sensitize the beta 1 adrenergic receptor within neutrophils. Beta 2 adrenergic receptors are repeatedly involved in splenic effects on neutrophil trafficking and in models of lung inflammation. There are not many studies testing the role of the beta 3 adrenergic receptor. One study shows that using a selective beta 3 adrenergic antagonist, SR 59230A, they could decrease the number of circulating neutrophils within a chronic stress model.[102] However, they did not see a difference in the number of circulating neutrophils in stress models genetically deficient for the beta 3 adrenergic receptor, complicating the implication of this receptor. Xue et al (2018) did not find expression of the receptor in neutrophils within their stress model but the effect could be mediated through cell extrinsic effects.[85] Supporting this possibility, Flach et al (2013) find that CL316, 243 agonism of the beta 3 adrenergic receptor increases neutrophils in adipose tissue and increases endothelial ICAM-1 and selectin expression in vivo but not in vitro.[103] Genetic ablation of E-selectin in mice abrogates the effect of beta 3 adrenergic receptor agonism on neutrophil infiltration. Overall, beta 1- and beta 2- adrenergic receptors have distinct roles in neutrophil recruitment whose sensitivity is dependent on the inflammatory context, and beta 3 adrenergic receptors have limited support to extrinsically promote neutrophilia.

ALPHA ADRENERGIC RECEPTOR STUDIES

Unlike the NK cell studies, neutrophil studies repeatedly implicate alpha adrenergic receptors. Compared to the aforementioned studies of beta-adrenergic receptors, there are not as many in number, but they highlight a clear impact of the alpha 2 adrenergic receptor in both promoting and dampening neutrophil activity based on geographical context.

I. Inverse Relationship Between Neutrophil Activity and Alpha Receptor Activity

Alpha 2 Adrenergic Receptors Inhibit Neutrophil Entry into the Lung

There are a number of studies showing that alpha adrenergic receptors preclude infiltration of neutrophils into the lung. In LPS induced acute lung injury (ALI) phentolamine increases lung infiltration while in LPS and other models of ALI (including intranasal IL-17 and ventilation injury), alpha 2 adrenergic agonists UK14304 and dexmedetomidine decrease neutrophil influx into the lung.[104–106] Notably, phenylephrine has no effect on the influx of neutrophils although it did in the case of Arcaroli et al (2002).[44,104] The effect of the alpha 2 adrenergic receptor signaling in positively regulating neutrophil number and infiltration can be extended to other models. UK14304 and xylazine increases neutrophil increases peritoneal infiltration in thioglycolate and air pouch models, an effect reversible by alpha 2 adrenergic antagonists.[107] Dexmedetomidine, a selective alpha 2 adrenergic agonist, decreases infiltrating neutrophil number in the context of subarachnoid hemorrhage.[42] One exception to these patterns in the lung is that influenza infected mice have fewer lung and BAL neutrophils with 6-OHDA induced sympathectomy.[34] An alpha adrenergic receptor mediated effect is implied as phentolamine but not propranolol improves survival in the model. It is not clear whether this is due to the alpha 1 or alpha 2 adrenergic receptor. These studies indicate that alpha 2 adrenergic receptors downregulate neutrophil infiltration into the lung.

II. Promotion of Neutrophil Activity Through Alpha Adrenergic Receptors

Alpha 2 Adrenergic Receptors Promote Neutrophil Infiltration in Inflammatory Contexts Outside of the Lung

The studies implicating a positive relationship between alpha adrenergic receptor function and neutrophil activity are more of a mixed bag in the type of models. Importantly, the majority of them are models of inflammation concerning organs other than the lung. A few studies have explored the effect of combining berberine, a dopamine receptor antagonist, and yohimbine, an alpha 2 adrenergic

receptor antagonist. In a cecal ligation puncture (CLP) model, berberine and yohimbine co-administered decrease lung and liver damage and neutrophil infiltration in an IL-10 dependent manner.[108] The study surmises that IL-10 dependent decreases in neutrophil CCR2 expression mediate this effect. One study supporting this finding shows that myocardial MPO is decreased by BRL 44408, another alpha 2 antagonist, in the CLP model.[109] This would suggest that yohimbine, by blocking the alpha 2 adrenergic receptor, blunts neutrophil infiltration . Two other studies using yohimbine find varying results with its co-administration with berberine. In a model of LPS-induced ALI, yohimbine has an independent effect and an additive effect to berberine, while in a model of LPS-induced ileitis, berberine decreases neutrophil infiltration, but yohimbine confers no added effect.[110,111] Thus, there could be an interaction between the inflamed organ and how other signals will sensitize the adrenergic signal. A couple of studies show that genetic ablation of alpha 2a and alpha 2c adrenergic receptors or sympathectomy results in decreased recruitment of neutrophils to excisional.[112,113] Although they did not test the alpha 2B receptor ,a different study shows that overexpression of the alpha 2B receptor in mice increases the number of infiltration neutrophils in the context of MSU induced peritonitis. Further, they demonstrate that this effect is cell-intrinsic by showing that neutrophil recruitment is increased when transferring these transgenic neutrophils irrespective of the host background genotype. One limitation here is that the forcing of alpha 2B adrenergic receptor could cause the receptor to become functional in a context where it would not normally be induced. Lastly, clonidine, an alpha 2 adrenergic antagonist, increases activation of neutrophils (CD18 marker) in local but not systemic circulation (CD18) in humans with forearm ischemia/reperfusion.[61] While it's difficult to generalize the contexts in which alpha 2 adrenergic receptors will play a role, it is clear that many times they promote neutrophilia in diverse contexts outside the lung.

Alpha Adrenergic Receptor in Neutrophils Summary

These studies together suggest that the alpha 2 adrenergic receptor plays a central role in the coordination of the neutrophil response. It seems that alpha 2 consistently plays a role to downregulate neutrophil infiltration into the lung (consistent with Arcaroli et al 2002). Outside of the lung, the alpha 2 adrenergic receptor tends to play the opposite role but the reason for this is unclear. As with beta adrenergic receptors, this difference might be explained by cell extrinsic effects acting in one place (in this case it might be the lung) and cell intrinsic effects playing a larger role in other organs. There are only a few studies considering the effects of the alpha 1 adrenergic receptor on neutrophil activity. Altenburg et al (1997) find that phentolamine and prazosin (but not propranolol) mitigates the induction of circulating neutrophils in response to intravenously administered LPS.[114] In opposition, prazosin increases neutrophil number in exercising mice while exercise alone decreases neutrophil number.[115] It seems that, like alpha 2 adrenergic receptors, alpha 1 adrenergic receptors may play a role dependent on the context of inflammation. Overall, the studies

concerning alpha receptor effects on neutrophil function repeatedly implicate the alpha 2 adrenergic receptor in multiple models and highlight the lung as an environmental context in which the function of this receptor changes compared to others.

Summary of the Adrenergic Code of Neutrophils

Following the model of Aracaroli et al (2002) the field conveys both differences in the effects of different adrenergic receptor subtypes on the neutrophil response and context-specific roles for these receptor subtypes. The beta 1, beta 2, and alpha 2 receptors have strong support for their role in controlling neutrophil recruitment while beta 3 and alpha 1 receptor have received limited attention. Generally, beta 1 receptors promote neutrophilia, beta 2 receptors inhibit neutrophilia, and alpha 2 receptors can promote or inhibit neutrophilia based on whether the site of inflammation lies outside or within the lung, respectively. Beta 1 receptors play an important role in the stress environment but also inflammatory contexts outside of the lung whereas beta 2 adrenergic receptors play an important role particularly in lung inflammation in addition to other inflammatory conditions. These studies add to the example of Arcaroli et al (2002) in that they show the differential effects of specific beta-adrenergic receptors and highlight effects outside the lung. It is unclear why the lung stands out in the patterns of effects on neutrophils. It could be biological in that receptor expression or regulation differs within the lung or it is likely that the abundance of studies within the lung masks circumstances where inflammation in other organs might exhibit similar patterns. The most informative studies in identifying these patterns are ones where selective agonist or antagonist are used or where genetic ablation of the receptor within models implicates a specific receptor. Human studies do well in helping to identify factors that cause receptor activity to differ within individuals, creating the opportunity for more targeted studies. Currently, human studies or clinical trials considering neutrophilic inflammation are in few but as adrenergic receptor intervention becomes tested more frequently, we might be able to ascertain whether differences between species exist. Together, these data tell us that in both research and potential clinical efforts care should be taken to establish which adrenergic receptor subtypes are the most pertinent within the disease context and that certain aspects of the inflammatory environment can help predict what receptor subtype that is and how one might target it to control the neutrophil response.

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Adrenergic Signaling and Monocytes

Monocytes are the final cell subset considered in this review. Monocytes have important antimicrobial effects and are gaining more attention in inflammatory diseases and cancer.[116] Monocytes can, on their own, adopt a pro-inflammatory or classical state of activation or anti-inflammatory (non-classical) profile determining their own function and cytokine production. Monocytes can become macrophages or dendritic cells adding to their flexible role in health and disease. Still, studies describing *in vivo* effects of adrenergic signaling on monocyte recruitment and function are sparse. This section explores what patterns can still be gleaned with the current state of the field.

Studies at Homeostasis Tend to Describe a Stimulatory Effect of Adrenergic Signaling on Monocyte Recruitment

There are only two human studies utilizing noradrenaline infusions to test the effect on monocytes, one showing that NE infusion increases monocytes in blood and the other finding no effect.[17,25] Adrenaline infusions have been done more frequently but not by much. Dimitrov et al (2010) found that adrenaline infusion increases nonclassical (CD14dimCD16+) but not classical monocytes(CD14+ CD16-) in volunteers.[117] Consistently, Kittner et al (2002) found that in female rheumatoid patients and healthy controls have increased circulating CD14+ monocytes with CD14+ CD16+ cells representing the largest increase in proportion.[23] Additionally, this study shows that there are little differences between patients and controls. Isoproterenol studies are like adrenaline studies where one study shows a promotion of monocyte number with adrenergic stimulation, and another shows no change. The latter study shows that isoproterenol administration does not change the number of monocytes in healthy or tumorized mice.[27] This study does not test whether monocyte subsets are changed. The other demonstrates that daily isoproterenol increases the number of monocytes within bone marrow, presumably by increased myelopoiesis.[118] It is uncertain whether these bone marrow monocytes are mobilized by isoproterenol alone. Another study, however, bolsters the idea that beta receptors can increase monocyte recruitment. Amphetamine promotes monocyte egress from spleen in rats, but can be prevented with propranolol further suggesting that beta receptors can induce monocytes to circulate.[119] Whether this is different between bone marrow and spleen requires further study. In addition to the dearth in studies at baseline, it is difficult to compare results among studies at homeostasis as the method for phenotyping and isolating monocytes is variable between studies. What we can gain from these studies is that adrenergic stimulation is capable of altering both monocyte number and activation state, although the specific effects of stimulation need more studies to clarify the relationship between adrenergic signals and monocyte activity at homeostasis.

Alpha 2 Adrenergic Receptors Tend to Promote the Recruitment of Anti-Inflammatory Monocytes

There are a few experimental studies considering the role of alpha-adrenergic receptors in altering monocyte activity and they tend to suggest that adrenergic signaling increases the number of anti-inflammatory monocytes. Beis et al 2018 found that noradrenaline infusion in humans undergoing a stress task increases the number of circulating monocytes. This is reversible by phentolamine. In contrast, a separate group demonstrates that prazosin increases the number of monocytes within mouse.[120,121] While it is possible that this difference is species-specific, it is more likely that the alpha 2 receptor is responsible for the effect demonstrated by phentolamine. In agreement with the functional relevance, there are 2 studies showing that dexmedetomidine agonism of the alpha 2 receptor within cancer patients promotes an anti-inflammatory phenotype in monocytes. The first shows that dexmedetomidine decreases NFkB activation in peripheral blood monocytes (Dong et al. 2017).[122] The second shows that dexmedetomidine increases the number of monocytic myeloid-derived suppressor cells with increased potential to downregulate T cells and produce vascular endothelial growth factor (VEGF).[123] In the infusion studies, adrenergic stimulation alone tended to skew monocytes toward a pro-inflammatory phenotype while the studies concerning alpha receptors within inflammatory/stress models show a trend where adrenergic stimulation results in anti-inflammatory skewing. This could be due to a difference in signals in the inflammatory milieu or a consequence of a difference between the action of alpha and beta receptors. The few studies here suggest that alpha 2 adrenergic receptors act in promoting the circulation of anti-inflammatory monocytes.

In Stress Models, Beta Receptors Augment Monocyte Circulation

There are a handful of studies concerning the role of beta-adrenergic receptors which show that stress induces an increase in monocyte number via beta adrenergic receptors. In human volunteers undergoing a social stress test, propranolol decreases transcriptional representation of CD16+(non-classical) monocytes.[124] Two studies in male rats demonstrate that social stressors can increase Ly6Chi macrophages and CD14+ (classical) microglia in the central nervous system, and can increase the number of monocytes in blood.[7,125] Both of these can be reversed by propranolol but not by adrenalectomy implicating sympathetic neurons in the expansion of the monocytic population.

A study in female rats found that chronic unpredictable stress results in increased metastatic colonization of cancer cells, and that this could be prevented with 6-OHDA, propranolol, or clodronate-mediated depletion of macrophages.[126] Isoproterenol administration alone recapitulates this effect, increasing the number of macrophages within, lung, blood, spleen, and bone marrow in a CCL2-dependent. By completing the picture, showing that CCL2 increases in chronic unpredictable stress can be abrogated with propranolol, the authors present a model in which beta-adrenergic receptor stimulation exacerbates cancer

metastasis by recruitment of monocytes via CCL2. The beta 3 adrenergic receptor is also implicated in the context of stress. Heidt et al (2014) demonstrates that in humans SR59230a decreases stress-induced Ly6Chi monocyte number. Interestingly, genetic ablation of the beta 3 adrenoreceptor has no effect. It is unclear whether the effects of propranolol act through beta 1 or beta 2 adrenergic receptors but one of these and the beta 3 receptor in the context of stress have been shown to increase monocyte recruitment. It has not been formally shown that these recruited monocytes exhibit a proinflammatory or anti-inflammatory phenotype; monocytes exacerbating metastatic colonization of cancer cells suggest an anti-inflammatory phenotype while Ly6Chi macrophages in the central nervous system tend to be inflammatory. Altogether, while it is clear stress models tend to promote the circulation of monocytes, the specific receptors at play and the inflammatory profile of recruited monocytes under these conditions is unclear.

In Various Inflammatory Models, Beta 2 Adrenergic Receptor Stimulation Increases the Recruitment of both Inflammatory and Anti-Inflammatory Monocytes Based on Context

A collection of inflammatory models, as with stress models, show a divide in monocyte skew toward inflammatory or anti-inflammatory but overall tend to demonstrate an increased recruitment of monocytes with beta adrenergic receptor stimulation. Kobayashi et al (2011) demonstrate that beta adrenergic receptors in burn patients mediate the recruitment of anti-inflammatory monocytes.[127] Propranolol treatment rescues and lowers CCL1 production in monocytes. The beta adrenoceptor blockade also reverses lowered IL-12 and increased IL-10 production in monocytes from burn patients. On the other hand, there are studies demonstrating that beta adrenergic receptors recruit inflammatory monocytes. In two separate studies of lung inflammation one shows that beta 2 adrenergic agonist salmeterol increases monocytes in circulation and the second shows that formoterol, another beta 2 adrenergic receptor agonist, decreases the recruitment of classical monocytes while promoting the recruitment of nonclassical monocytes. [63,69] The latter proposes that sympathetic neurons are responsible for this phenotype. In line with this, renal denervation decreases Ly6chi monocytes responding to ischemia/reperfusion injury.[60] In addition to these single stimulus studies, beta adrenergic receptor stimulation seems to be able to promote monocyte activity on longer time scales after pre-sensitization by an inflammatory stimulus. Saeed et al (2014) *Candida albicans* pre-sensitization protects against mortality with lethal *C. albicans* infection but not with the co-administration of propranolol.[128] The authors suggest this is due to abolishment of "trained" monocytes but more experiments need to be done to formally impute monocytes and their "training" in beta adrenergic receptor mediated protection against *C. albicans* infection. Finally, there are effects of the beta 2 adrenergic receptor that can promote both classical and non-classical monocytes. In humans, exercise induces the circulation of both monocyte subtypes and nadolol but not bisoprolol can strongly dampen this effect, implicating the beta 2 adrenergic

receptor as a stimulator of monocyte circulation.[11] The effects of exercise are likely due to a stimulation of immune cells within an otherwise non-inflamed milieu. These studies demonstrate that the environmental context will skew which types of monocytes are recruited although in most cases the beta 2 adrenergic receptor will recruit pro-inflammatory monocytes.

The Effect of the Beta 2 Adrenergic Receptor on Monocytes Can Change with Gender or Health Condition and the Beta 1 Adrenergic Receptor Can Explain Effects that Oppose its Function

Although the majority of studies show that beta adrenergic signaling, especially via the beta 2 adrenergic receptor, promotes monocyte recruitment, there are some factors that can cause adrenergic stimulation to inhibit monocyte recruitment. Ostensibly, one study selectively targeting the beta 1 adrenergic receptor within heart tissue describes an inverse relationship between beta adrenergic receptors and monocyte recruitment. Garcia-Prieto et al (2017) find that monocyte infiltration is increased with metoprolol in myocardial infarction in mice.[87] In another study bisoprolol slightly increases the proportion of intermediate monocytes in circulation in exercising humans.[11] These studies implicate the beta 1 receptor in negatively regulating monocyte recruitment. This is in contrast to the action of beta 2 adrenergic receptors which tend to promote monocyte recruitment. Still, beta 2 adrenergic receptors can also have the opposite effect under particular conditions. To start, obese models tend to show an opposite effect of beta 2 receptor stimulation compared that of lean models. Noh et al (2017) demonstrate in Zucker diabetic fatty rats that salbutamol decreases CCR2+ cells in blood and bone marrow.[129] Salbutamol also decreases NOS2 and CD68+ cells in heart and kidney, signifying that beta 2 adrenergic receptor stimulation in these rats precludes the recruitment of pro-inflammatory monocytes. Consistently, terbutaline decreases monocyte production of proinflammatory cytokines MCP1 and IL-8 and increases monocyte production of anti-inflammatory cytokine IL-10 in obese mice. Terbutaline has the opposite effect in lean mice, augmenting monocyte production of proinflammatory cytokines MCP1 and IL-6 and decreasing monocyte production of anti-inflammatory mediators TGFB and IL-10.[130] Following this trend, monocytes from lean mice treated with terbutaline have decreased expression of Arg1 (anti-inflammatory, M2-like marker) and TLR4 while in obese mice terbutaline decreases monocyte expression of iNOS (pro-inflammatory, M1-like marker) and TLR4. Thus, the beta 2 adrenergic receptor can have opposite effects on monocytes in both recruitment and inflammatory profile in obese and lean rodent models. Interestingly, in both models, acute exercise strongly induces monocyte expression of the beta 2 adrenergic receptor and ex vivo studies suggest that this switch the effect of the receptor on monocytes from obese mice.[131] Thus, the inflammatory milieu in an obese model could change the receptor expression in monocytes changing the effect of their stimulation on monocyte activity. Another strong effect, as with

both neutrophils and NK cells, the effect of the beta 2 adrenergic receptor is dependent on gender. In the previous section, formoterol was shown to promote monocyte entry into the lung but Murad et al (2017) demonstrates formoterol improves fluticasone mediated decrease in monocyte in bronchoalveolar lavage fluid in female mice. Consistently, propranolol increases the number of MHCII negative macrophage within a choroidal neovascularization model in female mice but not male mice. [132] These studies together highlight gender effects, obesity, and beta 1 adrenergic receptor stimulation as means by which beta adrenergic receptor stimulation can result in the inhibition of monocyte recruitment.

Summary of the Adrenergic Code of Monocytes

Analysis of the effect of adrenergic signaling on monocytes is limited by the number of studies dedicated to it but there are emerging patterns in the literature as of now. Both at homeostasis and in stress and inflammatory models, beta receptors tend to promote the recruitment of monocytes. Nuancing this picture, inflammatory models show that beta adrenergic signaling tends to promote a pro-inflammatory profile of monocytes but that can also recruit anti-inflammatory monocytes under certain conditions. As with neutrophils and NK cells there are not many studies describing the effects of adrenergic signaling on specific functions of monocytes *in vivo*. One might assume that with increased recruitment comes increased function but one study shows that propranolol increases monocyte phagocytic activity *in vivo* when most studies show that propranolol precludes monocyte recruitment (Shilov and Orlova 2000).[86] Although there are only a few studies concerning the beta 1 adrenergic receptors, it seems that its effect in monocytes is opposite to that of the beta 2 adrenergic receptor. This was also seen for neutrophils. Also, like neutrophils and NK cells, factors such as sex, health status, and the level of other mediators can act to switch the effect of beta-adrenergic receptor stimulation. Bedoui et al, showing a similar pattern for NK cells, demonstrate that Neuropeptide Y (NPY) increases the epinephrine-induced increases in monocytes at low epinephrine concentration but decreases monocytes at high epinephrine concentrations.[24] With medium epinephrine concentration, NPY increases monocyte number at earlier time points but decreases it after some time. Alpha receptor studies, although small in number, tend to demonstrate that alpha 2 receptors tend to promote the recruitment of anti-inflammatory monocytes while alpha 1 receptor might preclude monocyte recruitment. As for the source of stimulation, most studies impute sympathetic neurons rather than the adrenal gland although there are barely any studies testing or ruling out the role of the latter. There is also a lack of testing whether the effects of adrenergic stimuli can act through extrinsic means. This is likely because it is difficult to specifically target monocytes genetically but the lack of a difference of effects of adrenergic stimulation across various organs might suggest that extrinsic effects play a smaller role. The analysis of this review is limited in that it does not include data concerning macrophages and dendritic, and

it would likely clarify the effects of adrenergic on downstream monocyte activity but is outside the scope of this review. Despite the small number of studies describing the effects of adrenergic signaling on monocytes *in vivo*, the current literature demonstrates an important role for the beta 2 adrenergic receptor in stimulating recruitment and important contextual factors that can alter its function.

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What Analyzing the Adrenergic Code in Recruited Immunity Tells Us About the Field and the Nature of Neuroimmune Signaling

Taking a bird's eye view of the data of NK cells, neutrophils, and monocytes, there are more frequently utilized methodologies than others. An overwhelming majority of studies employ pharmacological techniques and of these, nonselective antagonists such as propranolol are used the most. This makes sense as the field progresses in creating more selective agents and, despite their limitations, nonselective agents have paved the way for further inquiry into the specific receptors responsible for neuroimmune interactions. For example, some of the first studies describing the relationship between adrenergic receptors were studies of infusing norepinephrine and epinephrine into volunteers and observing changes in immune cell number. In many cases there is a dominant adrenergic receptor in play and the nonselective agents will act on it to change immune cell behavior. There are cases shown here where a selective agent has a different effect compared to the nonselective agent within the same context. This is important as it contributes to the noise in the apparent relationship between adrenoreceptor activity and immune cell activity but also allows for intervention of immune cells in multiple ways within the same context. Comparatively, there are not as many genetic approaches employed. Pharmacological studies are more convenient but clearly have limitation in selectively targeting cell subsets, so genetic approaches are preferable. Still, it is impractical to start with conditional genetic models in the primary stage of determining whether adrenoreceptors play a role in a particular context, so pharmacological studies are best initially. Similarly, nonselective agents are helpful especially when there are multiple receptor subtypes that could potentially play a role (serotonin has more receptors than norepinephrine and epinephrine). The caveat here is that non-selective agents are more likely to have off-target effects, making follow-up with more selective agents almost a requirement for interpretation. Another reason that conditional genetic ablation is not great to start with is that it automatically precludes the analysis of cell extrinsic effects. For instance, adrenergic receptors in epithelia can control the ability of immune cells to infiltrate and can explain why in some contexts the same compound can cause opposing effects. Collectively, the majority of studies

to date concerning the role of adrenergic receptors in influencing immune cell activity are pharmacological, which has its limitations in not selectively targeting cell subsets but advantage in its potential to capture an effect of adrenergic signaling in the first place.

While both beta and alpha receptors have been imputed in each of these cell subsets, it is clear that beta adrenergic receptors receive much more attention. This could be a reflection of the biology, that beta adrenergic receptors are more important in orchestrating immunity but is more likely a consequence of the way adrenergic receptors are studied. There are many more compounds that selectively or non-selectively alter the activity of beta-adrenergic receptors and thus are easier to obtain and test. The effects of alpha receptors emerge in more recent papers as researchers come to realize their importance. This could also explain the lack of studies implicating the beta 3 adrenergic receptors. The most commonly used beta-adrenergic antagonists' propranolol and nadolol, although nonselective, do not target this receptor and thus its role could be ignored if selective agents are not used. A tool that has been better utilized to implicate these receptors initially is 6-hydroxydopamine (6-OHDA). 6-OHDA has been used to peripherally sympathectomize rodent models for decades now and is being increasingly used to test the role of neuroimmune circuits. This method is least selective and allows for the most adrenergic receptor subtypes to be tested. With that said, 6-OHDA ablates both dopaminergic and noradrenergic neurons, so co-administration of desipramine needs to be used to delineate the roles of each neuronal population. Further, this method is not going to pick up adrenergic signaling axes sourced from the adrenal gland. Still, the use of 6-OHDA is useful for many disease contexts where sympathetic neurons might play a role. Caution should be taken to interpret the results of 6-OHDA without downstream analysis of receptors as neurons secrete various neuropeptides and neurotransmitters. For instance, if one observes an effect of 6-OHDA this might be due to adrenergic signaling but could equally be likely to be due to NPY or RGM-A.

As may be apparent here, the majority of *in vivo* data detail the number of an immune cell subset within blood or an organ. The focus of these studies is not to create the "adrenergic code" so data such as inflammatory profiling of immune subsets may not be as pertinent to the story presented in these studies. Still, as we see context-specific differences in recruitment, one might also predict context-specific changes in the effect of adrenoceptors on immune cell functions and phenotypes. *In vivo* assays monitoring phagocytosis, cell-specific cytokine production, chemotaxis, etc. allow for the analysis of context-specific immune cell behavior that could only be tested *in vitro* before. Learning how adrenergic signals affect immune cells in the future will be aided by single-cell RNA sequencing where one might compare the transcriptomic changes due to adrenergic stimulation (could be extrinsic or intrinsic) across separate disease contexts. The major findings here are that even with primary focus on immune cell recruitment, there are stark differences and, importantly, predictable differences in receptor function based on subtype and environmental context.

This approach to understanding the context-specific effects of adrenergic signaling on orchestrating immunity has been revealing of the how one might navigate the complexity of the effects seen with the use of adrenergic axis-targeting compounds and of the approach to determining whether adrenergic signaling is important for a particular disease context. While the information here should not be used as clinical information, it can be used to think about how different patients might respond to future treatments intervening in adrenergic pathways. Furthermore, analyses like this can clue us into how other treatments such as antidepressants might 1) be repurposed for immunomodulation or 2) be already altering the immune system, which could, for instance, predispose someone to infectious disease or change the course of a pre-existing inflammatory condition. More broadly, putting these studies together has allowed for the analysis of the methods used in creating our current understanding in separate fields. There seem to be pockets of disconnected data that are saying the same thing and can be generalized to more than to, say, the asthma field or rheumatoid arthritis field or neutrophil field of study. In the same vein, human studies and rodent models' studies are undeniably performed in different ways. While not many species-specific effects were identified here, human association studies (e.g. patient-variable relationships) in the future concerning adrenergic signaling in inflammatory contexts might shed light on the potential to translate findings from model disease contexts to those of humans.

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Chapter 3 - Connecting C. difficile and the SNS

(Adapted from Thesis Proposal)

C. difficile **infection is prevalent, costly, and requires the development of novel therapeutics.**

Clostridioides difficile is a Gram-positive, obligate anaerobic, spore-forming bacterium responsible for the most common health-care associated infection.[1] In 2017, there were an estimated 462,100 cases of *C. difficile* infection(CDI) and at least 12,800 people died of the infection.[1–3] Currently the most common therapies for CDI are antibiotics. While antibiotics are the primary treatment for CDI, there is concern for the inevitable emergence of antibiotic-resistant strains (Miller, 2007). A more imminent concern for the use of antibiotics is for their tendency to increase the risk of recurrent infection. The rate of recurrent CDI is 13% and 20.9% for community-acquired and hospital-acquired CDI respectivelyClick or tap here to enter text.[1]. The Petri Lab, the lab where I worked for this dissertation work, identifies host-targeted approaches toward CDI having identified the immune system as a key modulator and biomarker of disease severity in mice and humans .[4–8] In looking to expand our view of host factors driving disease, we have considered that the nervous system could play a central role in disease as well as it densely innervates the intestine and has known roles in immune system orchestration and colitis.[9–12] Following preliminary data suggesting a role for the sympathetic nervous system (SNS) in the severity of CDI pathology in murine models, we decided to focus on this branch. We explored the contribution of the SNS to CDI and the experiments designed to dissect a mechanistic understanding of its influence in modulating disease.

Sympathetic nervous system contributes to colitis and infectious disease

The sympathetic nervous system has important roles in a number of inflammatory diseases such as colitis, inflammatory arthritis, neuroinflammation, cardiovascular disease, and cancer as well as in viral, bacterial, and parasitic infections.[10,13–21] The SNS responds on the scale of milliseconds, making it well equipped to respond to challenge and shifts in homeostasis, of particular note in intestinal barrier function and inflammation. [22] As such, many have found that ablation of the peripheral SNS with the neurotoxin 6-hydroxydopamine (6-OHDA) drastically shifts the course of disease in infectious and inflammatory contexts.[23–29] Despite our consistent ability to find a role for the SNS in disease, predicting the direction of its influence, whether it will function to ameliorate or worsen symptoms, is extremely difficult and seemingly case-by-case. This is likely because the SNS has multifactorial impact on factors influencing disease from effects on microbial growth and pathogenicity to direct modulation of immune system function.[30–33] With future studies it will be important to identify the direct targets

of the SNS that influence disease to find patterns in its role among different disease contexts.

Adrenergic receptors drive disease in a variety of contexts of inflammatory disease.

The SNS is capable of signaling to other neurons, immune cells, lymphoid tissue, and various cell types via an array of neurotransmitters and neuropeptides. The best studied influences of the SNS on disease are mediated through norepinephrine and epinephrine which bind adrenergic receptors (ARs) α1, α2, β1, β2, and β3. Though largely expressed in neurons, nearly all cell types are capable of expressing and responding to adrenergic stimulation. Following, adrenergic receptors have significant roles in various disease models. Relevant to this proposal, alpha ARs tend to promote inflammation in experimental colitis models whereas beta ARs do the opposite.[27,34–38] In viral and bacterial infections, the relationship between alpha ARs, beta ARs, and inflammation is similar yet with distinct mechanisms offered to explain the outcome. Namely, the alpha 2 and beta 2 ARs are repeatedly implicated in infectious and intestinal disease.[19,35,38–40] Still, the other adrenergic receptor subtypes and non-adrenergic neuromodulators have been shown to be critical in modulating disease. In line with the latter, cotransmitters neuropeptide Y and purinergic modulators have been shown to be important in IBD, experimental colitis, and *C. difficile* induced colitis.[41–44] In some cases, these cotransmitters can act synergistically with adrenergic receptors. Finally, some species of bacteria harbor norepinephrine sensors which modulate their growth, activity, and virulence in a form of transkingdom signaling.[18,23,30]

Sympathetic activity could influence CDI through local and non-local mechanisms

A central step in determining the role of the SNS in CDI is identifying the downstream effectors that mediate its effect but the locale of those effectors within the body is unknown. As a large variety of cell types respond to adrenergic stimulation, it follows that the sympathetic nervous system of organs outside the intestine could contribute to colitis. Studies have shown that the sympathetic nervous system is important in colitis, and further that the method of ablation of sympathetic neurons determine whether the intervention is beneficial or detrimental in disease progression.[27] Additionally, chemical sympathectomy has varying effects in infectious disease.[25,26,45–48] Thus, parsing out whether local sympathetic innervation of the colon is important for disease will not only enlighten likely downstream cellular targets mediating symptoms but also contribute to the broader understanding of the relative roles of the systemic sympathetic nervous system and local innervation to intestinal inflammation.

Potential Sympathetic Influence in *C. difficile*-related Immune Cells of Interest

C. difficile elicits a cascade of immune responses by toxin-mediated damage to the intestinal epithelia.[49–51] Following, these immune cell and effectors contribute to the clearance and resolution of the infection. Different cell types can have different effects on pathology and disease outcome as they affect the balance of inflammation and/or the ability to handle cell or toxin-mediated damage. Although adrenergic signaling has not been studied in the context of these different immune responses, the goal of this section is to connect immune pathways of interest in CDI to potential mechanisms by highlighting adrenergic influences on these pathways in other contexts (other tissues, other diseases, etc.).

Type 2 Immunity

Perhaps the most complete set of responses studied in the Petri Lab in the context of *C. difficile* is that of "type 2" immunity. Type 2 effectors generally are restorative, both dampening the level of inflammation and promoting repair of the intestinal barrier after insult.

Type 2 innate lymphoid cells (ILC2s) serve as a major mediator of type 2 immunity. ILC2s respond to the alarmin IL-33 and in response secrete type 2 cytokines including IL-13, IL-4, and IL-5. Depletion of innate lymphoid cells in total (not just ILC2s) with $Rag2^{-/-}$ $\gamma c^{-/-}$ worsens mortality in CDI mouse models.[7,52] This effect can partially be rescued by adoptive transfer of ILC2s into these mice.[7] In agreement, IL-33 stimulation is sufficient to increase ILC2 counts and improve survival and genetic ablation of the IL-33 receptor, ST2, worsens disease. Intestinal ILC2s, at least in the small intestine, can respond to adrenergic signaling as they express beta 2 adrenergic receptors (β_2AR). Stimulation of β_2AR in vitro or in vivo with salmeterol has a negative effect on ILC2 number and production of IL-5 and IL-13, type 2 cytokines also shown to be critical to CDI disease outcome.[53,54] Also importantly, β_2AR activity, though likely not directly as they do not highly express the receptor, affects the number of infiltrating eosinophils, another important cell type in CDI.[4,53] With these data taken together one could hypothesize that β_2AR activity in CDI would be detrimental if it has a negative effect on ILC2 function.

Eosinophils act downstream of ILC2 activity responding to cytokines such as IL-5. Both IL-5 and IL-25, a type 2 alarmin that activate ILC2s, is shown sufficient to increase eosinophil number and necessary for protection against CDI pathology.[4,6,55] Further, the effect of IL-25 in CDI mice is dependent on eosinophils as eosinophil depletion abrogates the effect of IL-25 administration on disease activity. The characterization of the effects of norepinephrine and epinephrine on eosinophils date back to at least 1950. Early studies demonstrated that intravenous administration of adrenaline was sufficient to reduce the number of blood circulating eosinophils.[56] Some studies determined that the effect of epinephrine on eosinopenia was far greater than that of norepinephrine.[57,58] Later studies up to the present have repeatedly implicated the β_2AR as a modulator of eosinophil number and function. Generally, β_2AR stimulation has a negative effect on eosinophil function as it decreases eosinophil adhesion to the vascular endothelium, degranulation, and respiratory bursts .[59–61] Eosinophils can also respond to α1ARs

and increase in number, of specifically Siglec-F^{hi} eosinophils, in response to stimulation.[62]

Macrophages, although not yet clearly implicated in CDI, can have a central role in type 2 immunity. Macrophages stimulated by type 2 cytokines can take on an "alternatively activated" or M2 phenotype and, in turn, secrete type 2 cytokines on their own. In general, macrophages are potently activated by adrenergic stimulation. A number of studies show that alpha 2 adrenergic receptor (α2AR) stimulation in macrophages increases their output of pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα.[63–67] Conversely, β_2AR tends to (but not always) have an opposite, anti-inflammatory effect.[34,67–73] Consistently, Gabanyi et al. found that muscularis macrophages have relatively increased expression of β_2AR compared to lamina propria macrophages and comparatively have an expression profile of M2 phenotype rather than the latter's M1 phenotype.[31] This means that sympathetic activity in CDI through macrophages could have a positive or negative effect on CDI outcome depending on which receptor subtype is dominantly active. If β_2AR activation is dominant in macrophages they will likely secrete cytokines such as IL-4 and IL-13 which would be beneficial to recovery.[55] On the other hand, if α2AR action dominates, macrophages might contribute to disease pathology by secreting norepinephrine-induced proinflammatory cytokines.

Adrenergic effects on type 2 immune effectors important in CDI will depend on both the cell type and adrenergic receptor type. If a dominant effect is found through α2ARs then it is likely that in suppressing the type 2 character of macrophages. If β_2AR is dominant, many other mechanisms are at play. Through macrophages the receptor can promote type 2 macrophages but on the other hand can have negative effects on both ILC2 and eosinophil activity. Determining the important receptors in CDI will give an idea on how likely these mechanisms are to play a role in disease.

Type 17 Immunity and T Cells

"Type 17" immunity is also an important set of immune responses to CDI. Generally, this class of responses is pro-inflammatory and promotes neutrophil infiltration into tissue. Type 17 cytokines such as IL-6 and IL-23 are associated with the severity of disease in CDI patients.[5,8]

Type 17 T helper (Th17) cells are central effectors of type 17 immunity. Th17 can promote neutrophilia and host defense by secreting IL-17, IL-6, and IL-23.[74,75] Saleh et al. demonstrated the importance of Th17 to CDI by using a mouse model treated with DSS (a colitis-inducing chemical) prior to infection to mimic intestinal bowel disease predisposition to severe CDI. Depletion of CD4+ T cells or blockade of IL-17A improves survival in CDI mice. Conversely, transfer of IL-17A+ CD4 T cells is sufficient to worsen disease. T cells express β_2AR though the role of the receptor on Th17 identity could be context specific. T cells from PBMCs from healthy patients respond to β_2AR agonist terbutaline by increasing expression of Th17 transcription factor, RORγ, and cytokine production of IL-17.[76,77] On the contrary, β_2AR stimulation in CD4+ T cells from a mouse arthritis model decreases

their IL-17 and IL-22 output.[78] The effect of β_2AR on T cell identity in CDI is unknown. Adrenergic stimulation can also affect T helper cell function broadly. For example, cell intrinsic- β_2AR agonism limits CD4+ T cell egress from lymph nodes and, during disease or secondary response, decreases their recruitment to peripheral tissues and impact on inflammation.[79] Stimulation by the SNS leads to adrenergic receptor crosstalk with chemokine receptors resulting in lymphocyte retention in the lymph node.

Neutrophils are the downstream effectors of the type 17 response and have a complex role in CDI.[80] On one hand the neutralization of neutrophils can lead to worsened disease.[81] In other cases, inhibiting neutrophil function and/or extravasation into the intestine is beneficial.[82,83] In patients, neutrophil activation markers are associated with increased disease severity.[84] Adrenergic signaling has a variety of effects on neutrophil activity. As with eosinophils, β_2AR stimulation decreases neutrophil number and function such as phagocytosis, chemotaxis, production of neutrophil extracellular traps, cytokines, and superoxides .[85–90] Again, this effect could be context-dependent as epinephrine can increase neutrophils trafficking or have little effect on neutrophil function.[91,92] Alpha adrenoreceptors seem to have the opposite effect on the number of circulating and tissue-infiltrating neutrophils with α-AR dependent stimulation. [40,93] The effects of adrenergic stimulation should be, however, predicted with caution. Among other context-dependent factors the outcome of adrenergic stimulation on neutrophil behavior can depend on sex and the cell-extrinsic effects.[94–96] As an example, β_2AR stimulation in endothelial cells can promote neutrophil egress from the bone marrow causing long-range effects.[97]

Not all type 17 responses are detrimental and that is exemplified by the role of type 3 innate lymphoid cells (ILC3s) in CDI. ILC3s secrete IL-22 to confer host protection against *C. difficile*.[52,98–100] ILC3s receiving β_2AR stimulation from the SNS during intestinal injury produce IL-22 to promote resolution of the epithelial barrier damage.[101] Interestingly, blockade of α2ARs abrogates homeostatic or stress-induced IL-22 and IL-17 production in ILC3s.[102]

As with adrenergic mechanisms and type 2 immunity, the effect of the adrenergic axis on type 17 immunity will depend on both cell type and receptor. The effect of adrenergic stimulation on Th17 cells seems to be context-dependent (e.g. state of inflammation). Neutrophil infiltration like eosinophil infiltration is generally dampened by cell-intrinsic β_2AR stimulation but cell-intrinsic mechanisms can cause the opposite effect. ILC3s can be stimulated by either β_2AR or α_2ARs and their effect on CDI depends on the cytokines they produce. Predicting the effects of adrenergic stimuli on type 17 immunity is just as complicated as for type 2 immunity. Here, a focus on whether the effects of adrenergic stimuli are on infiltrating or resident immune cells and whether those effects are cell-intrinsic can help parse out the most important mechanisms.

B cells

Antibody responses are essential to long-term protection against CDI and vaccine efforts in kind. Unfortunately, B cell activity and the quality of antibody response is hampered during infection.[103–105] Consequently, the ability to neutralize *C. difficile* toxins in subsequent infection is hindered. B cells, like T cells, are responsive to (nor)epinephrine through expression of the β_2AR. β_2AR stimulation can improve the amount and affinity of produced antibodies and B cell number.[106–108] For more information on the role of β_2AR in both B and T cells a review by Sanders discusses this at length.[109]

Investigating the role of the sympathetic nervous system in CDI will reveal therapeutic targets.

The study of the sympathetic nervous system in CDI is exciting not only for the future of its therapy but also in what it could mean for the study of other inflammatory and infectious diseases. In human and mouse studies of colitis, including our own study of *E. histolytica,* the alpha 2 adrenergic receptor has genetically been associated with disease.[110,111] In CDI, other infectious diseases, and IBD, depression and antidepressants both influence the risk for disease for reasons postulated but unknown.[112,113] Finally, it has been postulated that the SNS promotes disease in infections of gram-negative bacteria while ameliorating it for infections with gram-positive bacteria.[114] Our findings, presented in the next chapter, contradict this pattern, which suggests that unknown factors of SNS influence contribute to disease.

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Chapter 4 – The Sympathetic Nervous System Drives Hyperinflammatory Responses to Clostridioides difficile Infection (Published in Cell Reports Medicine)

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Summary

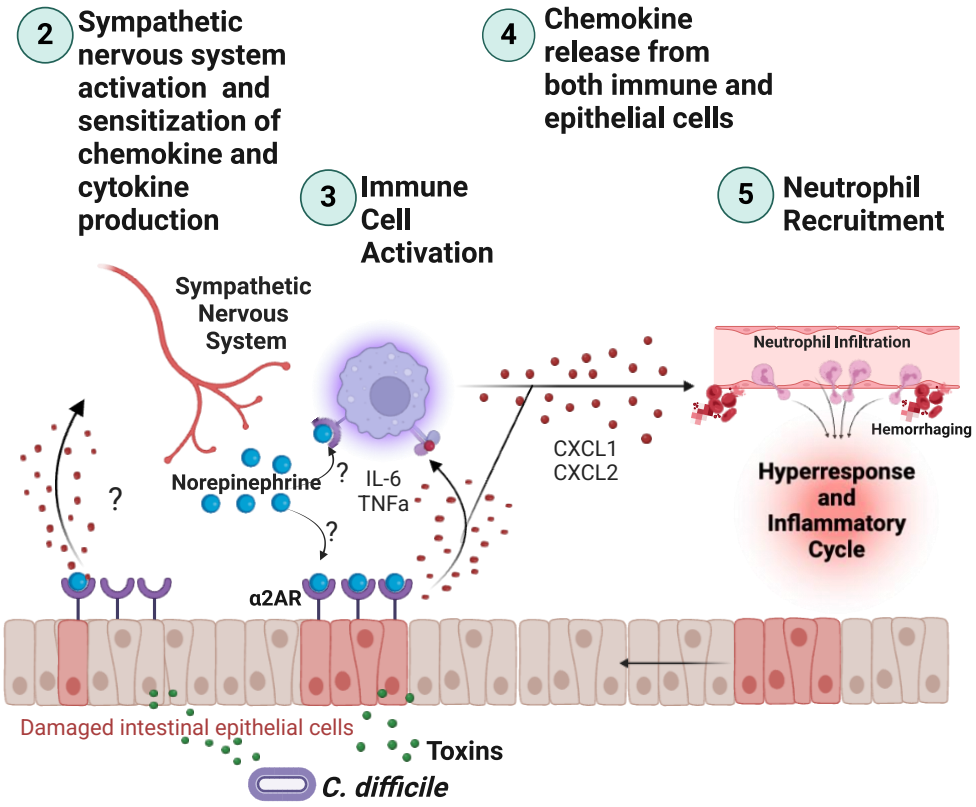
Clostridioides difficile infection (CDI) is a leading cause of hospital-acquired infections in the United States, known for triggering severe disease by hyperactivation of the host response. In this study, we determine the impact of the sympathetic nervous system (SNS) on CDI disease severity. Mouse models of CDI are administered inhibitors of SNS activity prior to *C. difficile* infection. Chemical sympathectomy or pharmacological inhibition of norepinephrine synthesis greatly reduces mortality and disease severity in the CDI model. Pharmacological blockade or genetic ablation of the alpha 2 adrenergic receptor

ameliorates intestinal inflammation, disease severity, and mortality rate. These results underscore the role of the sympathetic nervous system and the alpha 2 adrenergic receptor in CDI pathogenesis and suggest that targeting neural systems could be a promising approach to therapy in severe disease.

KEYWORDS

Colitis, *Clostridioides difficile*, sympathetic nervous system, adrenergic receptors, norepinephrine

Graphical Abstract



INTRODUCTION

Clostridioides difficile infection (CDI) stands as a prevalent cause of gastrointestinal disease, afflicting approximately 500,000 individuals annually in the US alone, and is recognized as a pressing public health concern by the CDC.[1] *C. difficile*, an opportunistic anaerobic bacterium, produces toxins that destroy the intestinal barrier leading to diarrhea, colitis, dehydration and, death. CDI poses a uniquely troublesome challenge not only for the severity of the disease, but because individuals treated with standard of care antibiotics frequently endure recurrent infection, at an estimated rate of 20% within 8 weeks post-treatment.[2] Microbiome-restorative therapeutics such as fecal microbiota transplant (FMT) offer promise as adjunctive alternatives though there is some concern for accessibility and safety.[3–5] More broadly, our limited comprehension of the biological factors underpinning CDI impedes the identification of at-risk individuals and of creating new strategies in managing severe disease.

Over the past decade, substantial advancements have highlighted the role of the immune system in the pathogenesis of CDI.[6–14] This progress was in large part facilitated by the development of murine models of CDI that mirror the intestinal damage and inflammatory responses of patients.[15] These models have helped pinpoint the specific immune cells and molecules modulating disease progression. Following, experiments with CDI mice can highlight other biological systems that influence CDI pathogenesis. Recently, Manion et al. underscored the central

involvement of afferent sensory neurons in regulating symptom onset and inflammation using CDI models, marking a high point on a foundation of studies hinting at the potential contribution of neural systems[16–22] to disease and simultaneously broaching the question as to the mechanisms of action of neurons and neuromediators in shaping CDI pathology.[23] Here we address specifically the role of sympathetic neurons and its downstream effectors in affecting CDI disease outcome.

Stress, infection, and colitis share a complex interplay. Stress, typically but not always, has been associated with heightened susceptibility to infection and exacerbation of symptom severity, a phenomenon also observed in CDI.[24–26] Paradoxically, both depression and certain antidepressants have been linked to increased CDI risk.[27–29] The sympathetic nervous system (SNS), colloquially known as a primary driver of the "flight or fight" stress response, has been increasingly recognized as a pivotal mediator of inflammatory diseases. SNS components, particularly norepinephrine and its downstream adrenergic receptors, have been repeatedly implicated in colitis pathogenesis both in animal models and patients.[30–35] Although the direction of influence of SNS involvement on disease outcome is multifactorial and heavily context dependent (e.g. tissue, infectious agent, age), adrenergic influence on immune, barrier, and microbial function is well documented and further in vivo examples will help the establishment of patterns across disease contexts.

In this study, we investigated the role of the SNS in CDI pathology. Through disruption of SNS activity in a CDI mouse model, we observed a significant reduction in intestinal inflammation and mortality. Furthermore, pharmacological inhibition of norepinephrine synthesis or of the alpha 2 adrenergic receptor (α2AR) recapitulated these effects. Finally, genetic ablation of the α2AR gene, *Adra2a*, mitigated disease severity, emphasizing the critical role of the sympathetic nervous system and its effectors in *C. difficile*-mediated pathology.

RESULTS

Chemical sympathectomy mitigates intestinal damage and mortality in *C. difficile* infected mice

To investigate the role of the sympathetic nervous system in CDI pathogenesis, we employed a mouse model of CDI infection and tested the impact of sympathetic nerve ablation on disease severity and mortality using 6-hydroxydopamine (6-OHDA). 6-OHDA is a neurotoxin that selectively ablates catecholaminergic terminals and has been used in a variety of contexts to implicate the sympathetic nervous system in disease.[36–41] Mice were infected with the R20291 strain of *C. difficile* after receiving either 6-OHDA or vehicle, and their clinical disease severity was monitored (Figure 1A). At the onset of symptoms (day 2), mice treated with 6-OHDA had reduced colonic norepinephrine concentrations compared to those receiving vehicle (Figure 1B). Importantly, 6-OHDA treatment significantly reduced clinical severity and mortality rates in *C. difficile*-infected mice (Figure 1C and Supplementary Figure

1A and 1B). Furthermore, early-stage CDI-induced weight loss was attenuated in 6-OHDA-treated mice (Figure 1D and Supplementary Figure 1C), although 6-OHDA did cause minor weight loss before infection (not shown). Importantly, the effects of 6-OHDA on clinical severity and weight loss was also apparent for female CDI mice (Supplementary Figures 1B and 1C).

In line with milder disease severity, we observed less intestinal tissue damage in 6-OHDA-treated mice, characterized by preservation of the epithelial architecture and fewer instances of hemorrhaging but not edema day 2 postinfection (Figure 1E and 1F). Interestingly, 6-OHDA did not impact day 2 *C. difficile* bacterial burden in cecal contents, which is consistent with previous findings indicating improved outcomes with pharmacological intervention without affecting *C. difficile* burden (Figure 1G, 1H, and 1I).[6] Nevertheless, we did find a nonsignificant reduction in the concentration of *C. difficile* toxin A/B in cecal contents (Figure 1J). Finally, 6-OHDA provided protection against the VPI 10643 variant of *C. difficile,* a strain expressing a varied toxin repertoire (binary toxin-deficient) (Supplementary Figure 1D). Together these data suggest that the sympathetic nervous system is necessary for disease pathology in CDI and its effect strain nonspecific.

Inhibition of noradrenergic signaling ameliorates CDI severity

Norepinephrine serves as the primary neurotransmitter of the sympathetic nervous system and is increased in the CDI mouse colon during symptom onset (Figure

2A). However, the system also releases epinephrine, dopamine, and various neuropeptides, all of which can exert significant effects on intestinal and immune functions. Further, 6-OHDA is taken up by both noradrenergic and dopaminergic neurons through norepinephrine transporters and dopamine transporters, respectively, resulting in the destruction of both noradrenergic and dopaminergic terminals.

To investigate whether 6-OHDA's protective effects acted via noradrenergic neurons, we administered desipramine, a norepinephrine transporter blocker, to mice. Desipramine blocks the uptake of 6-OHDA into noradrenergic neurons, sparing them, while leaving dopaminergic neurons vulnerable to 6-OHDA. Injecting desipramine 30 minutes before 6-OHDA treatment completely abolished the protective effect of 6-OHDA on mortality (Figure 2B and 2C), indicating that noradrenergic neurons are pivotal in driving CDI pathology.

Further exploring the putative role of norepinephrine, we examined whether inhibiting the conversion of dopamine to norepinephrine would also confer protection against CDI-induced mortality and clinical severity (Figure 2D and Supplementary Figure 1G). Nepicastat is an inhibitor dopamine beta hydroxylase, the enzyme responsible for norepinephrine and epinephrine synthesis. Oral administration of nepicastat substantially reduced mortality in CDI mice (Figure 2E). Nepicastat appeared to slightly but significantly mitigate the rate of CDI-induced weight loss (Figure 2F). Though nepicastat should deplete both norepinephrine and epinephrine, 6-OHDA does not deplete epinephrine in its

main producer, the adrenal medulla.[42] Together these data suggest that norepinephrine is the primary mediator of the sympathetic nervous system driving CDI pathology.

Blockade of the alpha 2 adrenergic receptor protects against CDI-mediated pathogenesis in a subtype-specific manner

Downstream of norepinephrine, there exist five major adrenergic receptor (AR) subtypes: α1, α2, β1, β2, and β3 ARs, all G-protein-coupled receptors (GPCRs) and expressed in a variety of cell types. To identify the receptor responsible for mediating CDI, we administered alpha- or beta-adrenergic antagonists to mice (Supplementary Figure 2; for subtype specific antagonists see Supplementary Figure 3A-3H). Blocking the α1 AR with prazosin showed little impact on mortality rate of CDI mice (Supplementary Figures 2A and 2B). Treatment with propranolol (a β1/β2 AR receptor blocker) or SR 59230A (a β3 AR receptor blocker) also did not confer protection against mortality in CDI mice (Supplementary Figures 2C, 2D, 2E and 2F). In fact, our findings suggest that beta AR blockade might worsen mortality rates in CDI mice.

Administration of the alpha 2 AR blocker RX 821002 (RX; Figure 3A) near fully recapitulated the protective effects of 6-OHDA (Figure 3B and Supplementary Figure 1H). While RX did not protect against CDI-induced weight loss (Figure 3C), it protected against intestinal epithelial cell damage and

prevented hemorrhaging (Figure 3D and 3E). This protective effect was not unique to RX, as another α2AR inhibitor, yohimbine, also reduced mortality rates in CDI mice while having no effect on weight loss (Supplementary Figure 4D and 4E). As with 6-OHDA, RX did not prevent edema or affect *C. difficile* burden in fecal pellets on day 1 as measured by plating or cecal contents on day 2 as measured by qPCR, albeit slightly decreased when measured by glutamate dehydrogenase (GDH) release (Figure 3F, 3G, 3H and Supplementary Figure 4B). The treatment did result in a modest decrease toxin A/B concentration in cecal contents (Figure 3I).

α2AR receptors can be further divided into subtypes: α2a (pharmacologically defined as α2d in rodents), α2b, and α2c. We systematically inhibited the three subtypes of alpha 2 adrenergic receptors in CDI mice with specific pharmacological antagonists. Inhibiting the α2b and α2c subtypes showed minimal to no effect (Supplementary Figure 3C-3F). Notably, we observed a small but significant effect on protection against mortality (compare 15% survival to 87% survival in RX 821002-treated mice) when using BRL 44408, an antagonist of the Adra2a subtype (Supplementary Figure 3A and 3B). Co-administration of the Adra2c inhibitor JP 1302 did not yield additional protective effect (Supplementary Figure 3G and 3H). While both rodent and human α2a receptors are encoded by the *Adra2a* gene, the α2a receptors in rodents have distinct pharmacological profiles. Due to these differences, rodent α2a receptors are classified as α2d receptors.[43,44] RX 821002 has higher affinity for α2d

adrenergic receptors compared to other alpha 2 adrenergic receptor antagonists and could explain its greater effect in CDI mice compared to the α2a-specific inhibitor BRL 44408 (Figure 3B and Supplementary Figure 3A). Thus, the results together suggest that α2a ARs (α2d by pharmacology) receptors mediate disease in CDI.

Genetic ablation of Adra2a reduces mortality in male CDI mice

After observing that pharmacological inhibition of the α2AR improves CDI severity, we were prompted to test if genetic deletion of the receptor would recapitulate the effects of small molecule inhibition. There are three subtypes of α2AR encoded by the genes *Adra2a*, *Adra2b*, and *Adra2c*. Given the known association of *Adra2a* with colitis[32,33] and based on our discovery that blockade of the Adra2a/Adra2d subtype of the receptor had the most significant pharmacological effect, we decided to evaluate the impact of *Adra2a* knockout (KO; Genotyping in Supplementary Figure 5B) on CDI disease severity.

Compared to mice heterozygous for *Adra2a* (and trending with WT), *Adra2a* KO mice showed markedly improved survival rates and clinical scores, although there was no effect on the rate of weight loss (Figure 3J and 3K and Supplementary Figure 3I). This effect was consistent with the pharmacological effects observed with α2AR blockade and was particularly evident in male mice but not in female mice (Supplementary Figure 4F, 4G, and 4H). This sex-dependent role of the alpha 2 receptor was also evident pharmacologically as

administration of RX demonstrated strong efficacy in male mice, it had no effect on female mice (Supplementary Figure 4C).

Chemical sympathectomy and alpha 2 AR blockade reduce intestinal inflammation in CDI mice

The sympathetic nervous system (SNS) plays a crucial role in regulating immune and barrier functions in inflammatory diseases. In CDI, immune cell infiltrates significantly impact disease outcomes. Neutrophil and Type-17-associated immunity are essential for protection against CDI, but an exaggerated response is linked to poor prognosis.[12,13,45] Conversely, eosinophilia and Type 2-associated immunity correlate with a better prognosis.[7,9,10]

To assess the SNS's impact on intestinal inflammation during CDI, we evaluated neutrophil, eosinophil, and monocyte numbers in the colons of 6-OHDA-treated CDI mice. We observed that while monocytes and eosinophils remained relatively unchanged, neutrophil numbers and proportions were significantly reduced in 6-OHDA-treated mice compared to untreated infected mice (Figure 4A; Gating strategy in Supplementary Figure 5A). Mice pretreated with desipramine (labeled "Des") before 6-OHDA treatment showed neutrophil counts similar to untreated mice and even higher numbers of monocytes and eosinophils, possibly due to desipramine's additive effect in increasing norepinephrine bioavailability.[46]

We considered that the lack of inflammation after SNS inhibition could be a result of enhanced barrier restoration by epithelial proliferation. However, we

found that cecal Ki67 staining was largely diminished on day 2 post-infection (especially in crypts) in 6-OHDA CDI mice as compared to vehicle-treated CDI mice (Supplementary Figures 1E and 1F). This would suggest that epithelial proliferation at the time of symptom onset is not likely to explain the protective effects of SNS blockade in CDI.

Considering the diverse immune effectors influencing CDI outcomes, we aimed to characterize the molecular inflammatory response using a Luminex cytokine array from cecal tissue lysates. Consistent with our cellular findings, we observed reduced levels of cytokines and chemokines associated with neutrophilic infiltration such as *KC and MIP-2* (also known as CXCL1 and CXCL2) in 6-OHDA-treated mice compared to untreated mice on day 2 post-infection (Figure 4B). Notably, cytokines related to Type 2 (and other types) immunity were also decreased or unchanged, further supporting the necessity of the SNS in the onset of inflammation in CDI. Similar results were obtained in mice treated with the α2AR blocker RX821002 (Supplementary Figure 4A and Table S2). Overall, these findings indicate that the SNS-axis initiates inflammation in CDI.

DISCUSSION

Here we have demonstrated the critical role that the sympathetic nervous system plays in driving disease in *C. difficile* infection in an in vivo context. We show that pharmacological ablation of SNS neurons, inhibition of norepinephrine synthesis, and pharmacological blockade or genetic deficiency of the α2AR

confer protection in the CDI mouse model. Further, we demonstrate these interventions halt the onset of inflammation and tissue damage.

A previous study by Manion et al. showed that sensory afferent neurons are crucial for the onset of CDI-induced inflammation.[23] Here, we have shown that sympathetic efferents are equally essential. Although there is crosstalk between sympathetic and sensory neurons, it remains unclear whether these systems work in concert to drive intestinal disease. Sensory activation may stimulate sympathetic output in a reflex arc, but many studies suggest that sensory stimulation reduces sympathetic activity and that α2AR stimulation reduces the sensory response.[47–50] Alternatively, sympathetic neurons might sensitize sensory activity, such as in the case of sympathetically maintained pain, a phenomenon mediated by alpha adrenergic receptors.[51]

The discovery of the α2AR as a major driver of disease was somewhat unexpected, considering that the α2AR is a well-known negative regulator of sympathetic activity. Blocking α2AR auto receptors on sympathetic neurons should theoretically increase sympathetic norepinephrine output. However, reconciling these results with those of 6-OHDA and nepicastat, we propose that RX's effects on CDI severity are mediated through postsynaptic α2ARs (heteroreceptors) rather than autoreceptors on sympathetic nerves. α2ARs have been implicated in sterile colitis conditions in both humans and mouse models.[32,33,52–54] Additionally, beta receptor activity has been shown to have anti-colitic effects, aligning with our observations.[55–57]

One question arising from this study is whether the effects of the SNS are local or extraintestinal. A prior study has shown that intervention of SNS at a local or systemic level can have opposing effects.[31] Future studies should determine whether local sympathectomy (e.g., surgically) can provide the same protection as systemic interventions like 6-OHDA. Both local and extraintestinal effects could explain the observed impact of SNS intervention as blood vessels and lymphoid organs are innervated by the SNS and influence immune cell trafficking.[58,59] A prior study injecting toxin A in the small intestine of mice showed that total extrinsic denervation of the small intestine could affect toxin A-induced epithelial damage and red blood cell accumulation, consistent with our own observations.[60] However, acute denervation was not sufficient to create these effects and the authors posited that changes to the enteric nervous system might dominate the onset of inflammation. These discrepancies might be due to differences in our models (intestinal location, toxin vs infection, timing, etc.) but it will be important to determine the relative roles of extrinsic and intrinsic nerves to CDI in addition to answering whether extraintestinal innervation contributes to disease.

Prior studies have shown that sympathetic innervation can limit type 2 effectors which could potentially limit inflammation in CDI.[61] However, we did not observe an increase in type 2 cytokines or eosinophilia with SNS inhibition. Instead, our observations are consistent with literature demonstrating the potent stimulatory effect of the α2AR on macrophage-produced TNFα and sepsis. [62–64] The largest effect on immune cell activity we observed was on neutrophil

infiltration. Others have demonstrated that norepinephrine can affect neutrophil mobilization though other mechanisms exist that might affect their recruitment to and activity.[58,59,65–70] More broadly, future studies will need to parse out the relative contribution to CDI of non-local vs local and non-immune vs immune mechanisms downstream of the SNS.

As *C. difficile* toxins are necessary for disease, a reduction in toxin A/B or their effects could explain the protective effects of SNS inhibition. Both 6-OHDA and RX interventions resulted in comparable bacterial burden with slightly reduced levels of toxins A/B. We observed higher water content in the cecal contents of RX-treated mice, which could potentially dilute toxin concentration. Adrenergic components and/or signals may affect toxin effects directly on the host or indirectly via the microbiome. The SNS influences intestinal epithelial cell proliferation via α2AR[71,72], potentially affecting toxin-induced disruption of the intestinal barrier. Additionally, adrenergic signaling can alter function in pericytes[73,74], recently recognized as *C. difficile* targets[23] and potential protectors against toxin-induced hemorrhaging[75] (Figure 1F and 3E). While the effects of *C. difficile* toxins on SNS health remain unclear, Xia et al. have demonstrated that toxin A can directly inhibit SNS release of norepinephrine in an alpha adrenergic receptor-dependent manner.[76] Whether *C. difficile* toxins bind the α2AR or another site of the SNS is unknown but their study combined with ours could indicate that these toxins target SNS and jumpstart the inflammatory response. As to the role of norepinephrine, a toxin-induced decrease of its output is perplexing

in the context of our finding that inhibition of norepinephrine signaling improves survival in CDI mouse models. This could mean that the host responds to toxin by decreasing norepinephrine output in benefit or, oppositely, that the role of norepinephrine is relative to timing (e.g. onset of inflammation vs resolution) and location (e.g. local vs extraintestinal). Lastly, adrenergic signals could also affect CDI pathogenesis indirectly through transkingdom signaling, as many microbes respond to norepinephrine and epinephrine. Pharmacological compounds targeting α2ARs can block sensor kinases in pathogens or commensals, affecting behaviors such as motility, adherence, and toxin production.[77–81] The effect seen in *Adra2a* KO mice and the lack of effect of RX 821002 in female mice would, however, suggest that α2AR blockers act directly on the host.

We found that the effects of RX 821002 and *Adra2a* KO were sex dependent. We predict that female and male mice may respond differently to alpha and beta adrenergic stimulation in CDI given the known sexual dimorphism in adrenergic receptor expression and sensitivity.[82–88] In general, *C. difficile* infection risk could be greater for female patients but a few different studies have shown an increased risk for mortality in male CDI patients. [89–93] Whether differences in the SNS network in males and females underly this observation will be an exciting course for future study. Related, other demographic factors such as age could also factor in the role of the SNS in CDI pathology. Advanced age is associated with both increased baseline sympathetic drive and exacerbated severity of CDI pathology.[94–99] Future research putting the SNS in the context of the factors that

influence its activity and downstream effects are necessary to predict the effect of intervention in CDI patients.

This study contributes to understanding the broader influences of the nervous system on CDI, broadening the scope of potential targets and biomarkers for CDI management. Prior to this study, there were indications of neural influence in CDI pathogenesis, but the central neural cells and molecules were unclear. Stress and antidepressants, which influence CDI outcomes, involve various neuronal systems and components, complicating risk assessment and molecular targeting. Additionally, previous mechanistic studies were often in toxin injection models rather than *C. difficile* infection models, potentially missing key aspects of neuronal influence on the course of infection such as effects on *C. difficile* behavior (e.g. growth, colonization and toxin production) and host responses to *C. difficile* antigens (e.g. balancing bacterial clearance with the risk of hyper response). In all, the results of this study demonstrate that SNS activation mediates CDI-induced hyperinflammation and intestinal damage and suggest that targeting the neural system or its downstream effectors could ameliorate the severity of CDI pathology.

Limitations of the Study

The major limitation of this study is that is does not yet uncover the mechanism downstream of α2AR responsible for driving CDI. Future studies should test the relative contributions of immune and non-immune adrenergic receptors in CDI

and determine whether those cell-specific effects are local to the intestine. Another limitation is that 6-OHDA could have off-target effects on sensory neurons and intrinsic dopaminergic neurons[100–102], effects of which are partially addressed by experimenting testing the components downstream of SNS firing. A central strength of this study is that we have tested multiple levels of the adrenergic pathway from neuron to receptor using different approaches from nerve terminal destruction to enzyme blockade to receptor antagonism with genetic confirmation and have observed consistent and sizeable effects on CDI mice. Next steps should make clear which cell types express *Adra2a* during CDI to determine which direct cellular targets of the SNS could explain the effects of intervention on the immune response and pathology.

STAR*METHODS

Detailed methods are provided in the online version of this paper and include the following:

RESOURCE AVAILABILITY

Lead contact

Materials availability

Data and code availability

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

C. difficile infection

Bacterial strains and culture

METHOD DETAILS

Pharmacological Agents

Single cell isolation and Flow Cytometry

Mouse histology and immunohistochemistry

QUANTIFICATION AND STATISTICAL ANALYSIS

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Author Contributions:

D.T. conceived, designed and performed the experiments and wrote the manuscript. J.L.L. helped conceive the project idea. D.T. conducted the data analysis with J.L.L, F.N., J.U., and B.T. provided valuable advice, reviewed the manuscript and helped with tissue processing. W.A.P. supported all aspects of the work.

Declaration of Interests:

W.A.P. is a consultant for TechLab, Inc., a company that produces diagnostic tests for *C. difficile.* W.A.P. and D.T. are listed as inventors on US Patent application PCT/US2023/062958 filed by the University of Virginia for alpha 2 adrenergic receptor blockade for the treatment of *C. difficile* colitis. The other authors report no conflicts of interest.

Figure 1

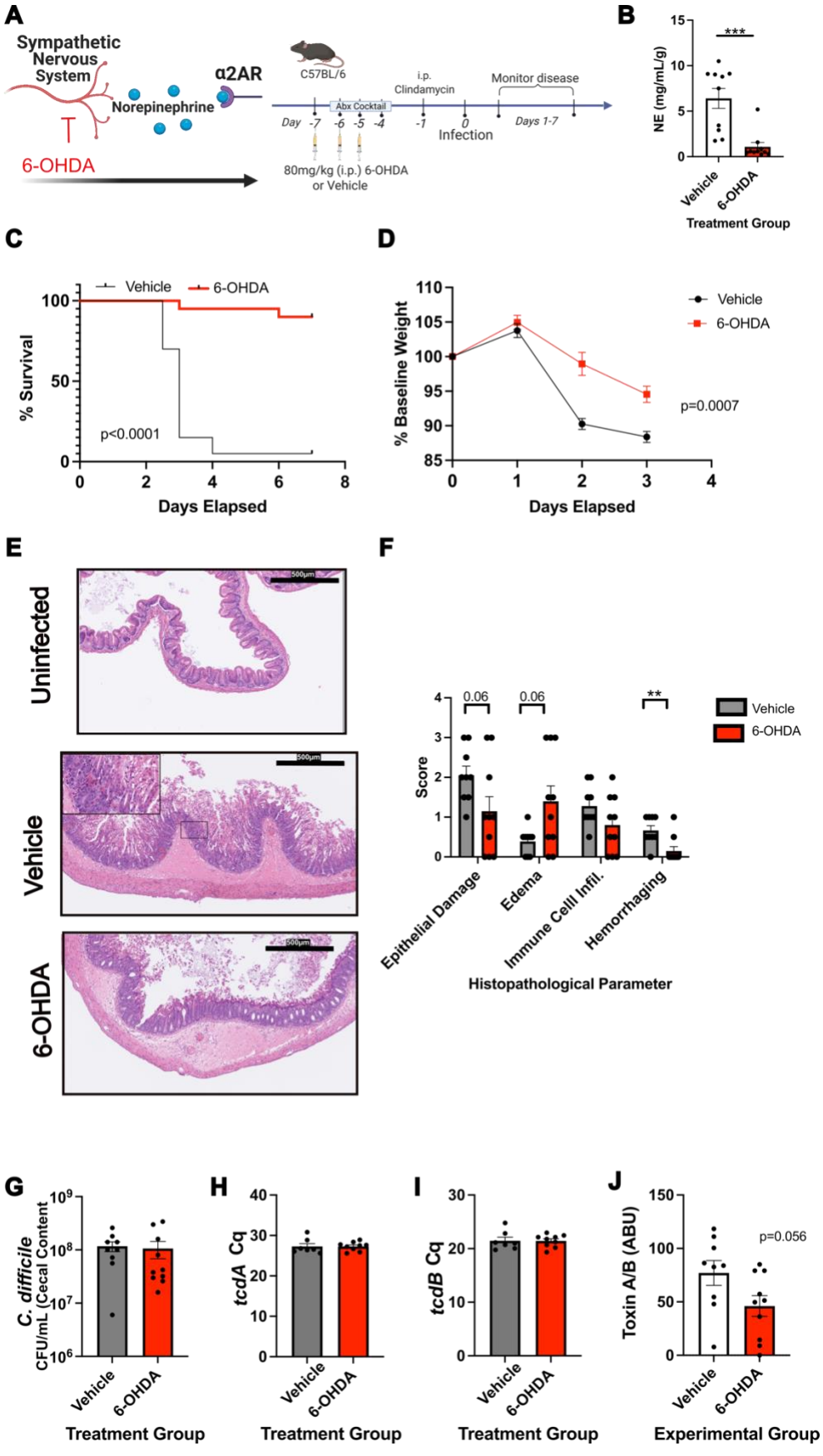

A
Sympathetic Nervous System
Norepinephrine
α2AR
6-OHDA
C57BL/6
Abx Cocktail
Day -7 -6 -5 -4
80mg/kg (i.p.) 6-OHDA or Vehicle
i.p. Clindamycin
-1
0 Infection
Monitor disease
Days 1-7
B
NE (mg/mL/g)
***
Vehicle
6-OHDA
Treatment Group
C
% Survival
Vehicle
6-OHDA
p<0.0001
Days Elapsed
D
% Baseline Weight
Vehicle
6-OHDA
p=0.0007
Days Elapsed
E
Uninfected
Vehicle
6-OHDA
500μm
F
Score
0.06
0.06
**
Vehicle
6-OHDA
Epithelial Damage
Edema
Immune Celll Infil.
Hemorrhaging
Histopathological Parameter
G
C. difficile CFU/mL (Cecal Content
Vehicle
6-OHDA
Treatment Group
H
tcdA Cq
Vehicle
6-OHDA
Treatment Group
I
tcdB Cq
Vehicle
6-OHDA
Treatment Group
J
Toxin A/B (ABU)
p=0.056
Vehicle
6-OHDA
Experimental Group

Figure 1: Chemical sympathectomy mitigates intestinal damage and mortality in *C. difficile* infected mice.

(A) (Left) 6-OHDA destroys nerve endings in dopaminergic and noradrenergic neurons. (Right) Mice were injected intraperitoneally with 6-OHDA (80mg/kg) or vehicle (0.2% ascorbic acid) on days 7, 6, and 5 prior to infection.

(B)The norepinephrine levels of cecal lysates were measured on day 2 post-infection from male CDI mice pre-treated with vehicle (0.2% ascorbic acid; open bar) or 6-OHDA (red bar) (n=10 per group)

(C) Survival curves and weight loss (D) after treatment with 6-OHDA (red) or vehicle (black) in male *C. difficile* infected mice. (n=20 per group)

(E) Representative epithelial barrier integrity day 2 post-infection (H&E; 5x) of treatment groups assessed by (F) scoring by two blinded observers of *C. difficile* infected tissue. The inset (20x) shows an area of hemorrhaging.

(G) *C. difficile* bacteria burden was measured on day 2 post-infection by plating cecal contents from male mice treated with 6-OHDA (red) or vehicle(grey). (n=9 Vehicle, n=10 6-OHDA)

(H,I) Toxin genes for *C. difficile* were measured as a proxy for pathogen burden day 2 post-infection. *tcdA* (H) and *tcdB* (I) qPCR quantification cycle (Cq) from

cecal contents in male mice infected with *C. difficile* after treatment with 6-OHDA (red) or vehicle (grey).

(J) Toxin A/B concentration was measured in cecal content from male mice 2 days post-infection by ELISA (TechLab, Inc.). Toxin A/B concentration for each sample was measured in arbitrary units (ABUs) where 100 ABUs is the measurement for the positive control standard from TechLab. (n=9 Vehicle, n=10 6-OHDA) Data represent mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by Student's t-test (B, F, G,H, I, J) Mantel-Cox log-rank test (C) and mixed effects model for the group factor (D). The data for (C) and (D) are pooled from two independent experiments.

Figure 2

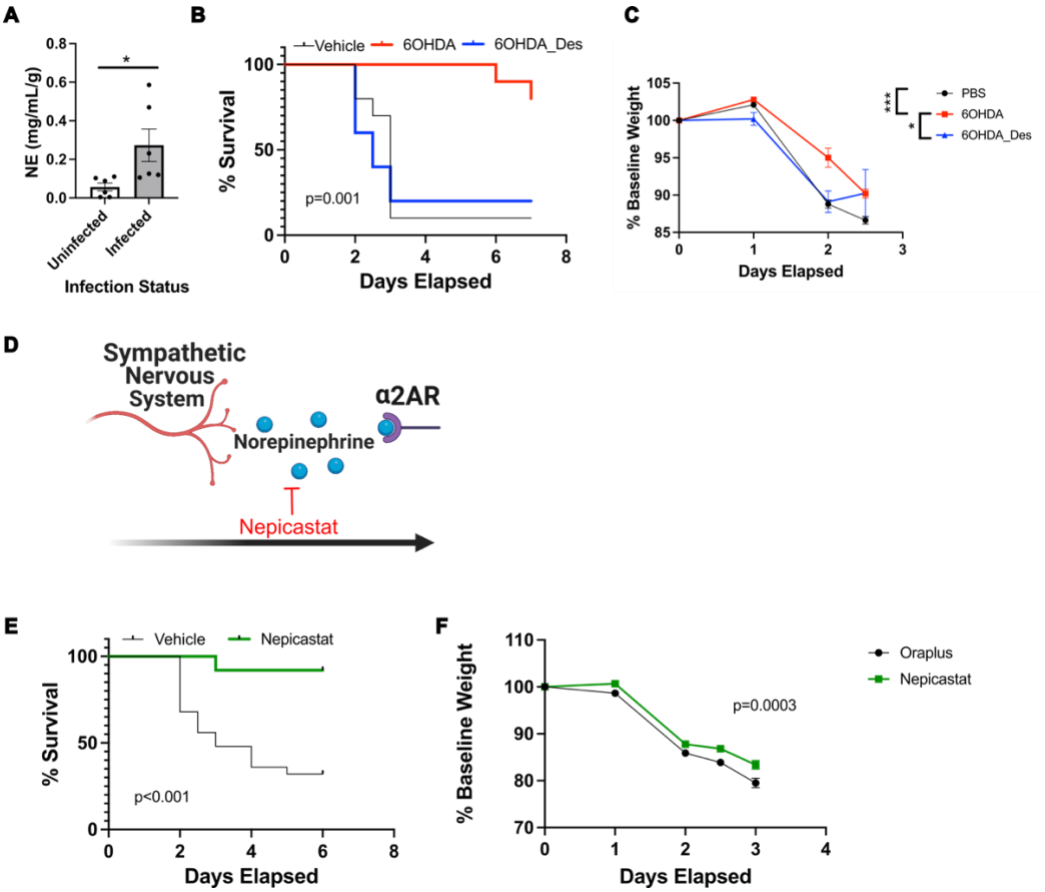

A
NE (mg/mL/g)
0.8
0.6
0.4
0.2
0.0
*
Uninfected
Infected
Infection Status
B
Vehicle
6OHDA
6OHDA_Des
% Survival
100
50
0
p=0.001
0
2
4
6
8
Days Elapsed
C
% Baseline Weight
105
100
95
90
85
***
*
PBS
6OHDA
6OHDA_Des
0
1
2
3
Days Elapsed
D
Sympathetic Nervous System
α2AR
Norepinephrine
Nepicastat
E
Vehicle
Nepicastat
% Survival
100
50
0
p<0.001
0
2
4
6
8
Days Elapsed
F
% Baseline Weight
110
100
90
80
70
p=0.0003
0
1
2
3
4
Days Elapsed
Oraplus
Nepicastat

Figure 2: Inhibition of noradrenergic signaling ameliorates CDI disease severity.

(A) Norepinephrine (NE) concentration in colonic lysates from male CDI mice was measured by ELISA on day 2 post-infection (n=6 per group)

(B) Male mice were intraperitoneally administered desipramine, a norepinephrine transporter inhibitor, 30 minutes prior to the administration of 6-OHDA to block the entry of 6-OHDA into noradrenergic terminals. Survival curves (B) and weight loss (C) after treatment with 6-OHDA (red), 6-OHDA and desipramine (6OHDA_Des; blue), or vehicle (0.2% ascorbic acid and PBS; black) in *C. difficile* infected mice. (n=10 per group)

(D) Nepicastat inhibits dopamine beta hydroxylase which converts dopamine to norepinephrine.

(E,F) Male mice were orally administered nepicastat (on days 0 and 1 post-infection), an inhibitor of dopamine beta hydroxylase, to inhibit the conversion of dopamine to norepinephrine. (E) Survival curves and (F) weight loss after treatment with nepicastat (green), or vehicle (OraPlus; black) in male *C. difficile* infected mice. Data represent mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by Student's t-test (A) Mantel-Cox log-rank test (B,E) and mixed effects model for the group factor (C,F). The data for (D) and (E) are pooled from two independent experiments.

Figure 3

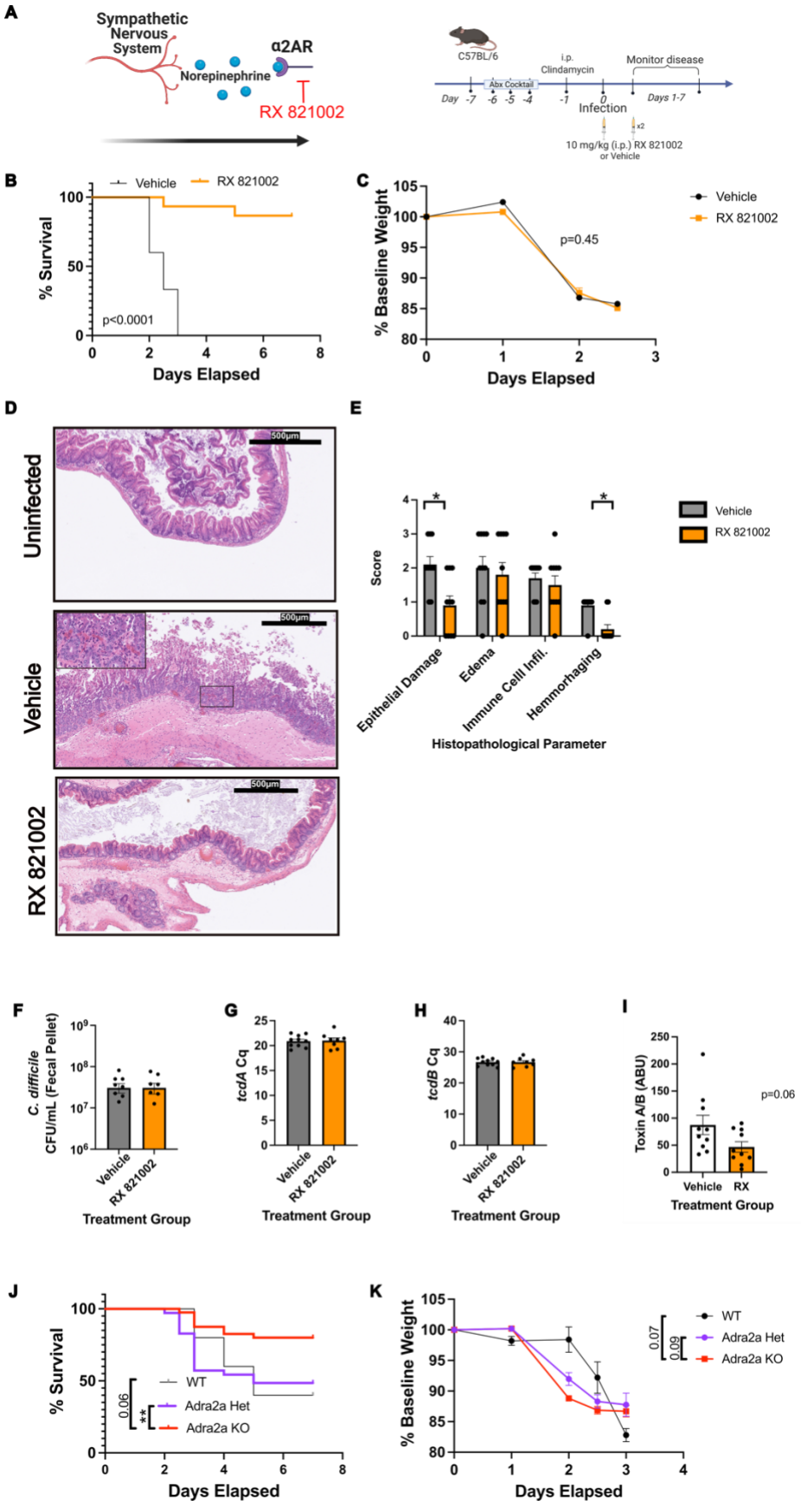

A
Sympathetic Nervous System
Norepinephrine
α2AR
RX 821002
C57BL/6
i.p. Clindamycin
Monitor disease
Abx Cocktail
Day -7 -6 -5 -4 -1 0 Days 1-7
Infection
x2
10 mg/kg (i.p.) RX 821002 or Vehicle
B
Vehicle
RX 821002
% Survival
p<0.0001
Days Elapsed
C
% Baseline Weight
p=0.45
Vehicle
RX 821002
Days Elapsed
D
Uninfected
Vehicle
RX 821002
500μm
E
Score
Epithelial Damage
Edema
Immune Cell Infil.
Hemmorhaging
Histopathological Parameter
Vehicle
RX 821002
F
C. difficile CFU/mL (Fecal Pellet)
Vehicle
RX 821002
Treatment Group
G
tcdA Cq
Treatment Group
H
tcdB Cq
Treatment Group
I
Toxin A/B (ABU)
p=0.06
Vehicle
RX
Treatment Group
J
% Survival
0.06
**
WT
Adra2a Het
Adra2a KO
Days Elapsed
K
% Baseline Weight
0.07
0.09
WT
Adra2a Het
Adra2a KO
Days Elapsed

Figure 3: Blockade of the alpha 2 adrenergic receptor protects against CDI-mediated pathology.

(A) (Left) RX 821002 inhibits alpha 2 adrenergic receptors. (Right) Mice were injected intraperitoneally with RX21002 on days 0 and 1 post-infection.

(B,C) Survival curves (B) and weight loss (C) after treatment with alpha 2 AR blocker RX 821002 (orange) or vehicle (PBS; black) in male *C. difficile* infected mice (n=30 per group).

(D) Representative epithelial barrier integrity on day 2 post-infection (H&E, 5x) of treatment groups assessed by (E) scoring by two blinded observers of male *C. difficile* infected tissue. The inset (20x) shows an area of hemorrhaging.

(F) *C. difficile* bacteria burden was measured on day 1 post-infection by plating fecal pellets from male mice treated with RX 821002 (orange) or vehicle(grey).

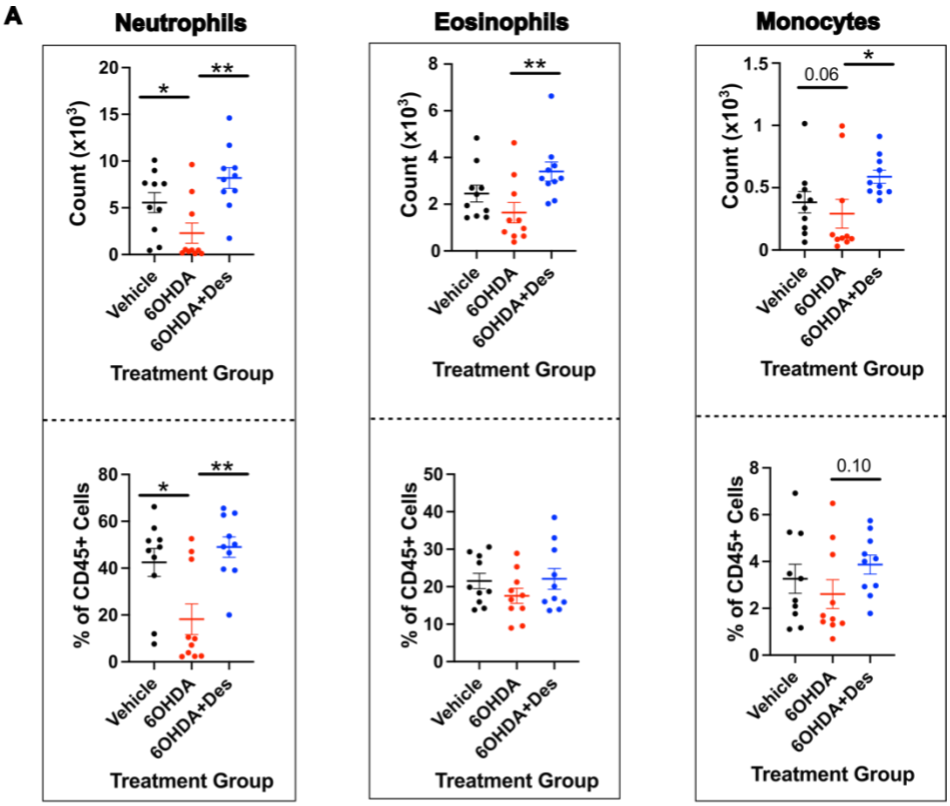
(G,H) Toxin genes for *C. difficile* were measured as a proxy for pathogen burden on day 2 post-infection. *tcdA* (G) and *tcdB* (H) qPCR quantification cycle (Cq) from cecal contents in male mice infected with *C. difficile* after treatment with RX 821002 (orange) or vehicle(grey).

(I) Toxin A/B concentration was measured in cecal content from male mice 2 days post-infection by ELISA (Techlab, Inc.). Toxin A/B concentration for each sample

was expressed in arbitrary units (ABUs) where 100 ABUs is the measurement for the positive control standard from Techlab.

(J,K) Male mice homozygous deficient and heterozygous for the *Adra2a* gene were infected with *C. difficile.* Survival curves (J) and weight loss (K) for *Adra2a* heterozygous mice (purple) or *Adra2a* homozygous KO (red) or WT (black) mice (n=35 *Adra2a* Het, n=40 *Adra2a* KO, WT, n=5). Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by Student's t-test (E,F, G,H,I) Mantel-Cox log-rank test (B, J) and mixed effects model the group factor (C, K). The data for (B, C, J, K) are pooled from three independent experiments.

Figure 4


A
Neutrophils
Eosinophils
Monocytes
Count (x10³)
% of CD45+ Cells
Vehicle
6OHDA
6OHDA+Des
Treatment Group
*
**
0.06
0.10

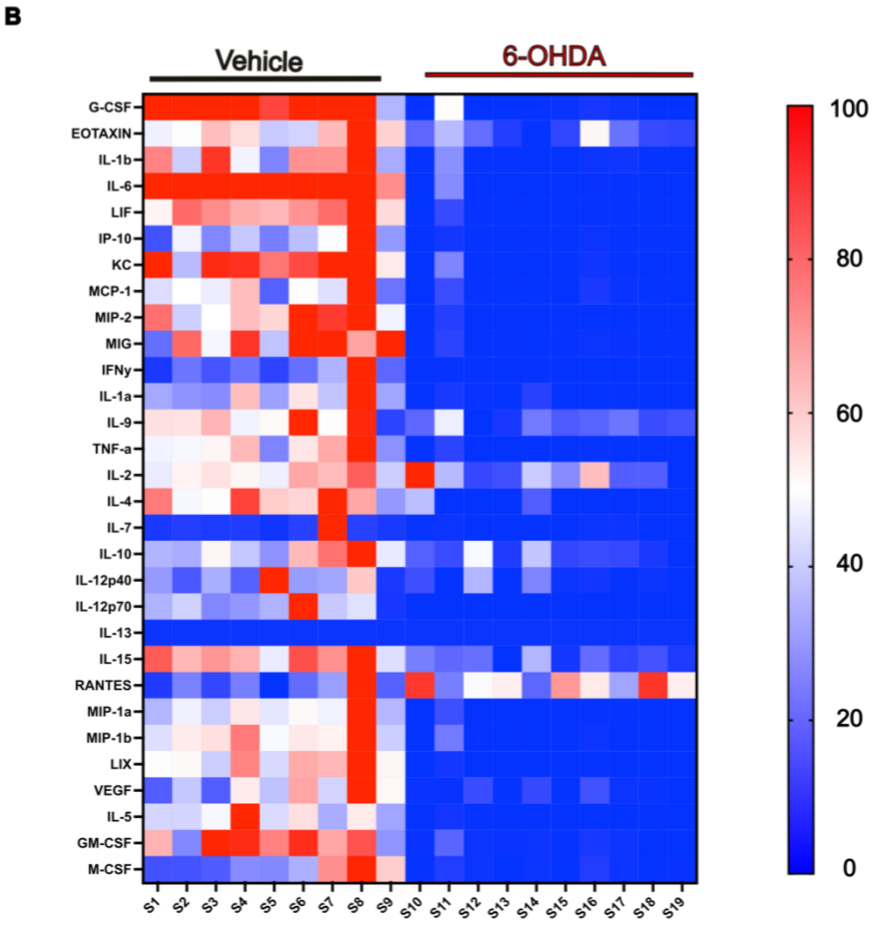
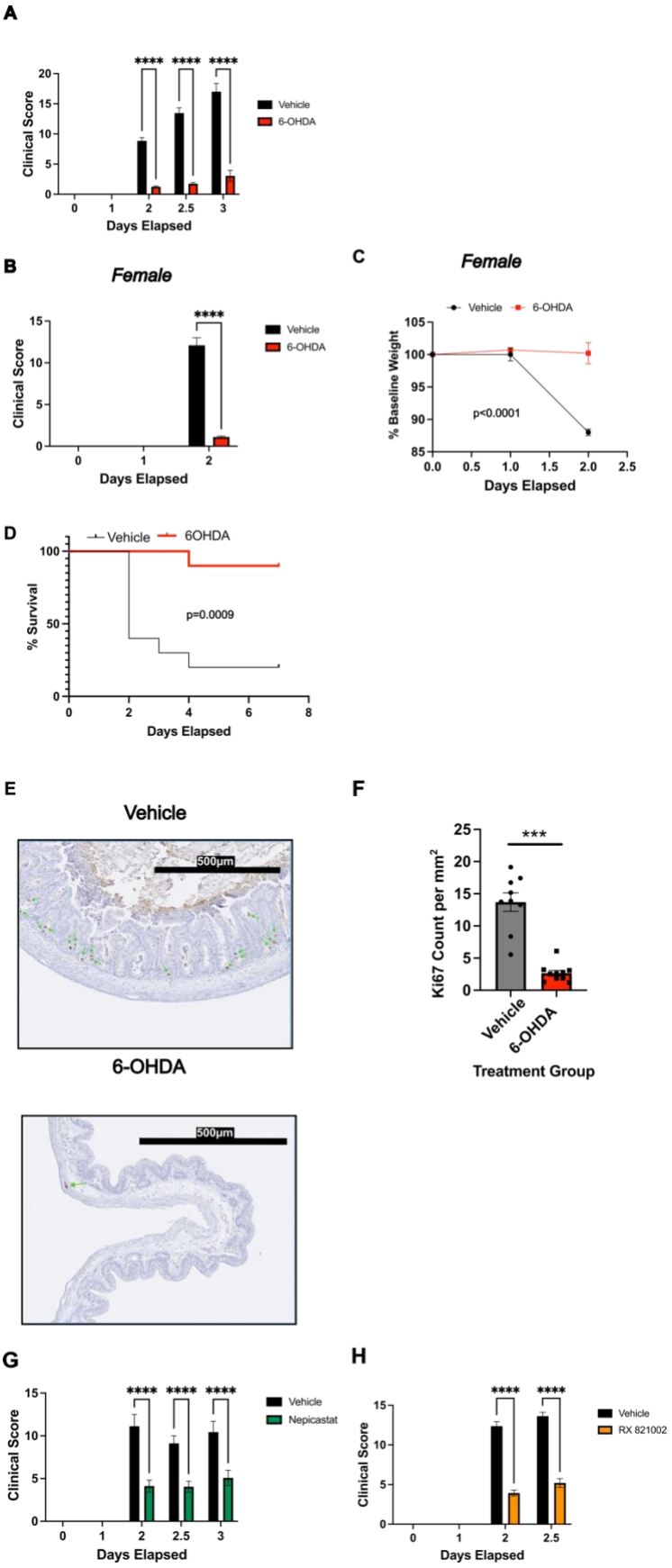

B
Vehicle
6-OHDA
G-CSF
EOTAXIN
IL-1b
IL-6
LIF
IP-10
KC
MCP-1
MIP-2
MIG
IFNγ
IL-1a
IL-9
TNF-a
IL-2
IL-4
IL-7
IL-10
IL-12p40
IL-12p70
IL-13
IL-15
RANTES
MIP-1a
MIP-1b
LIX
VEGF
IL-5
GM-CSF
M-CSF
S1 S2 S3 S4 S5 S6 S7 S8 S9 S10 S11 S12 S13 S14 S15 S16 S17 S18 S19
100
80
60
40
20
0

Figure 4: Chemical sympathectomy reduces intestinal inflammation in CDI mice.

(A) Colons from infected mice pretreated with 6-OHDA (blue), 6-OHDA and desipramine (green), or vehicle(0.2% ascorbic acid; black) were collected and processed for flow cytometry at day 2 post-infection. Quantification of cell count (top) and % of total CD45 cells (bottom) for neutrophils (CD45+ CD11b+ Ly6g+ Ly6c+), monocytes (CD45+ CD11b+ Ly6g- Ly6c+), eosinophils (CD45+ CD11b+ SiglecF+ Ly6g−) in the colon. (n=10 per group)

(B). Cytokine protein expression in cecal lysates from female mice were measured by 32-plex Luminex panel on day 2 post-infection (Samples 1-10: PBS, Samples 10-19: 6-OHDA) Each column of the heatmap is normalized to a scale between 0% and 100% where these quantities represent the lowest and greatest values in the column, respectively. See Table S1 for concentrations and comparisons. Data represent mean ± SEM. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ by Student's t-test (A).

Supplementary Figure 1



Supplemental Figure 1: Chemical sympathectomy protects in a *C. difficile* strain-nonspecific manner and without restorative proliferation.

(A) Clinical scores after treatment with 6-OHDA (red) or vehicle (0.2% ascorbic acid; black) in male *C. difficile* infected mice. (n=20 per group)

(B) Clinical scores and weight loss (C) after treatment with 6-OHDA (red) or vehicle (0.2% ascorbic acid; black) in female *C. difficile* infected mice. (n=10 per group)

(D) Survival curves after treatment with 6-OHDA (red) or vehicle (0.2% ascorbic acid; black) in mice infected with the VPI 10463 strain of *C. difficile* (n=10 per group)

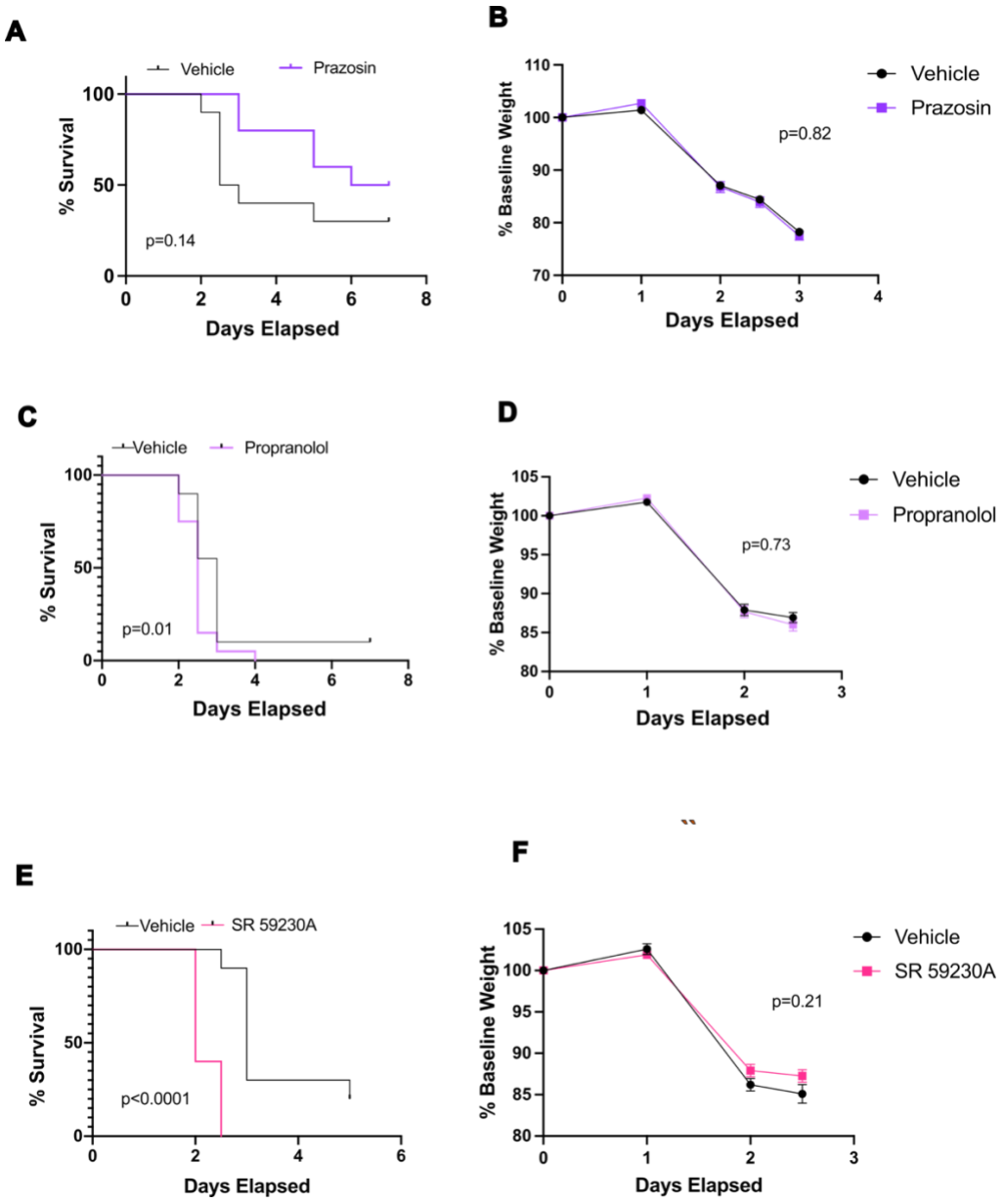
(E) Ki67 staining in cecal sections from CDI mice treated with 6-OHDA or vehicle (0.2% ascorbic acid) on day 2 post-infection. Quantification of the # of Ki67+ puncta per mm^2 tissue in (F) (n=10 per group).

(G) Clinical scores after treatment with nepicastat (green), or vehicle (OraPlus; black) in male *C. difficile* infected mice. (n=25 per group)

(H) Clinical (B) after treatment with alpha 2 AR blocker RX 821002 (orange) or vehicle (PBS; black) in male *C. difficile* infected mice (n=30 per group).

Related to Figure 1. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by Student's t-test (F), mixed-effects model for the group factor (C) with post hoc pairwise comparison in (A,B,G,H), Mantel-Cox log-rank test (D). The data for (A,G) and (H) are pooled from two and three independent experiments, respectively.

Supplementary Figure 2



Supplemental Figure 2: Blockade of alpha 1 or beta-adrenergic receptors does not protect against CDI-mediated pathogenesis.

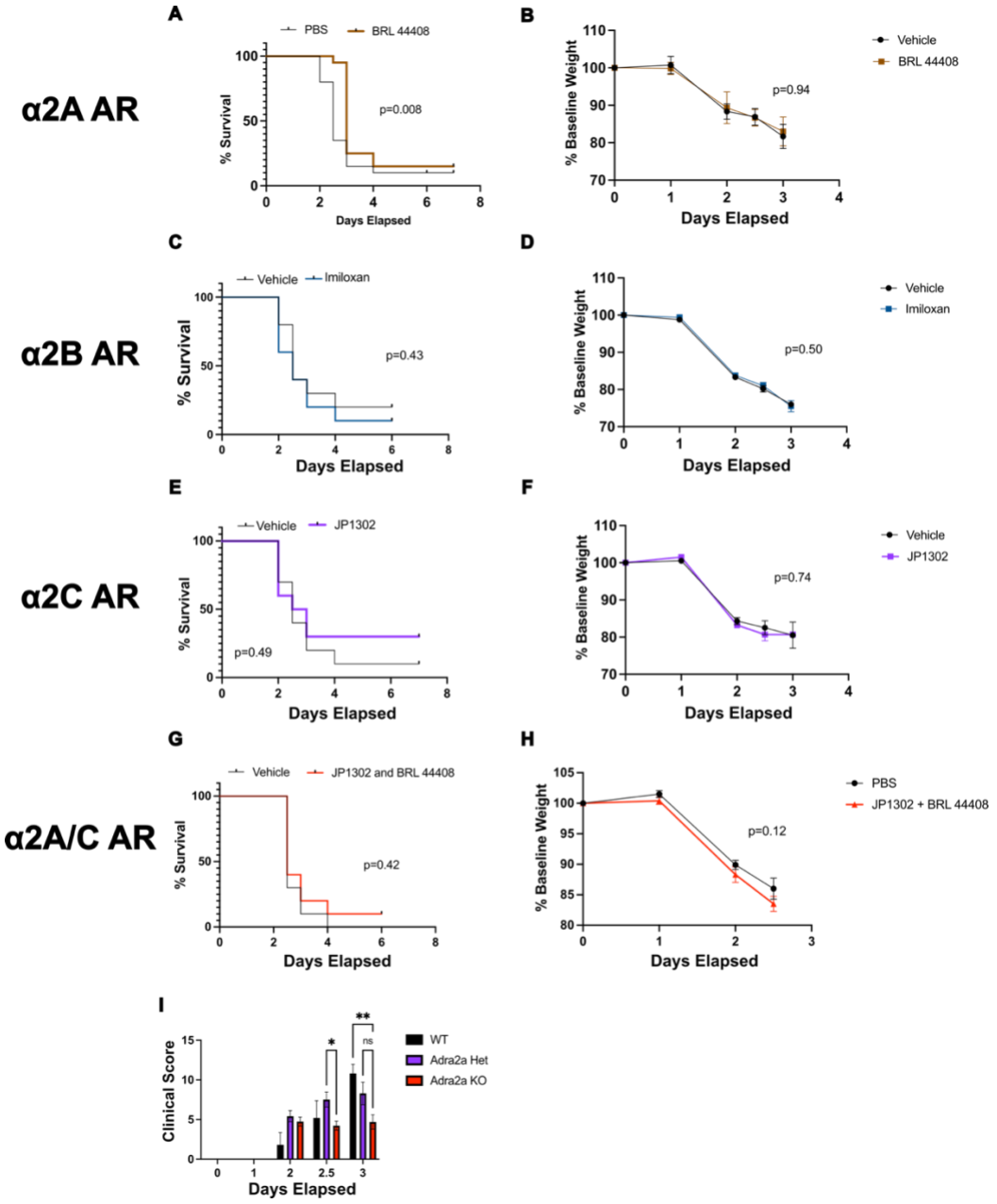
Mice were injected intraperitoneally with beta or alpha 1 AR antagonists on days 0 and 1 post-infection.

(A,B) Survival curves (A) and weight loss (B) after treatment with alpha 1 AR blocker prazosin (dark purple) or vehicle (PBS/DMSO; black) in male *C. difficile* infected mice (n=10 per group).

Survival curves (C) and weight loss (D) after treatment with beta 1/2 AR blocker propranolol (light purple) or vehicle (PBS; black) in male *C. difficile* infected mice (n=10 per group).

Related to Figure 3. Survival curves (E) and weight loss (F) after treatment with beta 3 AR blocker SR 59230A (pink) or vehicle (PBS; black) in male *C. difficile* infected mice (n=10 per group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by Mantel-Cox log-rank test (A,C,E) and mixed effects model for the group factor(B,D,F).

Supplementary Figure 3



Supplemental Figure 3: Effect of alpha 2 AR blockade on CDI-induced mortality is subtype specific.

Mice were injected intraperitoneally with alpha 2 AR subtype (a, b, or c) antagonists on days 0 and 1 post-infection. (A,B) Survival curves (A) and weight loss (B) after treatment with alpha 2a AR blocker BRL 44408 (brown) or vehicle (PBS; black) in male *C. difficile* infected mice (n=20 per group).

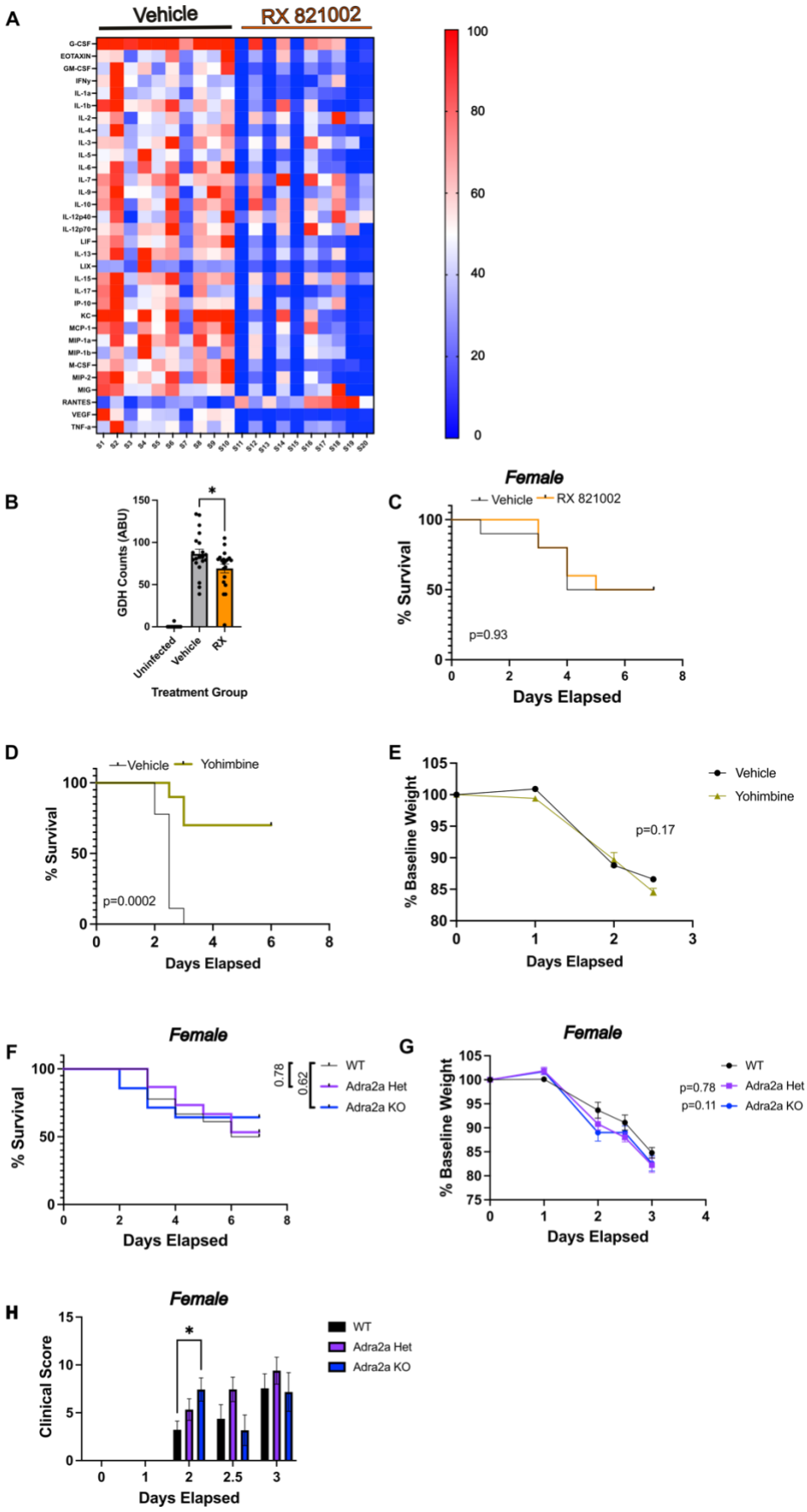
Survival curves (C) and weight loss (D) after treatment with alpha 2b AR blocker imiloxan (blue) or vehicle (PBS; black) in male *C. difficile* infected mice (n=10 per group).

Survival curves (E) and weight loss (F) after treatment with alpha 2c AR blocker JP 1302 (purple) or vehicle (PBS; black) in male *C. difficile* infected mice (n=10 per group).

Survival curves (G) and weight loss (H) after treatment with JP 1302 (purple), both BRL 44408 and JP 1302 (red) or vehicle (PBS; black) in male *C. difficile* infected mice (n=10 per group).

(I) Clinical scores for *Adra2a* heterozygous mice (purple) or *Adra2a* homozygous KO (red) or WT (black) male CDI mice (n=35 *Adra2a* Het, n=40 *Adra2a* KO, WT, n=5).

Supplementary Figure 4



Supplemental Figure 4: Alpha 2 AR blockade protects against CDI-induced inflammation and mortality in a sex-dependent manner.

(A) Cytokine protein expression in cecal lysates was measured by 32-plex Luminex panel on day 2 post-infection (Samples 1-10: Vehicle (PBS), Samples 10-20: RX 821002) Each column of the heatmap was normalized to a scale between 0% and 100% where these quantities represent the lowest and greatest values in the column, respectively. See Table S2 for concentrations and comparisons.

(B) *C. difficile* glutamate dehydrogenase (GDH) concentration was measured in cecal content 2 days post-infection by ELISA (TechLab). GDH concentration for each sample was measured in arbitrary units (ABUs) where 100 ABUs is the measurement for the positive control standard from TechLab. (n=10 Uninfected, n=20 Vehicle and RX)

Survival curve (C) after treatment with RX 821002 (orange) or vehicle (PBS; black) in *C. difficile* infected female mice (n=10 per group).

(D,E) Survival curves (D) and weight loss (E) after treatment with alpha 2 AR blocker yohimbine (gold) or vehicle (PBS; black) in *C. difficile* infected mice (n=10 per group).

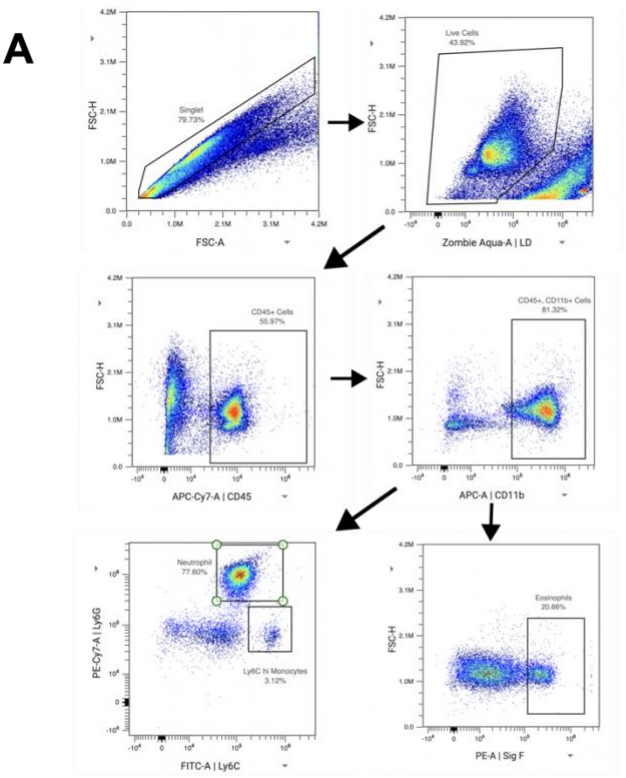
(F,G) Female mice WT, homozygous deficient, and heterozygous for the *Adra2a* gene were infected with *C. difficile.* Survival curves (F) and weight loss (G) for WT (black), Adra2a heterozygous mice (purple) or *Adra2a* homozygous KO mice (blue). (n=18 WT, n=15 Adra2a Het, n=14 Adra2a KO)

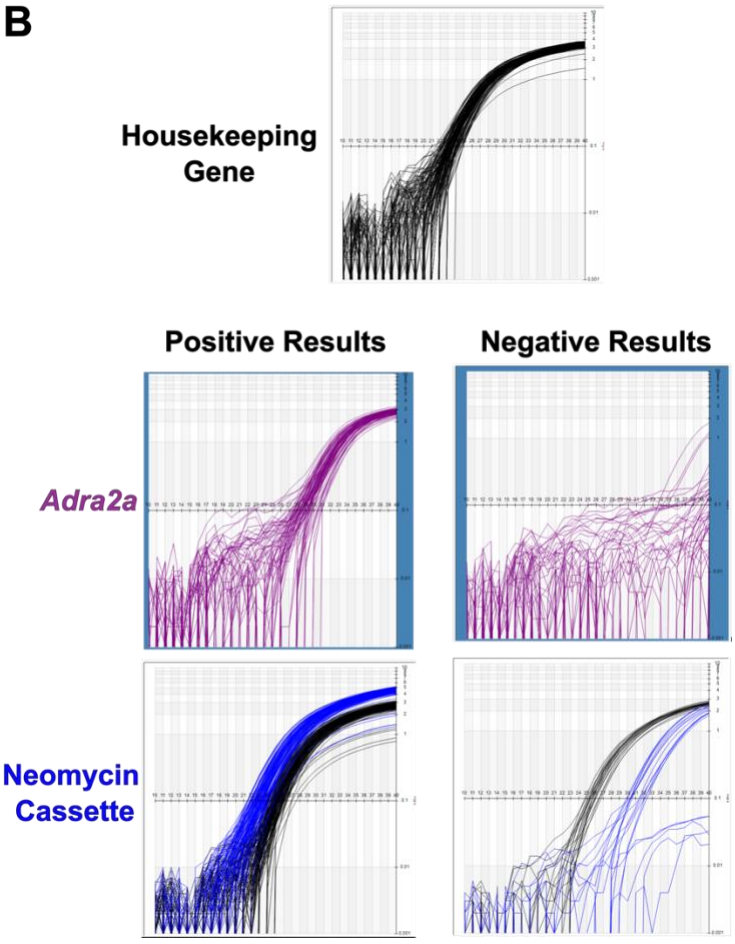
(H) Survival curves (F) and weight loss (G) for WT (black), Adra2a heterozygous mice (purple) or *Adra2a* homozygous KO mice (blue). (n=18 WT, n=15 Adra2a Het, n=14 Adra2a KO)

Related to Figure 3. Data represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by Mantel-Cox log-rank test (A,C,D,F) , 1-way ANOVA with post hoc comparison (B) or mixed

effects model for the group factor(E,G). The data for (B,F,G,H) are pooled from two independent experiments.

Supplementary Figure 5





Supplemental Figure 5: Gating strategy and genotyping

(A) Gating strategy for flow cytometry experiments. Cell immune populations from colon were identified as follows: neutrophils (CD45+ CD11b+ Ly6g+ Ly6c+), monocytes (CD45+ CD11b+ Ly6g- Ly6c+), eosinophils (CD45+ CD11b+ SiglecF+ Ly6g−). Related to Figure 4.

(B) Genotyping of *Adra2a* mutant mice was done by sending tail snip biopsies to Transnetyx. The results of genotyping were based on TaqMan probe based real-time PCR for probes against *Adra2a* and the neomycin cassette used to disrupt the genotype the *Adra2a* gene. The results shown are for the housekeeping gene (first row; black lines), positive and negative detection of *Adra2a* (second row; pink lines) and positive and negative detection of the neomycin cassette (third row; blue lines) based on the cycle threshold. Related to Methods and Figure 3.

Cytokine/Chemokine	Significant?	P value	Mean of Vehicle (pg/mL)	Mean of 6-OHDA (pg/mL)	Difference	SE of difference	Adjusted P Value
G-CSF	Yes	<0.000001	17147	1111	16035	1657	<0.000001
EOTAXIN	Yes	0.000057	2821	1244	1578	296.9	0.000743
IL-1b	Yes	0.000009	6688	325.9	6362	1019	0.000133
IL-6	Yes	<0.000001	20518	512.7	20006	714.7	<0.000001
LIF	Yes	<0.000001	2013	27.86	1985	123.2	<0.000001
IP-10	Yes	0.00034	2899	95.79	2803	627.7	0.003058
KC	Yes	<0.000001	15300	451	14849	1427	<0.000001
MCP-1	Yes	0.000032	9947	346	9601	1716	0.000451
MIP-2	Yes	<0.000001	14339	128.2	14211	1538	0.000001
MIG	Yes	0.000002	6870	163.9	6706	952	0.000034
IFNy	No	0.020554	85.98	1.013	84.96	33.27	0.084113
IL-1a	Yes	0.000095	155.7	4.388	151.3	29.86	0.001144
IL-9	Yes	0.000255	165.1	97.82	67.32	14.64	0.00255
TNF-a	Yes	0.000002	83.08	1.727	81.35	11.76	0.00004
IL-2	No	0.029873	1.692	1.23	0.4622	0.195	0.086968
IL-4	Yes	0.000001	1.304	0.603	0.7014	0.09468	0.000021
IL-7	No	0.181457	12.75	2.168	10.58	7.593	0.181457
IL-10	Yes	0.001056	38.62	17.44	21.18	5.376	0.007369
IL-12p40	No	0.017419	3.104	0.73	2.374	0.9015	0.084113
IL-12p70	Yes	0.000423	3.831	0.15	3.681	0.8435	0.003377
IL-13	-	-	ND	ND	ND	ND	-
IL-15	Yes	<0.000001	23.29	6.788	16.5	1.889	0.000002
RANTES	No	0.033867	12.78	23.13	-10.36	4.489	0.086968
MIP-1a	Yes	0.000001	410.4	9.736	400.7	54.29	0.000022
MIP-1b	Yes	<0.000001	270.2	13.58	256.6	29.19	0.000002
LIX	Yes	<0.000001	818.7	6.718	812	84.53	<0.000001
VEGF	Yes	0.000169	401.6	33.74	367.9	76.75	0.00186
IL-5	Yes	0.000002	323.8	3.725	320.1	45.18	0.000033
GM-CSF	Yes	0.000001	229.4	8.752	220.6	30.44	0.000026
LIX	Yes	<0.000001	818.7	6.718	812	84.53	<0.000001
M-CSF	Yes	0.002681	548.2	32.39	515.8	146.9	0.015981

Table S1. Cytokine protein expression in cecal lysates was measured by 32-plex Luminex panel on day 2 post-infection from mice administered Vehicle (0.2% Ascorbic Acid) or 6-OHDA. The adjusted p-value reflects a t-test result with multiple comparisons using the Holm-Sidak method. *ND stands for "not detected" as the concentration of the cytokine is below the limit of detection of the Luminex assay.*

Cytokine/Chemokine	Significant?	P value	Mean of Vehicle (pg/mL)	Mean of RX 821002 (pg/mL)	Difference	SE of difference	Adjusted P Value
G-CSF	Yes	0.00038	19597	8021	11576	2657	0.008317
EOTAXIN	No	0.011425	4686	2267	2419	858.9	0.118735
GM-CSF	Yes	0.000163	323.5	69.55	253.9	53.54	0.004381
IFNy	Yes	0.001881	183.5	51.79	131.7	36.19	0.027841
IL-1a	Yes	0.000304	418.4	83.88	334.5	75.02	0.007261
IL-1b	Yes	0.000587	6336	1867	4469	1074	0.011683
IL-2	No	0.382539	6.576	6.033	0.543	0.6066	0.618742
IL-4	Yes	0.000828	6.721	2.684	4.037	1.008	0.014793
IL-3	No	0.048972	3.368	2.332	1.036	0.4906	0.260122
IL-5	Yes	0.000423	352.1	87.47	264.7	61.42	0.008836
IL-6	Yes	0.000224	16051	3421	12629	2748	0.005589
IL-7	No	0.116013	7.599	5.994	1.605	0.972	0.443706
IL-9	No	0.017972	481.3	330.1	151.2	58.07	0.133695
IL-10	No	0.124736	67.17	53.58	13.6	8.444	0.443706
IL-12p40	No	0.584473	20.01	17.33	2.683	4.818	0.618742
IL-12p70	No	0.110674	25.01	16.86	8.145	4.855	0.443706
LIF	Yes	0.000018	2331	402.6	1929	334.7	0.000569
IL-13	Yes	0.000667	13.26	4.755	8.504	2.072	0.012591
LIX	No	0.01307	1886	389.3	1497	543.5	0.123272
IL-15	No	0.01582	65.52	38.51	27.01	10.14	0.133695
IL-17	Yes	0.000894	394.6	70.97	323.6	81.47	0.015096
IP-10	No	0.004098	1157	481.8	675.2	205.4	0.051981
KC	Yes	0.000357	15226	3858	11368	2593	0.008188
MCP-1	Yes	0.003116	10198	3520	6678	1958	0.042755
MIP-1a	Yes	0.000073	224	72.41	151.6	29.67	0.002051
MIP-1b	No	0.016477	121.1	55.03	66.08	24.99	0.133695
M-CSF	Yes	<0.000001	452.7	80.93	371.8	44.87	0.000005
MIP-2	Yes	0.000059	13561	2905	10656	2044	0.001758
MIG	No	0.006889	5513	2294	3219	1055	0.079605
RANTES	Yes	0.000196	5.783	15.34	-9.552	2.051	0.005075
VEGF	Yes	0.000061	241.9	19.43	222.5	42.8	0.001758
TNF-a	Yes	0.000925	105.6	33.66	71.91	18.17	0.015096

Table S2. Cytokine protein expression in cecal lysates was measured by 32-plex Luminex panel on day 2 post-infection from mice administered Vehicle (PBS) or RX 821002. The adjusted p-value reflects a t-test result with multiple comparisons using the Holm-Sidak method.

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RESOURCE AVAILABILITY

Lead contact

Further information and requests for reagents should be directed to and will be fulfilled by the corresponding author William Petri (wap3g@virginia.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice-All mouse experiments adhered to ethical guidelines and regulations for testing and research on animals, with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Virginia. Experiments utilized sex-matched C57BL6 and *Adra2a* mutant mice aged 10 to 15 weeks. C57BL6 mice were obtained from Jackson Laboratory, while *Adra2a* mutant frozen embryos (004367)[103] were procured from Jackson Laboratory. In experiments with *Adra2a* mutants, littermates were used as control. Mouse genotypes from tail biopsies were determined using real-time PCR with specific probes designed for the *Adra2a* gene and the neomycin cassette insertion for gene disruption (Transnetyx, Cordova, TN) (Supplementary Figure 5B). All animals

were housed in a specific pathogen-free environment at the University of Virginia's animal facility.

***C. difficile* infection**-Mice were infected and monitored following an established mouse model for CDI.[6] In this established protocol, mice were administered antibiotics to confer *C. difficile* susceptibility in the model (for effects of antibiotics on the microbiome see Moreau et al 2024).[104] Three days before infection, mice received an antibiotic mixture in their drinking water, comprised of the following antibiotics: 45 mg/L Vancomycin (Mylan), 35 mg/L Colistin (Sigma), 35 mg/L Gentamicin (Sigma), and 215 mg/L Metronidazole (Hospira). Subsequently, mice were transitioned to regular water and administered a single intraperitoneal injection (0.016 mg/g) of Clindamycin (Hospira) on day -1. On day 0, mice were orally administered *C. difficile* spores.

Mice were checked twice per day and health was evaluated by clinical scoring of the following parameters: weight loss, coat appearance, activity level, diarrhea, posture, and eye condition. Each parameter was scored by observation and added to a cumulative clinical score ranging from 1 to 20. Weight loss and activity scores ranged from 0 to 4, with a 4 indicating 25% or greater weight loss from baseline weight on day 0. Other parameters such as coat appearance, diarrhea type, posture, and eye condition were scored from 0 to 3. Mice with a clinical score of 14 or higher indicating severe illness were euthanized as per protocol. All experiments were done with *C. difficile* infected mice unless otherwise specified.

Bacterial strains and culture-*C. difficile* strains were prepared as in a previous study[7]. To prepare *C. difficile*, strains from frozen stocks were grown using BHI agar plates incubated at 37 °C overnight in an anaerobic chamber. A single colony was inoculated into BHI media and grown anaerobically overnight at 37 °C. The next day, cultures were centrifuged for 1 min at 6000 × g and washed twice in anaerobic PBS. Each mouse received 100 µl (1×10^2 -10^3 CFU for R20291 and 1×10^4-10^5 CFU for VPI 10643) of inoculum by oral gavage. *C. difficile* burden was quantified from cecal contents at day 2 of infection. Briefly, cecal contents were resuspended by weight in PBS. *C. difficile* burden was measured by toxin A (*tcdA*) and toxin B (*tcdB*) specific qPCR on the DNA isolated from cecal content using a QIAamp fast DNA stool mini kit according to the manufacturer's instructions. For the qPCR reaction, input DNA was diluted to normalize total DNA (100ng/uL total DNA) across samples. For the detection of *tcdA* and *tcdB* iQ multiplexing and target-specific probes (FAM/HEX) were used for the qPCR quantification. *C. difficile* toxins A/B and GDH were quantified using the *C. difficile* TOX A/B II and C. DIFF CHEK – 60 (TL5025) kits generously gifted from TechLab. For colony counts, either cecal contents or fecal pellets were resuspended and serially diluted for plating on BHI agar supplemented with 1% sodium taurocholate, 1 mg/mL D-cycloserine, and 0.032 mg/mL cefoxitin (Sigma), and anaerobically incubated at 37 °C overnight.[6]

METHOD DETAILS

Pharmacological Agents-6-OHDA Treatment*:* 6-OHDA from Sigma Chemical Co., St. Louis, Mo., was dissolved in sterile saline with 0.02% L-ascorbic acid as an antioxidant. It was administered intraperitoneally at a dose of 80 mg/kg once daily on days -7, -6, and -5 of infection. Desipramine HCl from Sigma was dissolved in PBS. In specific experiments, desipramine was injected intraperitoneally at a dose of 10 mg/kg, 30 minutes before each 6-OHDA injection.[41]

Adrenergic Receptor Blockers*:* Prazosin hydrochloride (Medchem Express; HY-B0193A) was diluted in PBS, and for control, diluted DMSO was used at an equivalent concentration. RX 821002 hydrochloride (Sigma; R9525), propranolol hydrochloride (Sigma; P0884), and SR 59230A (Tocris; #1511) were dissolved in PBS. Propranolol (10mg/kg), RX821002 hydrochloride (10 mg/kg) and prazosin (2 mg/kg) were injected intraperitoneally at a dose of 10 mg/kg once on day 0 and twice (AM, PM) on day 1 of infection.

Alpha 2 Adrenergic Subtype Blockers*:* BRL 44408 maleate (Sigma; B4559; 10 mg/kg), Imiloxan hydrochloride (Tocris; 0986; 3 mg/kg), and JP 1302 hydrochloride (Medchem Express; HY-103213; 3 mg/kg) were dissolved in PBS and injected intraperitoneally once on day 0 and twice (AM, PM) on day 1 of infection.

Inhibition of Dopamine Beta Hydroxylase*:* Nepicastat hydrochloride (Medchem Express; HY-13289A) was prepared by grinding the compound in a

methylcellulose-based vehicle OraPlus. Nepicastat was administered via oral gavage at a dose of 30 mg/kg once on day 0 and twice (AM, PM) on day 1 of infection.

Single cell isolation and Flow Cytometry- Colons were opened longitudinally and rinsed in Buffer A (HBSS, 25 mM HEPES, 5% FBS). The epithelial layer was separated from the lamina propria by incubating the colons in a dissociation buffer (HBSS, 15 mM HEPES, 5 mM EDTA, 10% FBS, 1 mM DTT) at 37 °C for 40 minutes in a shaking incubator. The lamina propria fraction (tissue left intact) was diced with scissors and further digested in RPMI 1640 with 0.17 mg/mL Liberase TL (Roche) and 30 µg/mL DNase (Sigma). Samples were digested for 40 minutes at 37 °C in the shaking incubator. Single-cell suspensions were achieved by passing each sample through a 100 µM cell strainer then through a 40 µM cell strainer (both Fisher Scientific). For flow cytometry, single-cell suspensions were prepared as described, and samples were stained with the following monoclonal antibodies: CD3 (145-2C11 BioLegend Cat No. 100328, dilution 1/100),CD11c (BioLegend Cat No. 117327, dilution 1/50), CD11c (N418, BioLegend Cat No. 117330, dilution 1/50), CD11b (M1/70, BioLegend Cat No. 101212, dilution 1/200), Ly6C (HK1.4, BioLegend Cat No. 128005, dilution 1/100), CD45 (30-F11, BioLegend Cat No. 103116, dilution 1/200), Ly6G (1A8, BioLegend Cat No. 127618, dilution 1/100), SiglecF (E50 2440, BD Cat No. 552126, dilution 1/100). For surface staining, 1×10^6 cells/sample were Fc-blocked using TruStain fcX (93,BioLegend, #101320, 1/200) for ten minutes at

RT then suspended in LIVE/DEAD Fixable Aqua (Life Technologies) for 30 minutes at 4 °C. Cells were washed two times with FACS buffer (PBS+ 2% FBS) and stained with the monoclonal antibodies for 30 min at 4 °C. Flow cytometry was performed on a Cytek Aurora Full Spectral cytometer and all data analysis performed via OMIQ.

Mouse histology and immunohistochemistry-Cecal snips from mice were fixed in Bouin's solution and switched to 70% ethanol after 24-hour fixation. Fixed ceca were sectioned, paraffin-embedded and stained with hematoxylin and eosin (H&E) by the University of Virginia Research Histology Core. Each histology slide was scored by two blinded observers. H&E tissue pathology was scored using a scale from 0 to 3 for multiple parameters: epithelial disruption, submucosal edema, inflammatory infiltrate, and hemorrhaging (0-1).[6,23]

For Ki67 quantification, mouse cecal tissue sections were fixed in 4% PFA and transferred to 70% ethanol after 24 h. Sections were embedded in paraffin by the University of Virginia Research Histology Core and stained for Ki67 (Abcam Cat #Ab16667) by the Biorepository and Tissue Research Facility.

QUANTIFICATION AND STATISTICAL ANAYLSIS

For mouse work, survival curves were created using the Kaplan–Meier estimate, and the Mantel-Cox test was used to ascertain statistical significance of survival between two groups. Comparisons between two groups in other experiments were done using a two-tailed t test, ANOVA, or mixed-effects model. Weight loss and

clinical scores of surviving groups were monitored until day 7 postinfection but statistical analysis was done up to day 3 (or earlier) as in many cases fewer than 2 mice survive in the control group. All n-values refer to biological replicates of mice. All statistical analyses were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

Chapter 5 – Future Directions and Suggestions

With these findings many questions are left. Here'd I like to outline the future steps that I think could build on the aforementioned work. I'll explore directions that could work in parallel to my findings (horizontal) and ones that directly build upstream or downstream of the role of the SNS in CDI (vertical).

Horizontal

1. Other Stress Mediators
    a. Adrenal gland
    b. Neuropeptide Y

Rationale/Related Data:

The discovery of the role of the SNS in mediating *C. difficile* mediated pathology begs the question as to what other stress components also play a role. Adrenal gland mediators such as epinephrine and glucocorticoids have been implicated in *C. difficile* contexts already though not tested systematically with a model of infection. Adrenalectomy in the toxin-A ileal loop model increases fluid secretion (modestly but especially in db/db leptin-resistant mice) and intestinal permeability.[1,2] The effects of adrenalectomy can be reversed by corticosteroid supplementation. [2] Related, KO of corticosteroid releasing hormone (CRH) ameliorates the effects of toxin A in the ileal loop model.[3] Importantly, CRH KO was also associated with a decrease of intestinal substance P, a neuropeptide shown to play a key role in CDI by Manion et al.[4] There is evidence that *C. difficile* toxins can have a repressive effect on glucocorticoid receptor activation. [5] The clinical data is less clear of the role of corticosteroids in CDI. Studies have shown that corticosteroids can increase the risk of severe CDI or no apparent effect.[6–8]

Additionally, co-transmitters might also play a role in modulating the effects of norepinephrine or could be important independently. Though there are many possibly important co-transmitters, neuropeptide Y (NPY) seems like a fitting candidate. NPY has already been implicated in intestinal bowel disease. [9,10] NPY's effect can be changed with catecholamine concentration giving a way to explain both the effects of beta and alpha adrenergic receptor effects downstream.[11] I also have found that 6OHDA cannot be effectively reversed by alpha 2 adrenergic receptor agonists (Fig 1 and 2). I do wonder whether lack of NPY in the system might also explain that result.

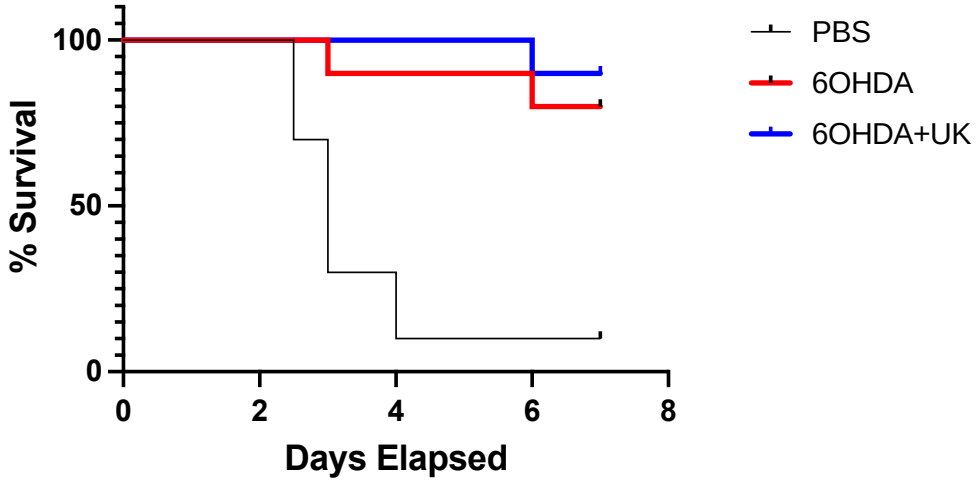


Fig 1. Survival curves after treatment with 6-OHDA (red), 6-OHDA and UK 14,304 (blue), or vehicle (black) in male *C. difficile* infected mice. (n=10 per group)

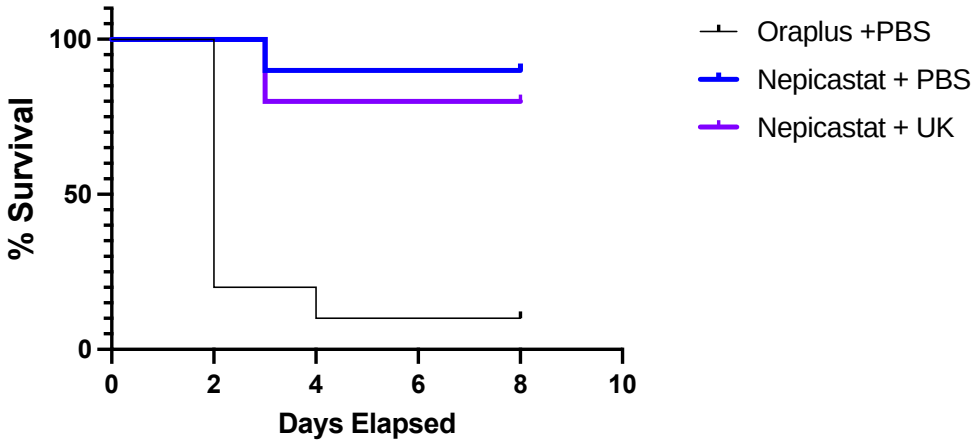


Fig 2. Survival curves after treatment with nepicastat (blue), nepicastat and UK 14,304 (purple), or vehicle (black) in male *C. difficile* infected mice. (n=10 per group)

Experiments:

To explore these hypotheses further, I propose a series of experiments to test the necessity of stress mediators in disease. I would conduct infections in adrenalectomized mice, CRH knockout, and mice treated with blockers of glucocorticoid receptors to determine their role in CDI pathology. Additionally, I would consider a restraint stress model to see if restraint stress exacerbates the severity of CDI, possibly dependent on the alpha-2 receptor and the SNS.

Pros:

Adrenalectomized mice from Jackson can be purchased for a simple infection experiment between them and sham surgery mice. There are easy ways to follow up adrenalectomy results (e.g. antagonism of glucocorticoid receptors, supplementation of corticosteroids, and epinephrine synthesis enzyme PNMT).

Cons:

Current data suggest that adrenalectomy will not have a protective effect (could worsen symptoms). One's effect size might not be as large if there is a mouse strain specific effect.[12] This direction is not incredibly novel but might have some surprising results.

2. Sympathetic – Sensory Crosstalk

Rationale/Related Data:

Sympathetically maintained pain is a phenomenon by which the SNS increases. There is evidence that sympathetically maintained pain works via alpha 2 adrenergic receptors. Manion et al shows that sensory neurons (and damage of them) leads to disease state in CDI.

Experiments:

One could start by looking for close apposition of sensory neurons to sympathetic neurons in the intestine with IHC and if so, whether those sensory neurons harbor the alpha 2 receptor.

As a test one could use sensory neuron specific ablation of the alpha 2 receptor (conditional KO) to test whether alpha 2 receptors in sensory neurons are important. Alternatively, or in supplement, one could try to reverse effects of 6-OHDA or RX 821002 with sensory neuropeptides Substance P or CGRP. I've tried already once with RX 821002 (Fig 3).

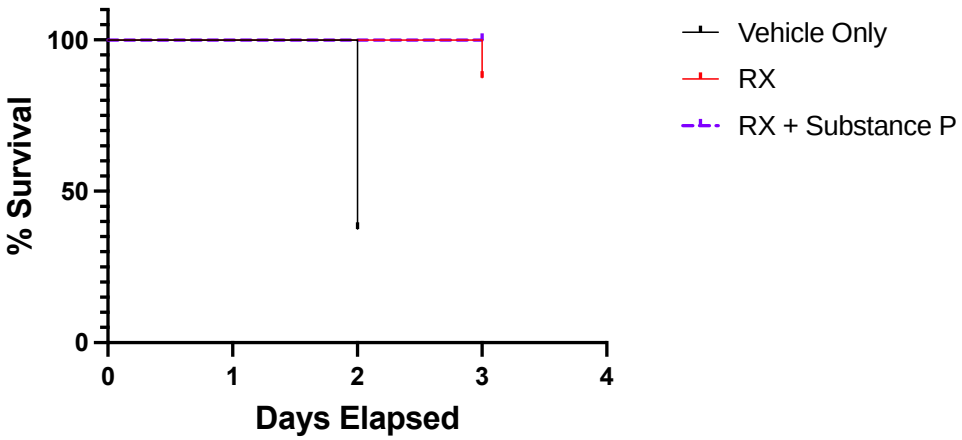


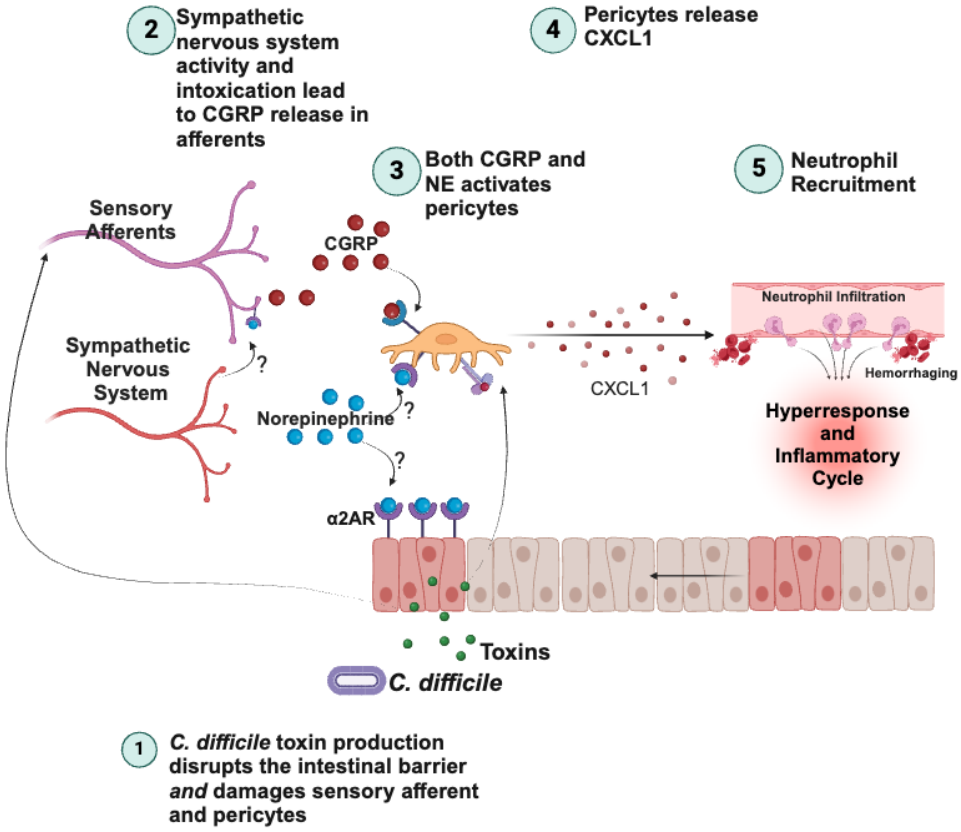
Fig 3. Survival curves after treatment with RX 821002 (red), RX 821002 and substance P (purple; dotted), or vehicle (black) in male *C. difficile* infected mice. (n=10 per group)

Pros:

This direction could be incredibly high impact synthesizing two major findings in neuroscience of *C. difficile*. Related, the major histological factor I am finding is a difference in hemorrhaging, a difference I see starkly with both 6-OHDA and RX821002. As pericytes can also respond to alpha 2 adrenergic receptor stimulation they might be a nice target cell to also probe.

Cons:

This is a very Neuroscience heavy project. Work with Campbell and Deppmann Labs for advice on circuits and visualization. Related, this phenomenon of alpha 2 in sensory neurons could be happening locally in the intestine or at the spine. Here's a graphical abstract of what crosstalk might look like:



3. Serotonin

Rationale/Related Data:

Depression and antidepressants have been implicated in CDI infection susceptibility.[13,14]

Experiments:

One could start with testing the effect of SSRIs in C. difficile mice (like fluoxetine; already tried a couple of times). I would recommend starting however, testing the necessity of serotonin using aTph1 inhibitor in infected mice. One would eventually have to show the neuronal Tph2 is not playing a role although it is much smaller of a source of serotonin and has not been implicated (to my

knowledge) in IBD. If there is a role of serotonin, serotonin receptor blockade experiments in infected mice would be the natural follow up.
Pros:
This direction is potentially high impact and a new direction for the *C. diff* field. Enterochromaffin cells are the well-defined cell source of serotonin making the known targets easy to find. I think Tph1 KO are available at Jackson.
Cons:
There are a large number of serotonin receptor subtypes. It would be more efficient to test the necessity of serotonin production first although Tph1 inhibitors are expensive. Fluoxetine experiments did not show a long-lasting effect in my first experiments. (Fig 4)

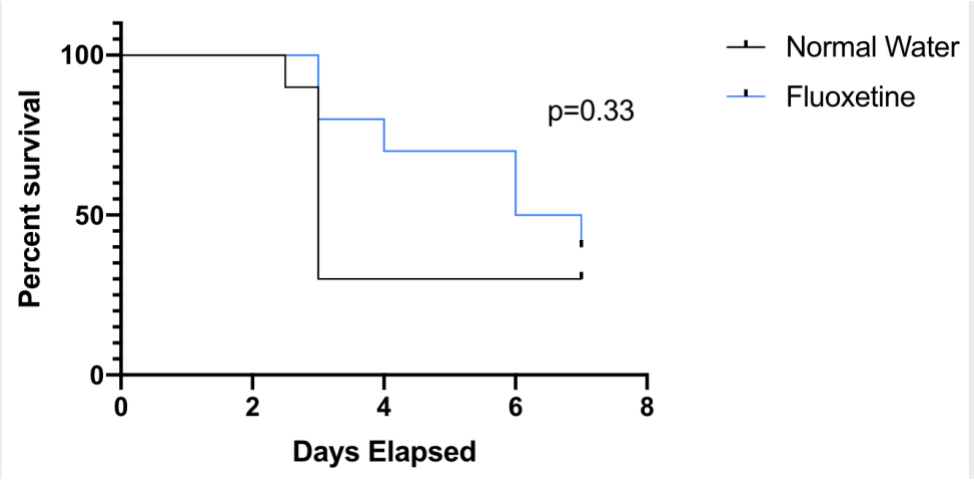


Fig 4. Survival curves of male *C. difficile* infected mice administered fluoxetine (blue) or normal drinking water (black). (n=10 per group)

(In this experiment fluoxetine was stopped after infection but in another similar results were observed when giving fluoxetine in drinking water during the full course even after infection).

Finally, with these experiments one will probably get a lot of microbiome questions.

Vertical

Upstream

1a. Central Control
Rationale/Related Data:
The SNS is under control by the central nervous system (CNS). The pituitary gland has been shown to be an important stress effector and important in

infection. Increasing evidence that *C. difficile* causes neural and cognitive changes.

Experiments:

I would start by trying to determine which brain structures are important by FOS and TRAP strategies (i.e. determine which brain regions activate with CDI) and might be connected to sympathetic activity.[15] Following, ICV injections of receptor inhibitors (you would need to know which system you are pining for) before infection could help with determining the important molecular components in the brain to infectious disease.

Pros:

This is a novel, interesting direction and opens up many more directions if any characterization is successful.

Cons:

There are many central nervous system structures and brain areas that could contribute to CDI.

Complicated surgical procedures and complex mice are likely needed. DSP4, which targets the locus coeruleus or the NE center in the brain, had no effect (Fig 5) meaning it is unlikely that CNS norepinephrine is as important (I still need validation that DSP4 worked as it should).

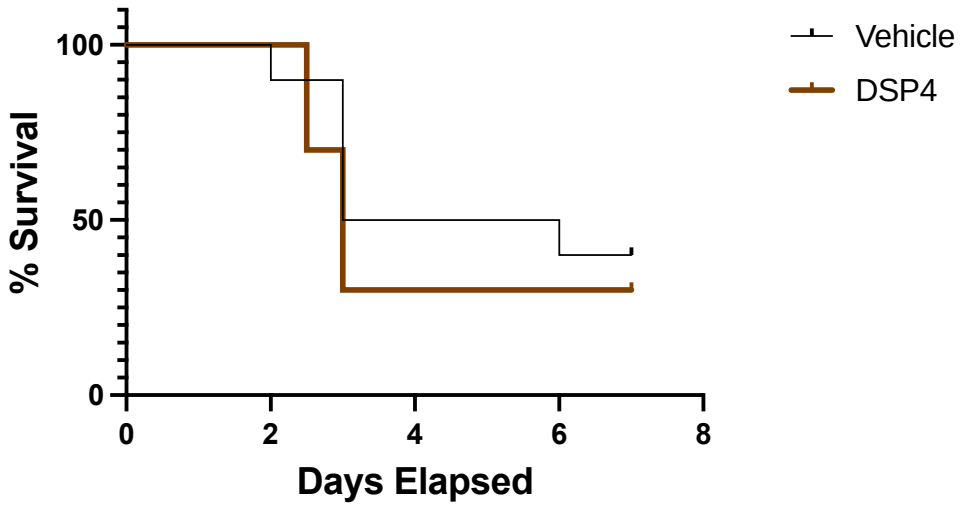


Fig 5. Survival curves after treatment with DSP4 (brown) or vehicle (black) in male *C. difficile* infected mice. (n=10 per group)

1b. Activators of SNS activity in CDI

Rationale/Related Data:

A paper showed that microbiome-sensing by sensory neurons can turn off sympathetic neurons (meaning with Abx-treated or GF mice sympathetic neurons were increased with activity).[16]

Experiments:

Start by measure CG-SMG activity after antibiotics, infection, and resolution to determine the activity of the SNS in each condition. As a later experiment (probably expensive), give monoculture or consortia of species to germ-free mice that do or don't affect SNS activity and compare the effect on CDI outcome.

Pros:

This direction is directly connected to a possible mechanism of the SNS and CDI paper.

The microbiome will always be of interest in the CDI field. The microbiome could play a part of the sensory-SNS crosstalk story.

Cons:

The CG-SMG is probably not something someone in the lab knows how to dissect but maybe someone in the Deppmann lab can. Otherwise, there is a paper that offers some guidance.[17] Related, it would be difficult/complex to do neuronal tracing and interventions with our setup.

2. Sex (and Strain)

Rationale/Related Data:

Sex: In Chapter 4, we observed that 6-OHDA protected both male and female mice against CDI-induced mortality. However, we also saw that alpha 2 blockade or genetic deficiency was only protective for male mice. I have not done any experiments in beta receptor blockade (or alpha 1) for female mice.

Strain: As mentioned, the effects of restraint stress is strain specific. Though these studies don't focus on the SNS, it could be that these effects are strain specific as well.

Experiments:

Gonadectomized males and ovariectomized females can be purchased from Jackson for simple infection experiments vs sham surgery mice. Use RX 821002 in surgically modified mice to determine the effects of sex hormones on the effects of the alpha 2 receptor.

Another easy experiment would be to use 6-OHDA and RX 821002 in other strains of mice to determine the effects of mouse strain on the role of the SNS and Adra2a. As a fairly simple characterization (also related to neutrophil hypothesis), determine whether there are Adra2a and/or beta 2 AR receptor expression differences in males and females. It could be also a good idea to do this for endothelial cells to see if any of these cells can explain potential sex differences in disease pathology, especially hemorrhaging. Another simple

experiment is to test beta receptor blockade in female mice. Added rationale: it could be that beta receptors have dominant or other cell effect in female mice downstream of adrenergic influence. This could also mean that adrenergic influence is more important from the adrenal gland in females where SNS is more important in females .

Pros:
All of these experiments are relatively easy and simple. These would be perfect for learning and working with trainees allowed to work with mice. Answers to these experiments could help narrow down the major mechanisms of the SNS in CDI.

Cons:
The biggest problem of this direction is that it alone is not high impact. As for experimental concerns, other strains may not be as susceptible to CDI making the signal of "protection" smaller. In determining sex-dependence, surgical manipulation sex organs might be too short-term if not done at the right age. These done earlier could make the experiment longer.

3. Local vs Non-Local SNS Activity in CDI
Rationale/Related Data:
The effects of the SNS depend on where it is acting. Surgical or optogenetic changes to intestinal innervation by the SNS can have different effects from 6-OHDA systemic ablation of the SNS. [18]
Lymphoid organs (e.g. spleen, bone marrow) are innervated by the SNS which could affect the state of inflammation.
Experiments:
The most direct approach would be to infect mice after intestinal sympathectomy procedure or sham to test the local. Alternatively, one could give local injection of RX 821002 or 6-OHDA then infect mice or give local cecal toxin, but they would need to verify that these drugs do not make it into systemic circulation.

Pros:
This direction can cut down possible mechanisms drastically as it gives a spatial idea of what cells could play a role downstream of the SNS. Local injection experiments have the same surgical procedure as the E. Histolytica infection, so the lab is capable.

Cons:
The intestinal sympathectomy is a very specialized procedure. I would suggest finding a collaborator who has already done so but the issue then is working with ACUC to ship mice that have undergone surgery. Many people assume that the effect is directly intestinal, so would not be surprising if it is. Higher impact if it isn't. Readouts to local injection of toxin would be difficult. H&E results were

unclear in my first tries (some pathology might be due to surgery/dehydration). Important: RX 821002 administration (systemic) will not allow for ketamine/xylazine anesthesia. Find another form when doing surgeries in the presence of RX.

Downstream

1. The Role of Immune vs Epithelial Cells

Rationale/Related Data:
Although it seems clear that the SNS is playing a major role. The cell type responsible downstream of the system is unclear. Our best guess is that immune cells or epithelial cells play a role.

Experiments:
A bone marrow transplant of Adra2a KO cells into WT recipient and vice versa will probably be the most expected way to test this hypothesis although it's not a perfect experiment. As a characterization (if local) one can determine which immune cells and epithelial cells express Adra2a by qPCR, IHC, or flow. As an alternative to the bone marrow transplant, Adra2a conditional KO mice (epithelial Vil Cre, Granulocyte or Neutrophil specific Cre) can be used.
As a more targeted approach, one could sort Adra2a KO neutrophils and transfer them to WT or vice versa. Use CD45.1 vs CD45.2 and compare phenotype (transcriptomics or qPCR or activation markers) and frequency (making it to the colon) to test both function and trafficking.
Testing intestinal permeability might give a functional idea of the role of the epithelia. As sick mice are dehydrated it is tough to get blood from them making FITC-dextran assays difficult. As an alternative one could determine the level bacterial translocation to the liver. Although differences not seen in RNAseq, you can try to look for changes in tight junction protein expression (e.g. ZO-1, claudins) with IHC to also get an idea if RX 821002 changes epithelial ability to maintain barrier function. Complementing in vitro studies might include determining whether alpha 2 AR stimulation increases primary epithelial cell culture cell susceptibility to *C. difficile* toxins.

Pros:
This is the most obvious and direct question following the paper. There are many avenues one could take (cell depletion, cell-specific ablation) with this direction and most will be high impact.
MyD88 KO experimental results suggest either or both are important (see discussion in "Tips" below).

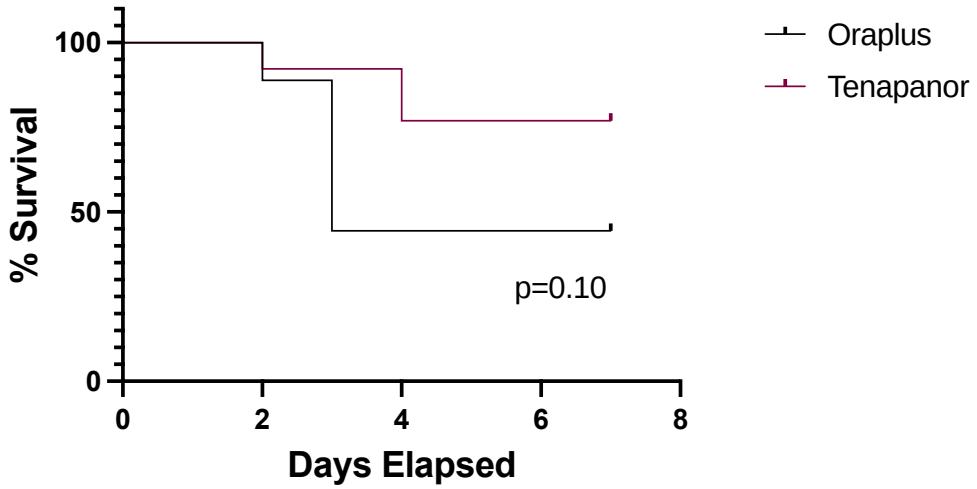
Cons:
There could be a major contribution of non-epithelial, non-immune cells. For example, platelets can play a major role in intestinal inflammation and are affected by alpha 2 adrenergic receptor stimulation. Also, these experiments will likely be expensive (time and money). Bone marrow transplantation is long, takes

expertise, and doesn't simply draw the line between immune and epithelial cell contributions. Finally, the effect of full body Adra2a KO isn't huge. The effects in just the immune or epithelial compartment are probably smaller.

2. Water (and Salt) Balance

Rationale/Related Data:

- Observation: Mice treated with RX 821002 or 6OHDA (RX 821002 especially) have very watery ceca. I wondered whether this might contribute
- Initial Test: Tenapanor



There is evidence suggesting that SNS activity affects salt/water homeostasis in the intestine. Additionally, sodium/hydrogen transporters such as NHE3 have already been implicated in CDI and general colitis.[19–21]

Experiments:

First one should, verify this tenapanor effect. Following, one could use inhibitors of NHE3 or other transporters to reverse the effects of RX 821002 in the infection model

Pros:

This experiment follows a direct observation and has a simpler testable hypothesis.

Cons:

The supposed downstream mechanism of water balance is unclear and multiple transporters could be at play. Increased intestinal fluid (especially in toxin ileal models) is usually a bad thing, so it might be hard to explain why it should be protective in this case.

3. Quorum Signaling

Rationale/Related Data:
There is a great wealth of literature demonstrating that norepinephrine can change microbe and pathogen behavior through quorum sensing receptors such as QseC. Initial test with a QseC inhibitor LED 209 shows a trend in protection (Fig 6).

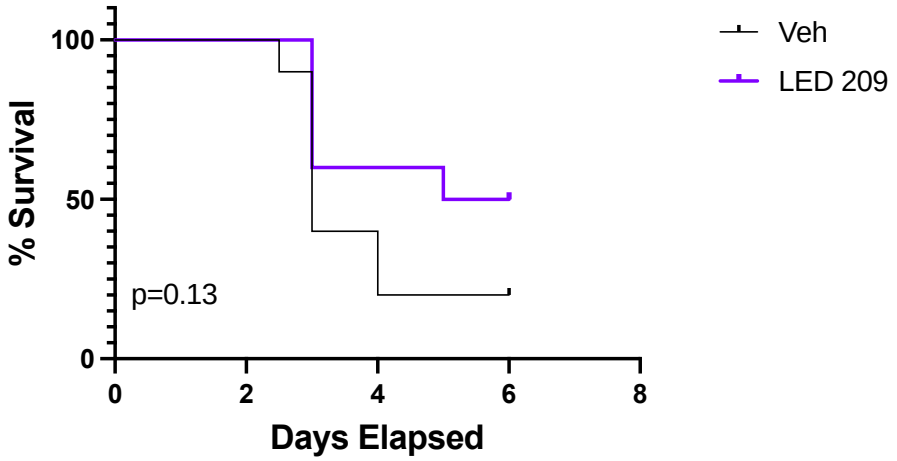


Fig 6. Survival curves after treatment with LED-209 (purple) or vehicle (black) in male *C. difficile* infected mice. (n=10 per group)

Experiments:
In vivo: Start by repeating the LED 209 experiment. LED 209 is an inhibitor of QseC, the bacterial receptor responsible for response to catecholamines. In vitro: Determine if *C. difficile* responds to catecholamines (growth and toxin production) by qPCR for toxin genes, OD/colony counts, and toxin (ELISA). To determine if *C. difficile* behavior is changing, use RNA in situ hybridization to visualize the localization of *C. difficile* in treated vs untreated mice. We tried this once but failed (too much background).

Pros:
This is a somewhat novel direction. There is evidence that *C. difficile* responds to norepinephrine (hard to find thesis on the internet). It is not known what receptor *C. difficile* would be using to respond to NE. This direction has easy ways to incorporate both in vitro and in vivo experiments.

Cons:
It will take extra experiments to verify that LED 209 is not acting on host alpha 2 adrenergic receptors (use the Adra2a KO). Even after it will be difficult at first to know if LED 209 is working directly on *C. difficile* or other members of the microbiome. And following that hurdle, it will be hard to confirm LED 209 effect with other means. In vitro non- effects might not reflect potential differences in

changes in potential for chemotaxis or adherence to epithelia by *C. difficile* as seen by other pathogens.[22–24] One work around could be to use qPCR for known genes of chemotaxis and adherence in the in vitro experiments.

Other

1. RNAseq analysis

Rationale/Related Data:

We took 4 groups of mice and sequenced distal colon: 1) Uninfected, 2) Uninfected + RX 821002, 3) Infected, 4) Infected + RX. All groups were given antibiotics, and the mice were sacrificed late on day 1 (something like 7pm that day) before symptoms occur.

Day 2 (onset of symptoms) was not chosen because we would likely see an inflamed vs non-inflamed signature which doesn't tell us much. We want to know what changes happen in the calm before the storm.

- Upregulated Pathways: Cell cycle, DNA replication

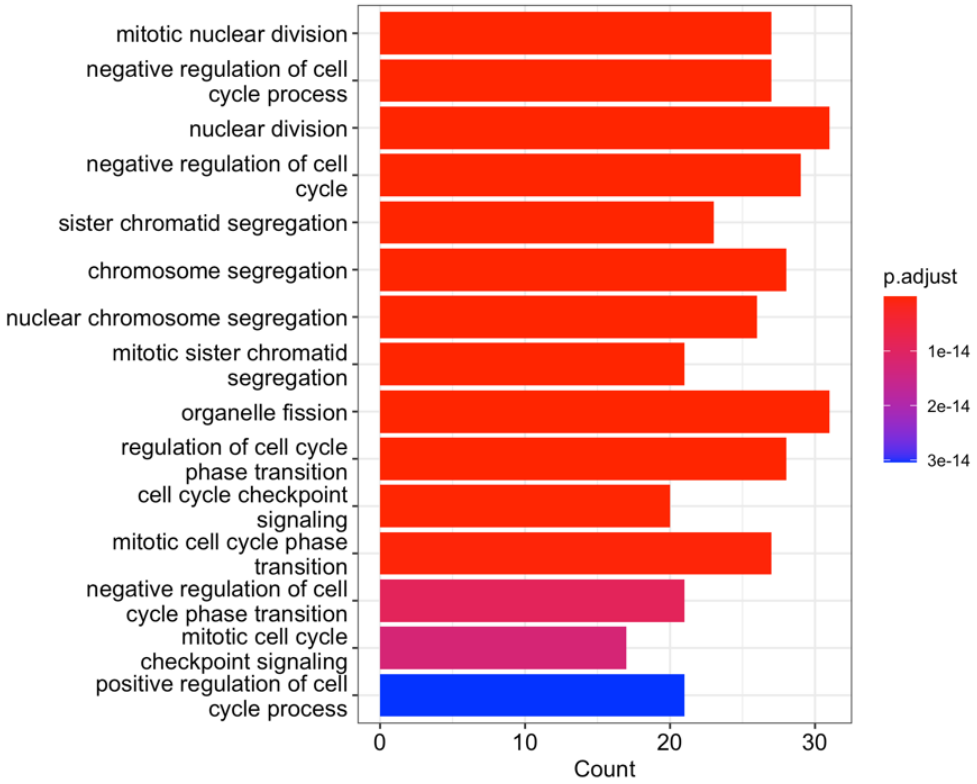


Fig 7. GO analysis of differentially expressed (upregulated) genes comparing RX 821002 treated mice to Vehicle (PBS) treated mice. All mice are uninfected.

(Comparing the same conditions in infected mice yields very similar results)

- Downregulated Pathways: Oxidative phosphorylation

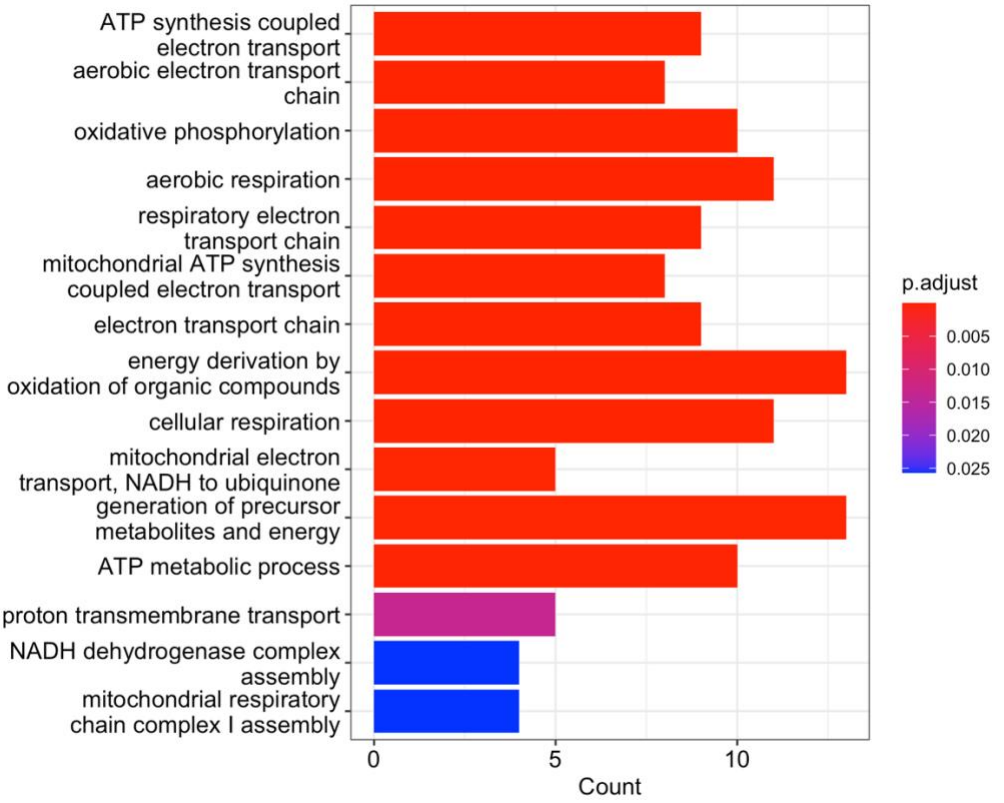


Fig 8. GO analysis of differentially expressed (downregulated) genes comparing RX 821002 treated mice to Vehicle (PBS) treated mice. All mice are uninfected.

(Comparing the same conditions in infected mice yields very similar results)

Targeted Analyses (DEGs) generally found no differences including, antimicrobial peptides (also verified with Jacob with qPCR), Tight junctions, Inflammasome, and cytokine genes.
There is a very pertinent paper that recently came out.[25] It shows that Enterococcus derived tyramine stimulates the Adra2a receptor and decreases intestinal stem cell proliferation. This is surprising because there are a few papers showing that Adra2a stimulation increases proliferation in intestinal epithelial cells although there are a few in support too. What's most striking is that tyramine treatment downregulates pathways that are very similar to ones upregulated with RX 821002 (a blocker) here. These suggest a similar mechanism could be important in our case as the most downregulated KEGG pathways for their studies are "Cell cycle" and "DNA replication". I think the best course is to follow suit with their other experiments to determine if the same is happening for our system.

Experiments:
I might start with some characterizations including day 1 staining of Ki67 after RX 821002 treatment. Alternatively, I would use a Lgr5-EGFP-IRES-CreERT2

tdTomato LS mouse - Lineage tracer tdTomato+ cells in crypt to see real time proliferation of crypt cells after tamoxifen (Visualize with IHC). Tyramine might work as an alternative way to test alpha 2 AR agonism.

Pros:

The results of RNAseq if tied to increased proliferation of the epithelium serves a strong explainer of early intestinal damage leading to large changes in inflammation. It is a very reproduced finding that intestinal crypt cells (especially amplifying ones) express Adra2a.

This could very well be the unifying mechanism in being important for both sterile colitis and *C. difficile* colitis. Enterococcus species have already been implicated in C. difficile infection severity. This can be a mechanism worth looking at for FMT as well. It gives something to look for in biopsies (intestinal stem cells) and 16s data (Enterococcus).

Cons:

We will have to prove that tyramine is or isn't also playing a role. Tyramine can also stimulate the SNS. A lot of the Enterococcus paper uses organoids. I don't think this is necessary, but it might be a reason to try intoxication assays in organoids with RX 821002. Fancy mice might need to be used to find stem cells and they could be difficult to target at the intervention stage.

2. Recurrent CDI

Rationale/Related Data:

The big and well-known problem in *C. difficile* is recurrence.

Experiments:

Administer SNS component inhibitors or use genetic KO in recurrent infection model of CDI.

Pros:

This direction is extremely high impact. If we had a recurrence model, the experiments would be simple. This could be a segway into testing the long-term effects of SNS activation (e.g. the effects on adaptive immunity and antibody production). [26] It might also relate to how FMT and the SNS as the other side of the coin.

Cons:

The biggest obstacle is that our lab has not been able to establish a recurrent CDI model with severe symptoms after reinfection. Note: SNS neurons grow back 14 days after 6-OHDA if another dose is not administered. Unfortunately, so far there is not much evidence currently to suggest that there will be a role.

3. Relating *C. difficile* infection and sterile colitis effects

Rationale/Related Data:
Adra2a has been implicated in ulcerative colitis (polymorphisms) and in mice (genetic and pharmacology) as it has in *C. difficile* colitis in this thesis. [27,28]
Experiments:
One could start by testing the mechanisms implicated by other papers of DSS, TNBS, etc. and determine whether similar cell types play similar roles in both contexts. Some papers suggest the importance of the alpha 2 AR in macrophages[29,30] Alternatively, compare the transcriptional response in DSS and *C. difficile* treated mice related to the SNS response or before and after SNS activity intervention as a comparative study.
Pros:
This direction is potentially very high impact. Also, this could be an important way to explain why the SNS doesn't work the same way in other infectious contexts (i.e. contrasting the role of SNS from other contexts).

Cons:
It will be difficult to know what to do in the beginning.

4. Vagus Nerve and CDI

Rationale/Related Data:
Surprisingly, there are no studies on the parasympathetic system in CDI. The vagus nerve is a major controller of inflammation. The parasympathetic system is like the Yin to the Yang of the SNS in that in many cases parasympathetic activity has opposite effects.

-

Experiments:
The most straightforward experiment would be to do a *C. difficile* infection in mice after cutting the vagus nerve. If the surgery is not possible then look to do a pharmacological vagotomy (would need to get advice on this). A complementary experiment would be to do a *C. difficile* infection in mice with vagal nerve stimulation (not sure if this is possible logistically in the BSL2).

Pros:
This direction would be of great public interest and novel. This study could potentially create a defining, opposing and complementing picture to the SNS story (or a surprising and unrelated one). One is more likely to find a collaborator who can do mouse vagal nerve stimulation or resection than one who can do intestinal sympathectomy

Cons:
There are not very many clean pharmacological approaches.

5. SNS in Human CDI

Rationale/Related Data:

People will want to know how these findings relate to patients with evidence presented in the SNS and CDI paper.

Experiments:

(Electronic) Determine if there is a relationship between heart rate variability (a proxy for SNS activity) and symptom severity (or WBC/neutrophils).

Characterize the relationship between NE and neutrophil count in human samples by measuring NE in intestinal biopsies to see whether local NE concentrations are associated with symptom severity, WBC count, or neutrophils.

Pros:

This would be the highest impact continuation of work.

Cons:

There are no easy intervention experiments for this direction. Most experiments will have to be characterizations. I do wonder if you can test the sensitivity of cells (probably PBMCs) to adrenergic stimulation from patients Note: Plasma NE did not stratify severe acute CDI from less severe. However, it seems that plasma NE is not the best indication of SNS activity.

Tips:

- Nepicastat (inhibitor of NE and E synthesis enzyme Dopamine Beta Hydroxylase)
    - Conversely, metyrosine (inhibitor of catecholamine synthesis enzyme Tyrosine Hydroxylase did not have as consistent an effect).
    - OraPlus works really well as a vehicle with crushed up (by mortar and pestle) compounds like nepicastat for oral gavage
- The effect of 6-OHDA was also seen with another strain of *C. difficile* VPI 10463which doesn't express the binary toxin. (See Chapter 4 of this dissertation)
- Desipramine (Norepinephrine Transporter blocker) might have an effect itself as I observed a restoration of immune cells past baseline when adding 6-OHDA + desipramine compared to 6-OHDA. This would make sense as the blocker should increase the bioavailability of norepinephrine (it's not being recycled as quickly).
    - I have the idea that where reversal of phenotypes with alpha 2 agonists fail that use of desipramine might work. It could be that the effect is greater and longer lasting.
- ***My most intriguing finding has to be the effect I saw with MyD88 KO mice (Fig 9). I found that Myd88 KO mice and WT mice untreated succumbed at similar rates. This would be surprising but they were all almost moribund right away meaning there is no way to see a worsening effect (which would be different in the infection where mice typically survive the whole course).[31] MyD88 KO were protected by RX but *very*

partially, as in an intermediate effect. As control WT mice given RX were completely protected through the infection course.

- MyD88 is necessary for neutrophil entry which is my biggest immune phenotype.
- This and the sex specific effects suggest that host mechanisms are more likely to be important than microbiome effects by RX.
- Begs the question as to why protection was partial. Are other DAMP/PAMP sensors compensating in some way? What mechanisms are ruled out if sensation of *C. difficile* (or the microbiome) is necessary for the effect of RX?
  - A hunch of mine is that MyD88 is necessary for neutrophil recruitment but that neutrophils entering the intestine are mis-activated by stimulation through the alpha 2 adrenergic receptor. [32,33] This mis-activation affects neutrophil ability to clear translocating bacteria and worsens disease. In a nutshell, RX works by giving infiltration neutrophils the necessary bacteria-clearing type they need but MyD88 KO limits the number of recruited neutrophils that could help.
    - Caveat to this hypothesis: I do see a lot fewer neutrophils in number in the WT-RX condition, but I wonder if that is because they were recruited quickly enough.

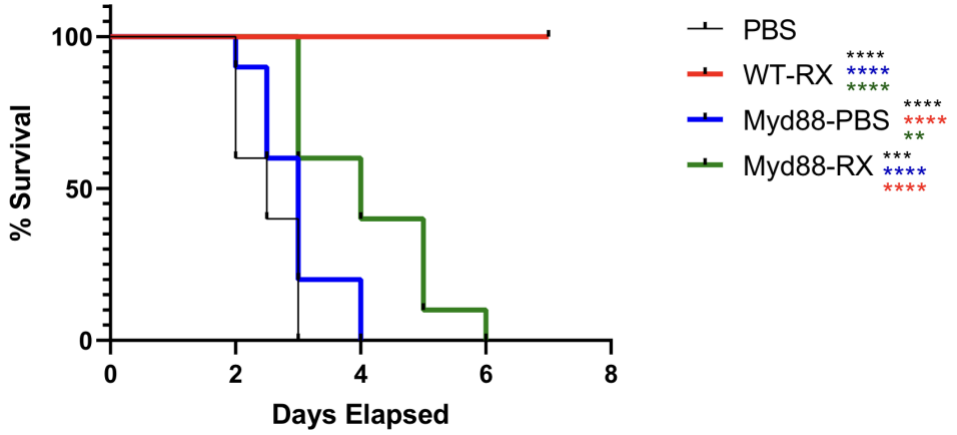


Fig 9. Survival curves in male WT *C. difficile* infected mice treated with RX 821002 (red) or vehicle (black) or Myd88 KO *C. difficile* infected mice treated with RX 821002 (green) or vehicle (blue). (n=10 per group)

- I didn't know where to fit it in here but DREADDs would be a really good way to go about confirmation of pathways and having ways to get around the agonism problem.
  - Use TH Cre specific DREADDs to inhibit or stimulate SNS neurons during CDI

- Another thing that I find difficult to fit in but is really important is probing the intracellular responses of intestinal epithelial cells to determine where the effect of RX 821002 "starts" during infection.
    - Alpha 2 AR downstream signals as that of the toxin's intoxication effects. It will be important to determine which if any are stopped
    - The *cleanest* determination of these molecular stops in toxin activity would be in vitro. Unfortunately, the alpha 2 AR receptor is not expressed in a lot of immortalized cell lines. There are cells lines that have been transfected with the gene that could be useful or primary culture would be another avenue.
    - I did try to see if there were differences in pERK when comparing RX 821002 to vehicle-treated mice with IHC but there didn't seem to be a clear effect (actually pERK looked pretty random even in uninfected controls).
        - Need to verify that in RNAseq there are no obvious differences in the intoxication pathway.
- Remember that the Adra2a is a subtype. Adra2b and Adra2c didn't seem to have a pharmacological effect when I blocked them, but I do wonder what might be happening in the Adra2a KO.
- WT littermate controls of Adra2a act very weirdly in terms of survival. It seems almost as if there is a cage-effect like delay in mortality and it's almost certainly due to some microbiome effect.
    - Also remember that SNS neurons are activated with a disrupted microbiome (antibiotics or germ free).[16] I believe this will be very important when considering how antibiotics might sensitize the host to severe disease and on the flip side, could explain some of the positive effects in FMT. This paper also shows FMT quiets SNS activity.
- Professor Harris is a really good resource for project and paper feedback (especially for infection/inflammation models).
- Don't use Mendeley. Use Zotero.

This is a non-exhaustive outline of the directions I think best pursued in context of the work shown here. For more details of the graphs shown here ask for my lab notebook which I am hoping to have prepped virtually.

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Chapter 6 - On Animal Models of Suffering

One thing that I've struggled with through the PhD is on the use of mice in infectious disease models. I figured the least I can do is share my thoughts tucked away in the back of this dissertation. Warning: these are opinions and not expert ones, just ones based on my likely-uninformed logic, more philosophy than science. I am working to inform myself on the matter and as you'll see I don't have all the answers. In fact, I am hoping that this will encourage those that do to do speak up and start a discussion.

In writing my first draft of this chapter, I learned that my opinions and feelings about the matter are more complicated and confused than I thought, so I turned to compare and contrast my thoughts to the champion of animal rights activism, Peter Singer. Singer's stimulating and coherent work *Animal Liberation* discusses arguments against using animals for research and food (Singer, 2009). Although I agree with Singer in many cases, I believe that our views on how to address the issue differs. In fact, to make my arguments clearer I will put myself in the awkward position of defending animal users against some of the arguments of Singer. With that said, Singer and I have the same end goal to mitigate the suffering of animals in research. I have repeatedly had to remind myself that he first wrote *Animal Liberation* in 1975 (almost 50 years ago!) as we still struggle to answer the questions and challenges that Singer poses. Here I'll talk about what makes animal models of suffering an issue, work backwards and explain why we can't get rid of them, and finally give a thought (yes only one) on how we might slow the spread of this festering wound of the human condition.

I might be species-ist (and still hate animal models of suffering)

The cornerstone of Peter Singer's arguments against the use of animals in research is that it is a form of "speciesism", a discrimination against other species. At a glance, it might seem that he is arguing that we should care about the lives of all species equally. However, Singer makes important nuances to the argument. For example, he states, "This does not mean to avoid speciesism we must hold that it is as wrong to kill a dog as it is to kill a human being in full possession of his or her faculties." (Singer, 2009, p. 18-19). And he does so more than once, nearly always specifying the last part that the human being must be in their "faculties" to be given any favor over other animals in exemption from experimentation. The label *"Homo sapiens"* doesn't give us the right to use animals as objects and does not give us the right to be free from experimentation where animals are used. There is some cognitive ability or capability (likely related to goal-making or self-consciousness) that Singer believes *would be* fair justification for using animals in important experiments instead of humans, but without it both organisms are on a level playing field. He writes, "So when experimenters claim that their experiments are important enough to justify the use of animals, we should ask them whether they would be prepared to use a brain-damaged human being at a similar mental level to the animals they are using."

(Singer, 2009, p. 83). I must reiterate that he is not calling for human subjects in experiments but admonishing the use of animals in experiments where we would never use a human. In another section he states, "If the experimenters would not be prepared to use a human infant than their readiness to use nonhuman animals reveals an unjustifiable form of discrimination on the basis of species, since adult apes, monkeys dogs, cats, rats and other animals are more aware of what is happening to them, more self-directing, and, so far as we can tell at least as sensitive to pain as a human infant." (Singer, 2009, p. 82). For me, these statements are logical but ring a little alarm in the back of my mind. I care more about infants and brain-damaged people than mice, but should I? I still think yes but it's difficult to give a logic-based rebuttal. Singer is challenging us to think about why we have drawn the line for objects of use at the level of species. It's a fair question (that I will touch on later) that I do not have a clear answer for and, in large part, agree with the sentiment of the question. We should respect other animals at a level close to how we respect other humans. However, I think that is a very different question to choosing whether to inflict pain on an animal model or human, regardless of intellectual capability. If given the choice I would have to go with the animal, but I say this only to carve out true thesis of this chapter: I value humans more than animals and I *still* believe that human-induced animal suffering is a major moral tragedy for our species.

My chief concern is not in this chapter is not in the general *use* of animals but in animal models where suffering is necessary. It is not that I think that breeding, eating, etc. of animals are not valid points of discussion but here I'd like to concentrate on the thing that I believe to be the currency of tragedy: *pain*. We care about pain because…well… Actually, now that I really think about it there is no *objective* reason why we should care about the pain of animals. The cold, utilitarian reasoning for why we should care about the pain of other humans might be because you might find yourself in their position of need. Many animals, especially those bred for science offer the general public very little in being sympathetic of their pain. I'll argue that it does not matter if there is an objective reason for caring about the pains of others, the fact of the matter is that many of us do. Whether it is motivated by religion, a feeling, an experience, the *subjective* value of relieving the suffering of others is important to us. This may come as a surprise, but I do not think that the subjective nature of the argument necessarily weakens it. Singer uses a ton of Pathos (as he should) in his arguments to illustrate the suffering of animals in heinous and unnecessary experiments. Dissenters may argue, people can make a compelling and sad story about any object. I cried for a melting Frosty the Snowman as a child. I agree the stories alone are not enough. But when we connect the stories to struggling and pain as Singer does, I think it makes it hard for us to ignore. Sure, one could argue that the nature of pain is different than it is in humans, but the outward appearance of their torment is much like our own. Without getting into the scientific details, the most parsimonious explanation is that we share enough aspects of pain and agony, aspects of which I would never wish on another human, animal, or snowman.

Where should the line of concern be drawn on a Kingdom level, Phylum, species level? Unfortunately, I am not *yet* extending my sympathies towards plants, bacteria, archaea, or single-celled eukaryotes. My reservations are limited to animals and more specified than that. My mind can certainly be changed if there is sufficient evidence that animals such as insects and small fish exhibit consciousness and self-awareness to an appreciable extent, but otherwise I am not concerned about these animals either. I am concerned for animals that exhibit *self-aware physical and/or social* pain. I am aware that insects experience pain, but I also know that people argue that plants also experience pain. As I have not been convinced that plants are self-aware (wow, would this change everything) I am under the impression that all "pain" is not equal across different organisms. One aspect of pain is an avoidance response. That seems to be the primary purpose of pain, to avoid present or future harmful stimuli. But avoidance behavior without "experience" is just reflex. But there are a lot of animal models we use that exhibit much greater than the reflex. Perhaps the most disturbing aspect of my PhD was observing the social pain of rodent models infected with *C. difficile*. It was quite obvious that mice would huddle closer to very sick or dead mice, even after separation they would try to go back even when the other mouse offered nothing in terms of warmth. Or on the other hand where it was obvious that a sick mouse couldn't make it to the huddle of healthier sleepers. At times, I wonder whether I am projecting or anthropomorphizing them. At other times, I wonder if that's even a bad thing.

As a related note (and I will likely ruffle feathers with this one) I am curious about where others draw the line. There are many practical reasons that rodents are used as models for science including their small size, genetic similarity to humans, breeding capability, and short development time. However, there's an implicit reason for why we are tolerant of the use of rodents over other species: they're pests not pets. They bite. They poop everywhere. In public, they carry disease. They behave in many ways that humans see as aversive and for that reason it's much easier for us to justify their use in research. Often when I hear a gripe about animal use (even in the case of mice) it is usually they lament the use of such "cute" animals. Cuter animals (much like cuter people) get more sympathy. While it's understandable that our emotional response to the cuteness of animals should factor into our opinions, from a moral position it does not hold much ground. I am not asking us to think about using cuter animals more in research but why we don't extend the same sympathy to "uglier" ones.

We shouldn't care about animals because they are cute or because we value them the same as we do humans. We should care about pain and finding ways to mitigate that. At any cost? Maybe not but that's what we'll explore in the next sections.

Is 'we don't know what we don't know' an argument for or against animal models?

The argument *for* animal models is simple: there is no alternative. There is no substitute. In that way I do not believe that if there were proper ways to receive the answers, we need without animal models, that anyone would have a convincing argument why they would need or want to use them. But this is an insurmountable "if" as it seems now. The answers we need that necessitate animal models are to a couple of questions:

1. How can we be *confident* that we know how the body works?
2. How can we be *confident* that we know a therapeutic will work and be safe?

Models of suffering are just that and they are necessary. They are necessary because we need *confidence*. With a more painful (and/or commonplace) a malady, there is a heightened need to be sure that they therapeutic approach is going to work. How will we know if something cures sickness if we cannot recreate the illness in the first place? The half-answer is to find a proxy for sickness that we can model without pain but with every degree of separation from modelling the illness we are magnitudes less confident in our findings and our ability to successfully implement them in the form of therapy. A failed argument against the use of non-human primates is that a small fraction of the therapeutics used in these studies fail to make it through clinical trials. If we were to only use the next best thing, rodents, the genetic dissimilarity to humans increases significantly along with the rise of the chance of failure in clinical trials. Organ-on-a-chip models are exciting advances that could bridge that gap and use human cells but I'm not sure that we'll be able to model multi-organ systems at scale very soon. And even if we do, how long will it take for people to trust it?

In this vein, we must also look to how well animal models are making us more confident in general. To be perfectly honest, I can't cite a study that suggests they are. Only anecdotally can I say that my colleagues tend to give higher importance to *in vivo* studies when it comes to translatability of findings. One hope I had when picking up *Animal Liberation* was to find some statistic that demonstrated animal models don't actually work all too well for saving lives. But one thing bothered me. Let's say it were the case that animal models give us a better chance at ascertaining the effectiveness and safety of future therapeutics (and now we're not even talking about basic science research). What % of confidence boost would be acceptable? Are we fine with a 10,000,000 mice:1 effective drug ratio? Someone's answer might change if I asked if we are fine with a 10,000,000 mice: 1 human life ratio. I'm not sure if there's a number, we could all agree on. To make matters worse, we are terrible at ascertaining how many animals we are actually using. Singer cites millions and millions of animals being used and makes an important point that these are only the *reported* numbers of animals being used, those making it to publication. Even now, science is horribly inefficient in animal use but that also leaves us with much room for growth in reducing animal suffering.

Singer cites many studies where many animals are used in a suffering paradigm, but the results of the experiment are indeterminant. Most of the experiments, I agree are irredeemable faults of the experimenters who have failed to put in place the proper controls to interpret multiple outcomes (or the only question was "how far can we go?") (Singer, 2009, p. 49 and p. 61). However, some experiments flagged by Singer and many of today are ones that we could not have known the utility of the experiment beforehand. There is a bit of hindsight bias in saying "see? You tried and it didn't work." Singer brings up "learned helplessness" models as a failed model of depression (Singer, 2009, p. 46-47). Excluding the experiments without clear controls or foresight, how else would one dive into the black box of depression, an ailment we clearly still don't understand, without building a model that looks like it? Singer also laments that animals were used to *disprove* the theory that these models are good models of depression. I understand the frustration, but I again ask, how does one disprove the usefulness or translatability of a model without using the model? It's going to be even harder to make any progress on what to do with animal models of suffering as these models have clear purpose regardless of the outcome and, sadly, regardless of the interpretability of the outcome. Rid the latter and how we deal with the rest?

So, in one sense, Singer gets it right. Sometimes progress in understanding looks like wasted time. But then again, is it worth the cost? Maybe if we weigh it against our gained understanding of the world or the potential lives it could have saved even negative, seemingly unproductive data can be useful. It's up to us to weight the scales of animal pain vs aspirations of humankind but I have little faith (sorry) that we would come to a consensus on the matter anytime soon. Not only are the views and motivations so varied on the subject, animal suffering, as it was at the time of Singer, is not a public eye issue. I would like to look for the solutions to the problem that work regardless of one's position on the weight of animal pain to human endeavors but I'm getting ahead of myself…

What if Thanos snapped and all animal models disappeared?

If we care so much about pain, we should just get rid of it, right? Well, it's not that simple. Even the champion of animal rights is not necessarily against all animal research as he writes, "But to be opposed to what is going on now it is not necessary to insist that all animal experiments stop immediately." (Singer, 2009, p. 40). He explains that animal models should only be used in experiments serving "direct and urgent purpose" and, like many efforts already in place today, to find alternative methods to do experiments that replace the need of animal models (Singer, 2009, p. 40). I agree but I do think we have trouble considering what experiments are "direct and urgent". Our uncertainty of the outcome, intrinsic to the scientific process, already blurs the line on which experiments might be useful and important. Since we don't know what we don't know we are leaving a lot on the table if we only do "direct" experiments. Replicating studies

isn't necessarily "direct and urgent" but is sure important to do. Even more, some experiments that *might* serve direct and urgent purpose, which would include many if not all models of suffering, still might require high numbers of animals. My concern is not that if we should be striving for more "direct and urgent" experiments but if that goal and our inability to define it is a hinderance to our progress. I wonder if we are lying to ourselves when we say we can pick the right experiments and number of mice (and please don't yell 'power analysis') to use under these goals.

The COVID-19 pandemic was a clear reminder of the power of medical science to protect us against deadly diseases. A large part of that effort was the development of a mouse model that recapitulated the symptoms of the human ailment. Without those mouse models it's likely that Covid-19 vaccines and therapeutics would have not been developed in a timely manner. This is a case where I believe Singer would be ok with using animal models as the path for direct and urgent models is clear. It's an emergency. But what about illnesses that are not currently or ever a threat to mankind? Would Singer rid of cancer models, atherosclerosis models, mouse models of rare disease like Cantu syndrome? And what of the basic science research that make advances for these diseases possible? If Thanos snapped and those models went away, I would predict that the effects short-term would not be catastrophic. Long-term, if we were to only use animal models for emergency infectious diseases, we would be certain to see increases in disease growth today but might not mean the end of mankind.

I think if we're being honest with ourselves there are other motivations for keeping animal models that supersede our perceived cost of animal suffering. One of those is the pursuit of knowledge, learning about the world and how it works. To Singer, this would not likely be worth it for animal lives and pain, but to many others it would be. Another motivation is money. We might come to the same conclusions about drug toxicity and even therapeutic interventions through in vitro studies, but animal models allow for that increased confidence in investment. For animal models of suffering, money might not play as large a role, but confidence is still king. Regardless of that step of confidence, we'll stick to these models. The pain of millions of rodents will likely always be worth to save a single human life. Do we have the right? Singer would probably say no. The person sitting at the deathbed would say yes. I ask readers to use their humanity to care about animal suffering but not to necessarily make a philosophical decision what is the magic ratio of animal suffering to human suffering that is right and just. I think it's a lost cause. We must find a solution that maintains the life-saving power of animal models of suffering while slowly chipping away at our current ratio of animal to human pain.

Singer's solutions and Tyus's thoughts

As I have asked before, where do we go from here? We no longer have the relatively easy task of pointing out ridiculous experiments that harm animals.

Thanks to the work of Singer and others we have ethics committees and a general culture of respecting the animals we work with (whether that is so we are not punished or actually respect them). The problem now is understanding how to mitigate the number of ultimately necessary animal models of suffering. Getting rid of animal models in a snap worked well for the cosmetic industry but the risk to human lives is magnitudes greater if we think to do the same for animal models of suffering (Singer, 2009, p. 58-59). Even animal models not related to suffering are not easy to rid of because of other motivations like the pursuit of knowledge.

In this bleak outlook on the future of replacement of animal models of suffering, I have to come to one conclusion and long-term solution: master data collection and interpretation as a form of animal model use reduction, reuse, and rethinking. I envision a research community that is effectively able to use one mouse where we needed 50 yesterday to make the same conclusions. A large part in making this a reality is enhancing molecular biology techniques that are able to capture more biological information. I am excited to live in a time where single cell RNA sequencing is no longer the cutting-edge and -omics come in countless flavors. What I would like to see in the future is a community that is better at using all the data we generate. The big data problem has been an issue for a while, but some obstacles already seem to be addressed. No longer are we overwhelmed, in most cases, by the sheer amount of storage or compute to work with these datasets. What I see lacking (and maybe I have just not looked hard enough) is in the general ability to work with data that is not our own. Sure, there are databases like the GEO Omnibus that store loads of sequencing and other biological data, but I would like to see people more readily using that data whether it shows up in a figure or discussion. I have been seeing recent publications make publicly available data as part of Figure 1 in hypothesis generation and I think that is the right direction. I would like to see that happen more of this and, perhaps, at other steps in studies such as downstream mechanisms or future directions. In the literature I would love to see more meta-analyses integrating data from many sources. Yes, batch-effects and differences in experimental procedures complicate these analyses but complicated doesn't mean impossible. Lastly, my hope is that genomic (and other big data biological fields) take inspiration from other disciplines. I hope that they take on the feature analysis strategies of data science and the modeling capabilities of ecology and systems biology. Overall, I hope that scientist can become better detectives as we share data and strategy and, in turn, offput the load on a bunch of animal models that we might have predicted with a little more work up front.

If I were to do my PhD again, I wouldn't do this project. Yes, I would likely not use animal models in whichever direction I chose but not because I think it solves the problem. I have no illusions that I am absolved of responsibility or blame of animal models of suffering just because I don't use them. I know that necessary work is being done with these models to alleviate the suffering of many patients and that any work done with cell culture, computational modelling, etc. can only have been made possible by in vivo work. Someone has to do it, and the high horse mindset only undermines the unflashy and grueling work done by

researchers working with animals, infectious agents, etc. Making animals models of suffering harder to access would only make the work of researchers tougher and vilifying researchers for working with animal models of suffering is like suing a firefighter for water damage. Let's make our future focus on working with the making the most of the models we have and facilitating the interpretations of scientists to be more productive, so they don't have to use as many.

I am said to have availability to my UVA email for eternity of so please educate me with your studies, logic, opinions, concerns, and solutions at dt4tx@virginia.edu.

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