ENTERIC GLIAL MODULATION OF IMMUNE ACTIVATION DURING INFLAMMATORY STRESS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Physiology–Doctor of Philosophy

2020

ABSTRACT

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Gastrointestinal (GI) disorders such as inflammatory bowel disease (IBD), irritable bowel syndrome, and other functional GI disorders are major health concerns and account for about $26.4 billion in yearly costs in the United States alone. Many of these GI disorders manifest symptoms such as GI dysmotility, intestinal secretion and absorption dysfunction, and abdominal pain. Inflammation plays a major role in the pathogenesis of these diseases, and current therapies for many GI disorders aim to regulate the activation and progression of the inflammatory cascade. However, due to a gap in knowledge in how the immune system is regulated within the GI tract, there remains a lack of effective treatments for these common GI disorders. Interactions between the nervous system and immune system point to neurons having important roles in immune modulation, but the mechanisms of neuro-immune regulation in the gut is not completely understood.

The enteric nervous system (ENS) consists of enteric neurons and enteric glia arranged in plexuses embedded in the gut wall. This neural network is responsible for the normal secretomotor functioning of the GI tract, and the disruption of the ENS network alters GI functioning and underlies pathological GI symptoms. As part of the ENS, enteric glia work in tandem with enteric neurons to coordinate GI functions. In addition to their contributions to maintain normal secretomotor functioning of the GI

tract, enteric glia are activated by immunomodulatory signals, they can secrete and respond to cytokines, can exert immunosuppressive effects, and share characteristics with antigen presenting cells. Therefore, we hypothesize that enteric glia play an active role in immune regulation in the ENS.

In this dissertation, we specifically examine the role enteric glial cells play as an antigen presenting cell to regulate immune activation. Our results show that enteric glia have the machinery necessary to act as an antigen presenting cell and can express major histocompatibility complex (MHC) type II molecules during inflammatory stress to interact with T-lymphocytes. Enteric glial MHC II expression has functional relevance, as it modulates the activation in T_h17 and T_{reg} subtypes, but not T_h1 or T_h2 T-lymphocyte subtypes. Although MHC II molecules are typically associated with the expression of phagocytosed extracellular antigens, our results show that enteric glia do not readily phagocytose extracellular antigens. Instead, MHC II expression in enteric glia is mediated by autophagy. The activation of autophagic pathways is necessary, but not sufficient in eliciting enteric glial MHC II expression. Finally, although enteric glia regulate T-lymphocyte activation, cytokine levels at the whole organism or regional tissue levels remain unchanged, suggesting that enteric glial cytokine effects primarily operate at the local microenvironment level.

Our findings provide support for enteric glial cells having an active role as an immunomodulator. Specifically, we show that enteric glia modulate T-lymphocyte activation via autophagy-mediated MHC II expression and propose a novel mechanism of neuro-immune modulation in the gut.

ACKNOWLEDGEMENTS

I would like to first thank my mentor Dr. Brian Gulbransen for the guidance he has given me in this journey. His support and mentorship on how to become an efficient and successful scientist has brought me to where I am today in my research career. His positive outlook on any given situation has encouraged me to never give up even when faced with discouraging experimental results. Brian always had time to sit down to discuss any concerns I had whether it was science-related or otherwise, and he was never too busy to talk about data. I am especially grateful for the patience and understanding Brian has for the DO-PhD program – the sheer length of the program, the extra obligations I have that pull me away from experiments, and the times during medical school where I spent studying and wasn't very productive in lab.

I would also like to thank the past and present members of the Gulbransen Lab who have made being part of the lab such an enjoyable experience. Jonathan, David, Vlado, Siomara, Isola, Ninotchska, Wilmarie, Luisa, Christine, Mustafa, RJ, Becca, and Erika, thank you for all the help in lab and experiments when I overestimate how much I can manage in one day. Thank you for your insights and and helping me develop as a scientist, and our time spent hanging out outside of lab as well. Your help and friendship have made my experiences here very enjoyable.

I would also like to thank the DO-PhD program who made my graduate career here at MSU possible. Thank you, Dr. Justin McCormick and Ms. Bethany Heinlen, for admitting me to the DO-PhD program and allowing me on this wonderful journey. Thank you to Dr. Brian Schutte and Ms. Michelle Volker who are the current DO-PhD

administrators who continue to run the DO-PhD program and who are supportive of my journey. I would also like to thank the BMS program, especially Becky and Jasmine, who have been extremely patient and helpful as I navigate the intricacies of graduate school.

It is true that time flies when you're having fun, and I would like to thank the friends that I have made along the way here in Michigan that have made it feel like I moved to Michigan only a few short weeks ago. I would like to especially thank Briana, Laura, Sean, Scott, and Yajing – the times that we have shared together and friendship that we have makes this long journey so much fun.

I would like to thank my friends and family back in Canada as well, who have been ever supportive of my studies and studies here in the United States, no matter the physical distance that separate us, or the sheer number of years that I have spent as a student. They have never ceased supporting my endeavors here and have been my biggest supporters, reminding me during stressful times that it will all be worth it in the end.

I would also like to thank my committee members Dr. Julia Busik, Dr. James Galligan, Dr. Linda Mansfield, Dr. Adam Moeser, and Dr. Narayanan Parameswaran who have helped me in refining my project, giving me valuable feedback, and your attention during the countless hours of committee meetings. Your support has helped guide my development as a scientist.

I could not have been able to accomplish this journey without the help and support of family, friends, staff, faculty, collaborators, and countless others that are not mentioned here. Thank you from the bottom of my heart.

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KEY TO ABBREVIATIONS

3-MA – 3-methyladenine

6-OHDA – 6-hydroxydopamine

CD – Cluster of differentiation

CNS – Central nervous system

DMEM – Dulbecco's Modified Eagle Medium

EDNRB – Endothelin B receptor

ENS – Enteric nervous system

FACS – Fluorescence-activated cell sorting

GDNF – Glial derived neurotrophic factor

GFAP – Glial fibrillary acidic protein

GI – Gastrointestinal

GSNO – Glial derived S-nitrosoglutatione

IBD – Inflammatory bowel disease

IBS – Irritable bowel syndrome

IFN-γ – Interferon gamma

IL – Interleukin

IP – Intraperitoneal

KO – Knockout

LMMP – Longitudinal muscle-myenteric plexus

LPS – Lipopolysaccharide

MAPK – Mitogen-activated protein kinase

MHC – Major histocompatibility complex

NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells

PBS – Phosphate buffered saline

TGF-β – Transforming growth factor beta

TLR – Toll-like receptor

TNBS – Trinitrobenzene sulfonic acid

TNF – Tumor necrosis factor

VIP – Vasoactive intestinal peptide

WT – Wildtype

CHAPTER 1:

Introduction

Overview

The gastrointestinal (GI) tract is an important organ system responsible for the movement, digestion, absorption, excretion, and barrier defense against ingested particles (60, 128). The proper functioning of the GI tract is coordinated primarily by the enteric nervous system (ENS) (60), with modulating inputs from the central nervous system (CNS) (60). The ENS is a part of the autonomic nervous system and is embedded within the walls of the GI tract, and dysfunction in the ENS is a large component to the development of varying GI disorders (57). The loss of neuronal inputs into the GI tract leads to uncoordinated GI function, dysmotility, improper GI secretion, absorption, and improper regulation in GI blood flow (56). ENS neuropathies can be due to congenital causes, effects from the surrounding environment or disease, or a combination of factors (56, 57), leading pathological GI findings in diseases such as in Hirschsprung's disease, Parkinson's disease, Inflammatory bowel disease (IBD), Diabetes mellitus, Chronic intestinal pseudo-obstruction, Chagas disease, among others (56, 57, 148, 202).

Inflammation plays a major role in neuronal damage and death in the ENS, and is the hallmark finding in IBD. Given that the lumen of the GI tract is continuous with the outside environment, the constant exposure to bacteria, viruses, parasites, and other foreign particles are major sources for potential inflammatory stimuli (60, 192). Therefore, the GI tract plays a critical role in pathogen defense and is a major site for immune regulation (60, 143, 149). Although components such as the microbiome has been the focus of many studies examining GI immune regulation, there is growing support that the ENS has a major role in the modulating immune activity (114, 219).

Increasing evidence show that both enteric neurons and enteric glia, which are non-myelinating peripheral glia in the ENS, play substantial roles in immune regulation as well (22, 114). Specifically, enteric glia have the prerequisite cellular machinery to directly interact with the immune system as an antigen presenting cell and modulate its activity (4, 53, 90, 201). However, many of the studies have been performed *in vitro*, or have not shown functional effects on the immune system. Therefore, the specific roles that enteric glia play in immune regulation is not well understood. Here, this dissertation examines enteric glial function and responses in inflammation and its role in immune modulation in the ENS.

The Enteric Nervous System

Intrinsic ENS innervation

The ENS, also referred to the “brain of the gut”, is part of the autonomic nervous system (48) and is embedded along the entire length of the GI tract. The ENS receives inputs from the CNS but is functionally independent (48, 97). In fact, the GI tract maintains coordinated activity and does not cease to function when completely separated from the CNS (6, 7). The ENS is composed primarily of two ganglionated plexuses, termed the submucosal, or Meissner’s plexus, and the myenteric, or Auerbach’s plexus (82, 185, 194). These two plexuses regulate major functions in the GI tract. Anatomically, the submucosal plexus is located between the muscularis mucosae and circular muscle layers in the GI tract (Fig.1.1). The submucosal plexus is the smaller of the two plexuses and consists of fewer neurons (82). It is primarily involved in controlling absorption, secretion, and local blow flood to the gut (174, 198,

206). In contrast, the myenteric plexus, the larger of the two plexuses, is located between the circular and longitudinal muscle layers of the GI tract (185)(Fig.1.1). The role of the myenteric plexus is primarily motility focused, as it is responsible for the coordinated contraction and relaxation within GI tract (89, 97, 182). This is an important process, as it allows for the proper transit of ingested food particles through the GI tract, a process known as peristalsis.

The ENS develops from neural crest progenitors expressing gene markers Sox10, FoxD3, p75 neurotrophin receptor, Phox2b, RET, and endothelin B receptor (EDNRB) (32). As these progenitors differentiate into enteric neurons that populate the GI tract, they retain expression of Phox2b and RET, but lose expression of other progenitor cell markers. Enteric neurons also gain the expression of PGP9.5, β3-tubulin, and HuC-D (32). As they mature, enteric neurons further differentiate into different types of neurons and can be further classified by immunohistochemical labeling of neuronal markers and neurotransmitters they express. Classification of these enteric neurons falls into three major types: motor neurons (both excitatory and inhibitory), interneurons, and sensory neurons (49). Excitatory motor neurons elicit their excitatory effects primarily via release of acetylcholine, and to a lesser degree, takykinin proteins such as substance P (49, 61). These neurons can also be identified by their expression of calretinin and γ-aminobutyric acid. In contrast, inhibitory motor neurons primarily utilize nitric oxide as a neurotransmitter, along with vasoactive intestinal peptide (VIP), ATP, and carbon monoxide (48, 214). Interneurons (ascending and descending) present along the length of the GI tract responsible for reflex pathways utilize acetylcholine as a neurotransmitter, along with a combination of other markers such as serotonin,

somatostatin, nitric oxide, VIP, and calretinin (48). Finally, intrinsic sensory neurons utilize acetylcholine, calcitonin gene-related peptide, and tachykinins as neurotransmitters, and can be further identified via labeling for calbindin, calretinin, and isolectin B4 (48).

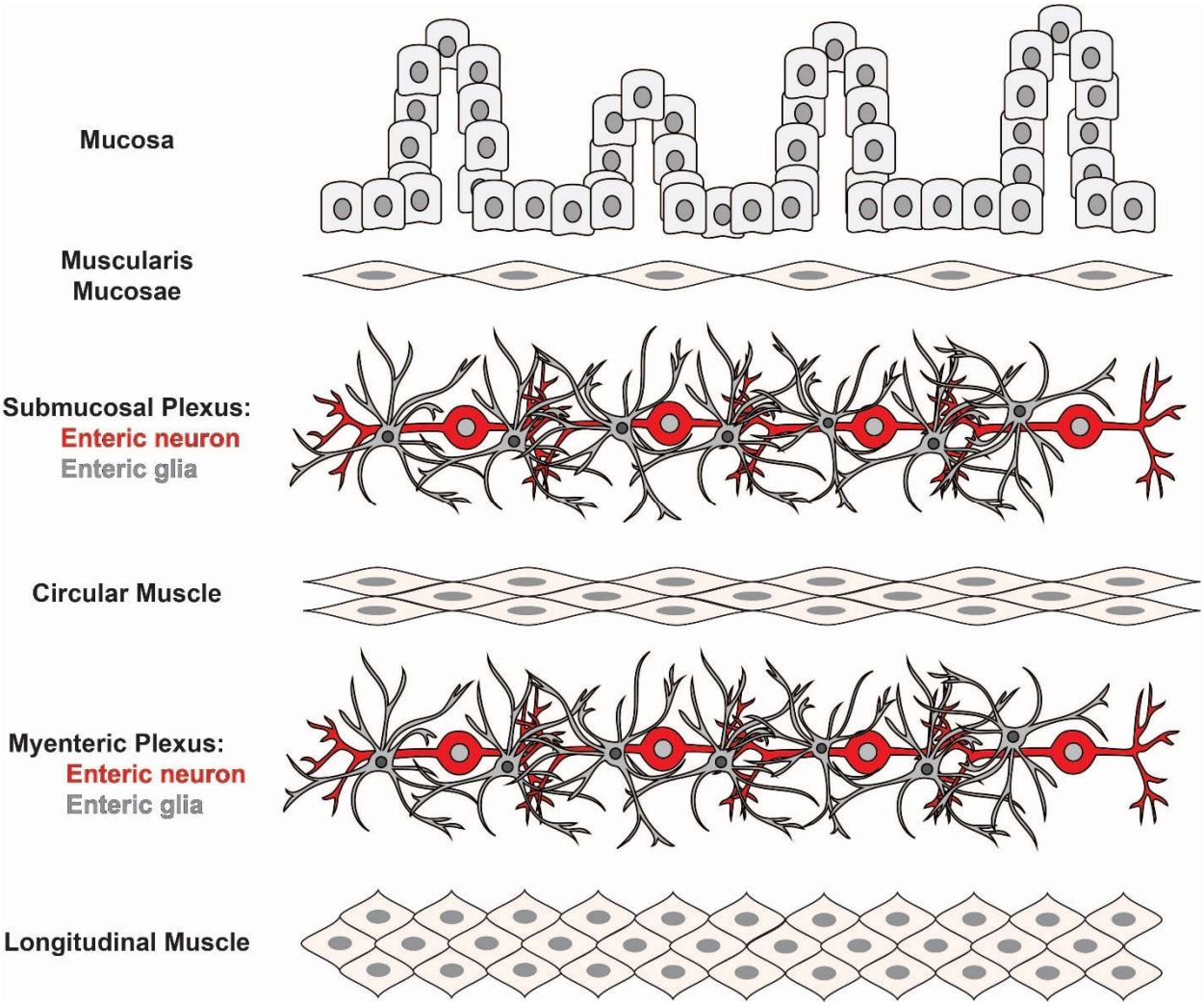

Figure 1.1: Arrangement of the ENS within the GI tract. The ENS is distributed within the walls of the GI tract in primarily two plexuses, the myenteric plexus and submucosal plexus. The submucosal plexus is located between the muscularis mucosae and circular muscle, while the myenteric plexus is located between the circular and longitudinal muscle layers.

Extrinsic inputs into the ENS

Despite extensive intrinsic neuronal inputs into the ENS to control GI function, the ENS also receives extrinsic inputs from the CNS to modulate GI activity (128, 150). CNS inputs into the ENS consist of sympathetic, parasympathetic, and sensory innervation arranged in vagal, thoracolumbar, and craniosacral pathways (60). The sympathetic and parasympathetic inputs to the ENS can modulate GI function by stimulating GI motility and digestion with parasympathetic stimuli, and inhibiting GI motility and digestion with the sympathetic pathway (73, 128, 150). GI motility is modulated by parasympathetic efferent motoneurons that innervate the GI tract and are derived primarily from the dorsal motor nucleus of the vagal nerve for the upper regions of the GI tract, and preganglionic fibers from lumbosacral regions of the spinal cord in the distal regions of the colon (19). These preganglionic efferent motoneurons release acetylcholine to bind nicotinic receptors and activate interstitial cells of Cajal and neurons in the myenteric plexus to elicit muscle contraction in the GI tract (19, 168). However, vagal activation of non-adrenergic, non-cholinergic postganglionic pathways also allows for release of nitric oxide and vasoactive intestinal polypeptide in order for the relaxation of intestinal smooth muscle (19). In contrast, sympathetic modulation of GI activity originate from thoracolumbar regions of the spinal cord to several sympathetic ganglia in order to supply sympathetic innervation to the GI tract. Postganglionic fibers from the celiac ganglion supply sympathetic innervation to the proximal GI tract such as the stomach, while neurons from the superior mesenteric ganglion and inferior mesenteric ganglion provide sympathetic innervation to the small and large intestines, respectively (19). Unlike parasympathetic nerves, the preganglionic

sympathetic neurons primarily utilize norepinephrine as a neurotransmitter (19). Sympathetic modulation of intrinsic ENS neurons contributes to decreased motility in the GI tract, and increased tone in intestinal sphincters (106).

In addition to modulating GI motility, sympathetic and parasympathetic nerves are distributed to lymphoid organs and immune tissues in the GI tract and have immunomodulatory effects on the GI system (24, 118, 186). Broadly speaking, parasympathetic activation is associated with anti-inflammatory effects in the gut, while sympathetic activation can lead to either pro- or anti- inflammatory effects, depending on levels of released neurotransmitters (16, 119, 186). Sympathetic signaling through α-adrenergic receptors can induce release of TNF-α from macrophages, promote T_h2 T-lymphocyte activation, and increase T_h17 T-lymphocyte activation through increased expression of IL-23 by antigen presenting cells (3, 91). In contrast, cholinergic signaling from parasympathetic pathways can act directly onto enteric neurons that interact with nearby macrophages. Release of acetylcholine by these enteric neurons can bind α-7-nicotinic acetylcholine receptors present on macrophages to inhibit the release of pro-inflammatory cytokine TNF-α (16). Interestingly, vagal efferent nerves can also interact with splenic sympathetic nerves within the celiac ganglion to elicit anti-inflammatory effects via the splenic nerve. Activation here leads to acetylcholine release in T-lymphocytes and thereby bind α-7-nicotinic acetylcholine receptors on splenic macrophages to inhibit release of TNF-α (16).

In addition to parasympathetic and sympathetic nerves, even sensory neurons have immunomodulatory properties and can inhibit immune activation in neutrophils, monocytes, and macrophages (28). Therefore, the contributions from the CNS in

immune modulation play important roles in homeostasis and can contribute to intestinal inflammation, ENS damage, and the pathogenesis of IBD.

ENS damage and disease

Damage to the ENS can arise from a multitude of sources and can lead to neuronal death. Since the ENS regulates GI secretomotor function, injury to the ENS can manifest as symptoms such as constipation, diarrhea, nutrient malabsorption, or abdominal pain (60, 108). ENS dysfunction can be due to malformations congenitally, direct modifications to neuronal structure and function, inflammatory effects, exposure to chemicals, changes in microbiota, or as a part of normal aging (57, 71, 120, 166).

In congenital ENS malformation, dysfunctions in the ENS arises due to inappropriate development of enteric neurons. For example, Hirschsprung's disease is due to the improper migration of neural crest cells during gestation and leads to aganglionsis in the distal portions of the GI tract, primarily affecting the colon (191). Genetic abnormalities in multiple genes such as RET, endothelin receptor type B, and Sox10 contribute to the pathogenesis of Hirschsprung's disease (17, 21, 148). Without the development of a proper ENS in sections of the gut, aganglionic regions lack enteric neurons and enteric glia. These regions do not have proper motility functions and typically cannot relax, leading to fecal stasis, bacterial overgrowth in the GI tract, and potentially lead to sepsis (120, 195).

Diseases causing neuropathies, such as diabetes, can lead to ENS damage by modifying neurons and neuronal function. In diabetes, elevated glucose levels in the body leads to the covalent linkage of glucose molecules, termed glycation, of neurons.

This occurs through a process known as non-enzymatic glycation, which can hinder neuronal functions and trigger nerve degradation (93, 202). Furthermore, diabetes may also activate other pathways such as the polyol, protein kinase C, mitogen-activated protein kinase (MAPK), TNF-α, NF-κB, cyclooxygenase, interleukin (IL), Wnt, and autophagy pathways, among others, to elicit neuronal damage and pathogenesis (38). This can lead to a host of GI symptoms (42) and can contribute to motility disorders and gastroparesis seen in diabetes patients (38, 202). Enteric glial cells within the ENS can act to limit neuronal degradation (109), but diabetic changes in enteric glial cells, such as changes in glial morphology and number, can limit their ability to protect enteric neurons and further contribute to diabetes-associated ENS dysfunction (144, 153).

Inflammation in the GI tract is a very common cause of enteric neuropathies, but the pathogenesis varies significantly between diseases. For example, inflammatory damage to the ENS can be caused by infection, such as in Chagas disease caused by *Trypanasoma cruzi* infection. The inflammatory response stimulated by this infection can lead to megacolon or megaesophagus due to destruction of ENS segments (81, 180). In IBD, pathogenesis is thought to be due to the inappropriate immune activation against the microbiota in genetically susceptible patients (29, 179, 210, 222). This can lead to inflammation in the GI tract, a hallmark of IBD. Inflammatory stress on the ENS is distributed differently in the two forms of IBD: Crohn's disease and ulcerative colitis. While tissue inflammation is limited to the superficial layers of the colon in ulcerative colitis (225), Crohn's disease can elicit inflammation anywhere along the length of the GI tract. In addition, inflammation in Crohn's disease can affect the entire thickness of the GI tract and not only the superficial layers as in ulcerative colitis (225). Inflammatory

damage in the GI tract can lead to the reorganization, damage, and death of both enteric neurons and enteric glial cells, thereby contributing to a loss of GI secretomotor functions and symptoms seen in IBD patients.

Inflammatory stress causes neuronal death through the activation of caspase-dependent apoptotic pathways in enteric neurons via activation of purinergic receptors and pannexin channels (63, 129). Neuronal death due to inflammation does not target specific subpopulations (129), but instead leads to a generalized decreased neuronal density in the ENS. However, inflammatory stress can also contribute to changes in neuronal behavior, such as the development of hyperactivity in intrinsic primary afferent neurons during, and after inflammation, thereby contributing to greater afterhyperpolarizing potential (104, 105, 129). The change in neuronal activity in these intrinsic primary afferent neurons suggest alterations in normal intestinal sensation and changes in motor reflexes that rely on these afferent nerves. In addition to alterations in neuron activity, IBD elicit changes in neurotransmitter expression in enteric neurons. For example, IBD patients have an increased proportion of neurons expressing substance P (134), neuropeptide Y (10), and changes in number of neurons expressing vasoactive intestinal polypeptide (10, 173).

In addition to inflammatory stress, ENS exposure to certain chemicals in the body can lead to detrimental effects. For example, chemotherapy treatment has many effects on the GI tract, but chemotherapy-induced neuronal death in the ENS can lead to serious dysmotility disorders (2, 43, 121, 208). Furthermore, chemical-induced ENS damage and alterations in neuronal and glial function can persist for extended periods of time, such as in Gulf War veterans exposed to pyridostigmine bromide decades ago

(70, 71). In this way, chemical damage to the ENS can have lasting effects even after offending chemicals are removed.

The interplay between the ENS and the microbiota in the GI tract also has significant implications on GI disease and secretomotor function. Many groups have shown interactions between the microbiota, the ENS, and the CNS (35, 171, 204). Changes in the microbiota composition has profound effects on GI function, and GI diseases such as IBD have been linked with intestinal dysbiosis and alterations in microbiota (1, 139). Due to the complex nature of microbiota interactions, the specific effects due to changes in microbe distribution are not completely known and is a topic of study.

Finally, the process of normal aging causes increased neurodegeneration overall, but neurons in the ENS are especially susceptible (166). Despite the plasticity of the ENS and its capability of neuronal outgrowth, aging leads to the reduction in the number of neurons and glia in the ENS (166). Furthermore, aging causes alterations in neuronal morphology and arrangement, suggesting alterations in neuronal behavior and function, and may contribute to GI motility disorders seen in old age, such as fecal incontinence and constipation (26, 52). Taken together, ENS neuropathy can be due to numerous reasons ranging from congenital malformations, direct neuronal modifications, inflammation, the exposure to chemicals, changes in microbiota, just from normal aging, or a combination of any of these factors. ENS neuropathy, irrelevant of cause, leads to GI secretomotor dysfunction by causing damage to both enteric neurons and enteric glia, thereby disrupting the ENS network.

Enteric Glia

Enteric glia, together with enteric neurons, form the ENS. They are a type of non-myelinating peripheral glia that share similar characteristics as astrocytes of the CNS (50, 55, 84). They are in extremely close contact with each other (15) (Fig.1.2), and were historically thought of being merely a 'support' cell to enteric neurons by providing nutritional and structural support to the ENS. However, enteric glia have since been shown to have an important impact on enteric neurogenesis, activity, and survival (32, 65, 135).

Enteric glia are derived from neural crest cells and begin development and colonization of the intestinal tract within the first two weeks in utero (55, 88, 163). During development, enteric glia express markers such as Sox10, FoxD3, B-FABP, glial fibrillary acidic protein (GFAP), and S100β, making them a distinct population compared to developing enteric neurons or neural crest progenitor cells (32). Therefore, current identification of enteric glia within the ENS is achieved by the labeling of glial-specific markers S100β and GFAP (32, 135, 164), and by morphology, as they have distinct astrocyte-like star shaped morphology with long and highly branched processes (50, 164, 165) (Fig.1.2). However, despite their similarities with glial populations in the CNS, enteric glia are a distinct population. This is evidenced by their embryonic origins from the neural crest, in opposed to astrocytes being derived from the neural tube (8, 55). Due to the differences in embryologic origin, enteric glia also express the Sox10 transcription factor, unlike astrocytes (127).

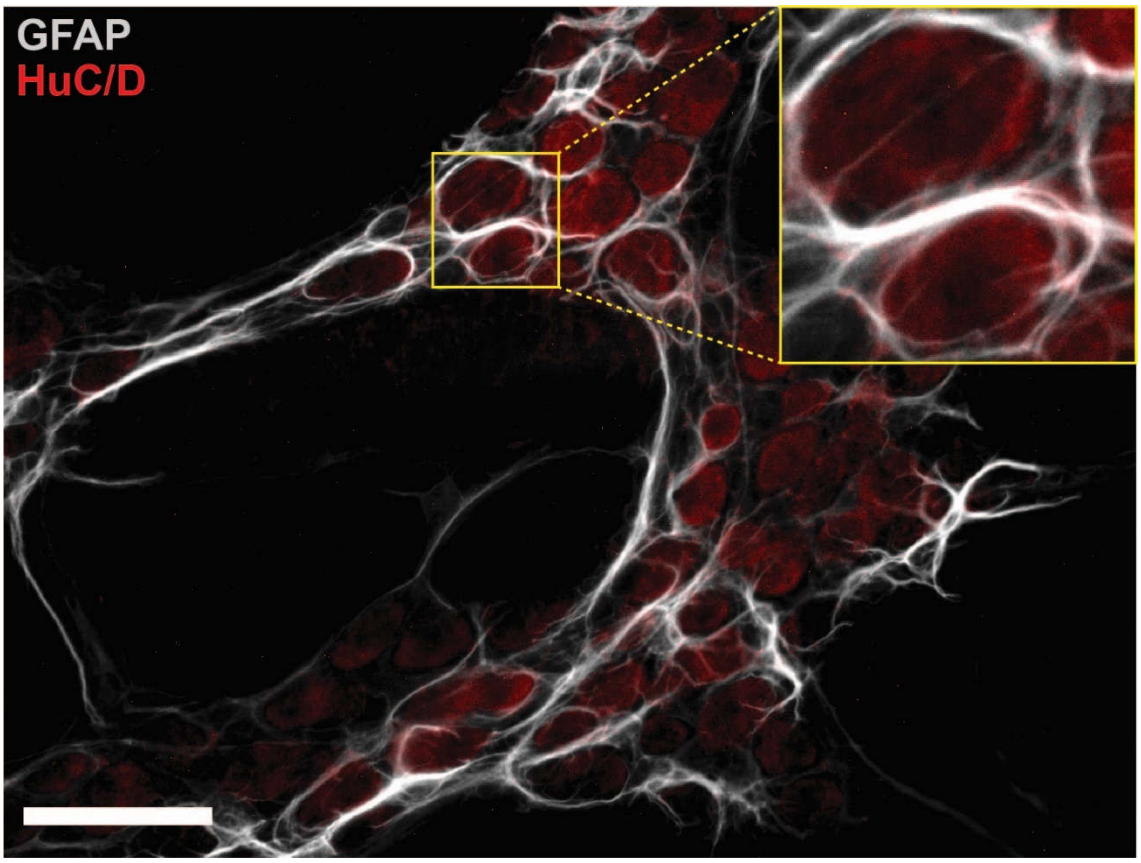

Figure 1.2: Enteric glial proximity to enteric neurons. Immunofluorescent labeling of enteric neurons with HuC/D (red) and enteric glia with glial-specific marker GFAP (grey). Enlarged area further demonstrates the proximity of glial processes to enteric neurons. Scale bar represents 50μm.

In the established ENS, enteric glia communicate with enteric neurons and other cell types such as intestinal epithelium to play an active role in GI secretomotor function. To interact with neuronal signaling, enteric glia express receptors for numerous neurotransmitters such as cholinergic receptors (27, 36), adrenergic receptors (62, 132), purinergic receptors (58, 64, 92), serotonin receptors (14), among others (130, 175, 224). This allows for enteric neurons to respond to nearby neuronal activity and be targets of innervation. In addition, enteric glia can also modulate neuronal signals in the ENS, thereby contributing to the control of GI motility (32, 65, 135) (Fig.1.3). In addition to interactions with enteric neurons, enteric glia can interact with non-neuronal cell types as well. Their interactions with intestinal epithelial cells play a role in the control of intestinal secretion and barrier function (110, 111, 170), and their ability to secrete and respond to cytokines can affect immune activity (90, 162) (Fig.1.3). A more in-depth examination of enteric glial cell characteristics that support their role in immune modulation is presented in chapter 2 of this dissertation. Taken together, enteric glia play an active role in normal ENS signaling as well as secretomotor and immune modulation activities in the GI tract.

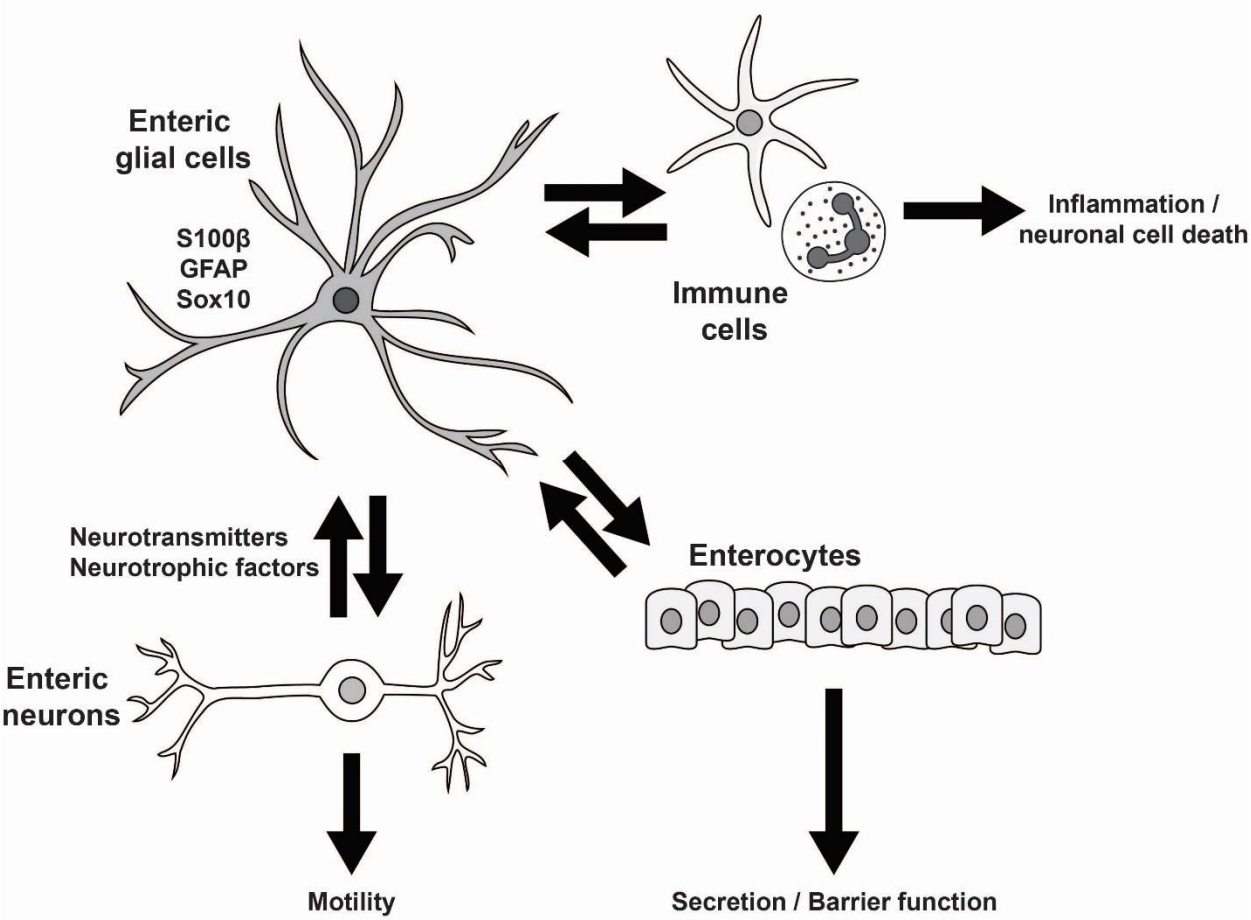

Figure 1.3: Overview of enteric glial functions. Enteric glia play an active role in the communication and interaction between enteric neurons, enterocytes, and immune cells in the intestinal tract. These interactions are through neurotransmitters and soluble molecules such as nitric oxide or secreted cytokines in order to affect GI secretomotor function, barrier function, and immune activity.

In addition to their role in ENS signaling, enteric glia also play a role in neurogenesis and neuronal maintenance in the ENS. Studies demonstrate that enteric glia help maintain the health of the ENS and recover from injury via the expression of neurotrophic factors that can stimulate neuron outgrowth and regeneration (11, 66, 181). However, during oxidative stress and neuronal damage, enteric glia can also play the role of executioner and contribute to neuronal death (18). In mice, there are roughly equal numbers of enteric glia and enteric neurons in the GI tract, but the ratio between glia and neurons increases in higher order animals, reaching roughly seven-to-one enteric glia-to-neuron ratios in humans (51, 74). This suggests that enteric glia can effectively monitor neuronal health and function, and that increasing numbers of enteric glia are necessary for the increasing complexity of GI functions in higher organisms.

The Immune System

The ENS in the walls of the GI tract does not exist in isolation. In fact, many immune cells are in the surrounding microenvironment of both the submucosal and myenteric plexus. Dendritic cells, muscularis macrophages and memory T-lymphocytes are all normally distributed within the mucosa, submucosa, and muscular layers of the GI tract (5, 33) to monitor the local microenvironment for any potential pathogens (Fig.1.4). The proximity of immune cells to the ENS lends to interactions between cells of the ENS and the immune system (138, 209). While having immune cells in close proximity to the ENS is beneficial in eliminating nearby pathogens, immune activation or dysregulation can theoretically have immediate effects on the ENS.

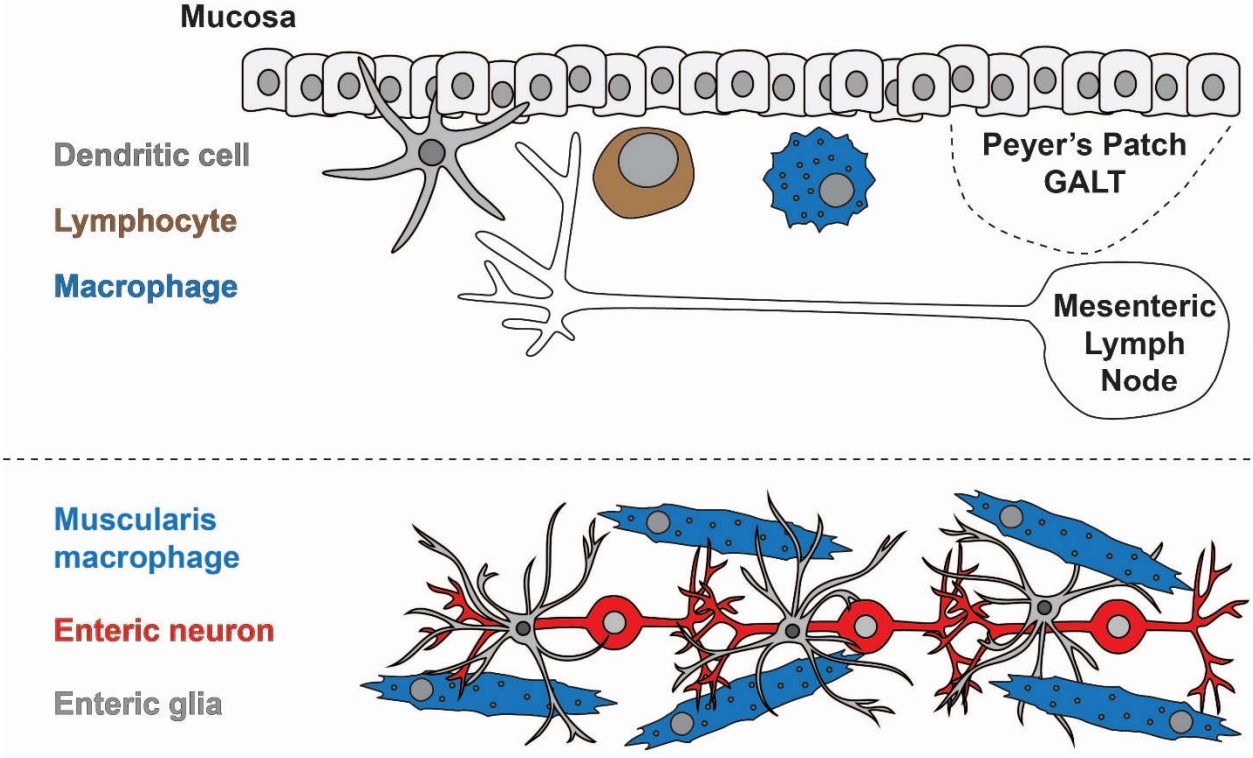

Figure 1.4: Overview of typical GI immunity. Spread within the mucosa, submucosa, and muscular layers of the GI tract are dendritic cells, lymphocytes, and macrophages. Nearby GI-associated lymphoid tissues (GALT) and regional mesenteric lymph nodes contain additional immune cells to respond to pathogens. In the submucosal and myenteric plexuses, muscularis macrophages are present in close proximity to the ganglia.

Broadly, the immune system is responsible for host defense and is involved in the recognition and removal of pathogens, foreign molecules, and damaged cells and tissues. It is divided into two main components, the innate and adaptive immune systems. The innate immune system is responsible for a rapid response to an invading pathogen by limiting its spread, phagocytosing and destroying pathogens, and communicate with the adaptive immune system to mount an additional response to a given pathogen (169). Innate immunity relies on molecular patterns common on many pathogens to generate a generic immune response (83, 145).

In contrast, the adaptive immune system does not respond with the same speed as the innate immune system and relies on activity in the innate immune system to convey antigens to direct it to the proper target. In addition, the adaptive immune system has the ability to generate a 'memory' of a previous immunologic insult such that subsequent exposure to the same insult elicits a stronger and more rapid adaptive immune response (145, 169). Activation in either the innate and adaptive immune systems can lead to inflammation within the ENS, contributing to the pathogenesis of ENS disorders and IBD. Currently, numerous therapeutics have been developed to target specific steps in each arms of the immune system (75), and there continues to be rigorous study into immune regulation as a means to limit inflammatory damage to the ENS.

Inflammation

Inflammation has been recognized since ancient Roman times. The cardinal signs of inflammation were described by Aulus Cornelius Celsus as being *rubor* (redness), *tumor* (swelling), *calor* (heat), and *dolor* (pain) (193). The process of

inflammation represents a broad response to limit the spread of pathogens and deliver immune cells, among other products, to help eliminate threats. However, inflammation is also a major driver for neuroplastic changes in the ENS (207). Changes to neuron survival and the overall ENS network leads to functional alterations in the ENS and contributes to secretomotor symptoms in diseases such as IBD.

The overall inflammatory process is accomplished by first increasing blood flow and vascular permeability to the site of infection or injury. During infection, the increase in fluid to the area helps to isolate a pathogen to prevent spread (122). Furthermore, the increase in blood and lymph flow to the area helps bring leukocytes to the site of injury or infection to help progress the immune reaction (122). Initial immune cells recruited to the inflamed area primarily consists of neutrophils and monocytes, but progressively expands to include macrophages and T-lymphocytes over time (122, 152). If the recruited immune cells are incapable of clearing the pathogen, granulomas may form to sequester and isolate the offending agent from spreading to other regions of the tissue or body (122). The inflammatory pathway resolves once the pathogen or inflammatory agent is cleared from the body, and tissue repair and regeneration is activated to reverse damage incurred by the inflammatory process.

One of the ways immune cells carry about effector functions is through the expression of cytokine molecules. Cytokines, which are soluble chemicals in the bloodstream used for cell signaling, and chemokines, which are chemotactic cytokines, contribute either to the development or resolution of inflammation, depending on the molecule (41, 78). They are expressed by both the innate and adaptive arms of the immune system, and the expression and levels of different cytokines are relevant in the

pathogenesis of inflammatory diseases such as IBD. They cytokine landscape in IBD is very diverse and includes a multitude of pro-inflammatory and anti-inflammatory cytokines and chemokines in its pathogenesis (47, 136, 199). Among them, therapeutics have been developed and are being tested to neutralize a portion of those cytokines and chemokines, such as Fontolizumab targeting IFNγ, Infliximab (tumor necrosis factor), Tocilizumab (IL-6), Ustekinumab and briakinumab (IL-12 and IL-23), Anrukinzumab (IL-13), Secukinumab (IL-17), Vercirnon (chemokine receptor CCR9), Eldelumab (CXCL10), and Smad7 anti-sense oligonucleotides (CCL20) (47, 136, 199). Furthermore, the therapeutic administration of recombinant IFNβ, recombinant IL-10, or recombinant IL-11 is also being studied as a means to limit inflammatory burden in IBD (136). Therefore, the regulation of cytokine and chemokine levels is an important area to regulate inflammation and is the current strategy to limit inflammatory damage to the ENS and surrounding tissue in IBD.

The inflammatory pathway represents a destructive process as recruited neutrophils and monocytes phagocytose and destroy and clear foreign pathogens and cellular debris. However, their destructive nature and release of reactive oxygen species can be non-discriminant and cause collateral damage to the surrounding tissue (124, 142, 152). The prolonged activation of inflammation in a tissue can lead to fibrosis and scar formation, leading to the replacement of damaged tissue with non-functional scar tissue. In the context of GI inflammation, intestinal fibrosis presents a very serious complication in IBD patients. The combination of inflammation-induced neuron loss in the ENS and increased intestinal fibrosis leads to GI secretomotor dysfunction in IBD patients and leads to an increased morbidity and mortality (76, 99).

Despite its destructive properties, the process of inflammation can be beneficial to tissue repair and regeneration. Inflammatory recruitment of immune cells, especially macrophages, play a major role in wound healing and tissue regeneration. Specifically, M2-subtype macrophages function to remove neutrophils and apoptotic cellular debris from inflammatory regions, to promote angiogenesis, extracellular matrix synthesis, stimulate progenitor cell activation, and fibroblast proliferation (80, 94, 142). Furthermore, at the resolution of inflammatory processes, the release of mediators such as resolvins by endothelial cells inhibits neutrophil recruitment and extravasation to damaged tissue, and stimulates macrophages to clear apoptotic neutrophils and other cell debris from the inflammatory site (46, 176). Many of these inflammatory functions, especially during the early stages of inflammation, are carried out by the innate immune system, and this arm of the immune system represents a major player in the GI inflammation.

The Innate Immune System

The innate immune system represents one of the first lines of defense against infectious agents and pathogens, the first line of defense being epithelial barriers that separate our bodies from the external environment and potential pathogens. The innate immune system consists of fast responding immune cells such as dendritic cells, macrophages, polymorphonuclear cells (including neutrophils, basophils, and eosinophils), and mast cells (83). These cells are positioned in areas of close proximity with potential pathogens, such as the mucosal layer of the intestinal tract. Innate immune cells recognize pathogens by common molecular patterns expressed on their cell surface, termed pathogen-associated molecular patterns (67, 83). Binding of these

pathogen-associated molecular patterns onto ‘pattern recognition receptors’ expressed on immune cells activates the immune cell, leading to phagocytosis and clearing of the pathogen (67, 83). Activation of innate immune cells such as dendritic cells can also lead to the migration of the dendritic cell to nearby lymph nodes to stimulate and activate the adaptive immune system. Depending on the severity of pathogen invasion, tissue inflammation may also ensue.

Pattern recognition receptors are divided into 3 major types: Toll-like receptors (TLR), NOD-like receptors, and RIG-I-like receptors (96). These pattern recognition receptors bind pathogen-associated molecular patterns typically consisting of bacterial, viral, or fungal components. However, pattern recognition receptors can also bind molecules that represent cellular damage, termed damage-associated molecular patterns (59). For example, TLR1&2 recognize bacterial cell wall components like peptidoglycan, and triacyl lipopeptides, TLR3 recognize dsRNA molecules, TLR4 recognize lipopolysaccharide (LPS), TLR5 recognize flagellin molecules, TLR7&8 recognize ssRNA molecules, and TLR9 recognize dsDNA (83, 96, 226). RIG-I-like receptors recognize different viral pathogens, while NOD-like receptors a variety of bacterial, viral, and fungal pathogen-associated molecular patterns, along with non-microbial damage-associated molecular patterns (96, 226). However, binding onto pattern recognition receptors does not always elicit the destruction of the microorganism. Pattern recognition receptors have also been shown as a means to monitor and recognize commensal microorganisms in the microbiota (23, 95). Binding of TLR molecules by commensal organisms is necessary for intestinal homeostasis, is a way to modulate microbiota populations, can induce anti-inflammatory responses in the

intestines, and modulates intestinal epithelial metabolism (23, 30, 95, 154, 218). In the context of the ENS, enteric glial cells can express pattern recognition receptors such as TLR4 to detect LPS molecules, and can release nitric oxide via TLR4 binding (107, 201).

As described above, the activation of innate immune cells can trigger phagocytosis of potential pathogens. The process of phagocytosis represents an important pathway in innate immune cells to both clear pathogens and to prepare antigens for presentation to the adaptive immune system (67). Once recognized by innate immune cells, restructuring of the cellular cytoskeleton allows for the engulfment and internalization of foreign molecules into phagosomes (44). The maturation of the phagosome into a phagolysosome involves the fusion with lysosomes, the acidification of vesicle contents, and the accumulation of reactive oxygen species and hydrolases including various proteases, lipases, nucleases, glycosidases, and phosphatases to degrade internalized particles (44, 203). In the GI tract, dendritic cells resident macrophages, and recruited neutrophils and monocytes are the primary cell populations carrying out phagocytosis activities.

Antigen Presenting Cells

While phagocytosis in neutrophils is primarily to clear pathogens and not to present antigens to the adaptive immune system, dendritic cells and macrophages play an additional role as professional antigen presenting cells to load phagocytosed particles onto major histocompatibility complex (MHC) II molecules for presentation to the adaptive immune system (145, 183). Antigen presenting cells form an important transition between the innate and adaptive immune system and can direct an immune

response towards specific targets. In addition to their phagocytic ability and constitutive expression of MHC II molecules, professional antigen presenting cells are also capable binding and activating T-lymphocytes (145, 169). This is accomplished by the expression of T-lymphocyte co-stimulatory molecules such as CD40, CD80, and CD86, along with activating cytokines to stimulate T-lymphocyte proliferation and differentiation (169).

Although antigen presentation to T-lymphocytes is primarily carried out by dendritic cells and macrophages as professional antigen presenting cells, growing evidence supports the presence of 'atypical' antigen presenting cells as well (87, 137, 183). These atypical antigen presenting cells are characterized by the ability to express MHC II molecules in an inducible manner, as opposed to constitutive expression of MHC II in professional antigen presenting cells (87, 183). Furthermore, although atypical antigen presenting cells can express co-stimulatory molecules, they are generally not as effective as professional antigen presenting cells at triggering T-lymphocyte activation, differentiation, and proliferation (87, 177). In fact, there are conflicting studies debating whether atypical antigen presenting cells are functional at inducing T-lymphocyte activation (87, 157, 177). Currently, cells such as mast cells, basophils, eosinophils, and type 3 innate lymphoid cells have been classified as atypical antigen presenting cells (20, 40, 45, 69, 87, 112, 141, 151, 220). However, due to conflicting findings on their capabilities to effectively function as an antigen presenting cell, the true role of atypical antigen presenting cells remains to be elucidated.

Within the ENS, enteric glial cells show promise to also act as an atypical antigen presenting cell. Enteric glia have been shown to express MHC II molecules, T-cell co-

stimulatory molecules, and can interact activate T-lymphocytes *in vitro* (4, 53, 201). Moreover, astrocyte cell populations in the CNS that are related to enteric glia show phagocytic capabilities and can uptake (85). A more detailed examination into the characteristics of enteric glial cells that supports their potential role as an immune modulator is presented in chapter 2 of this dissertation.

Type II Major Histocompatibility Complex

As described above, the MHC II molecule is constitutively expressed on professional antigen and functions as a crucial component by which self-derived or phagocytosed antigens are presented on the cell surface to T-lymphocytes (145, 183). The identity of presented peptides activates specific T-lymphocytes, and therefore modulates immune activation. Slight changes in the peptides presented can drastically change the resultant immune reaction, as simple post-translational modification in presented peptides can change a non-existent immune response into a complete autoimmune cascade (13, 72). Inappropriate peptide loading onto MHC II molecules and dysfunctional antigen presentation are contributing factors in the development of multiple diseases, such as IBD, systemic lupus erythematosus, and multiple sclerosis (25, 113, 123, 211, 216). Therefore, the proper loading of peptides onto MHC II molecules is crucial in directing appropriate immune responses. In the mouse (the animal model used in this dissertation), the MHC II molecule is a heterodimer composed of α1, α2, β1 and β2 chains (77, 184, 189) (Fig.1.5a). These are expressed on two functional α loci (*Aa* and *Ea*), and two functional β loci (*Ab* and *Eb*) on chromosome 17 (77, 189) (Fig.1.5b). Gene products from either the *Aa* and *Ab* pair or *Ea* and *Eb* pair combine to form functional MHC II molecules.

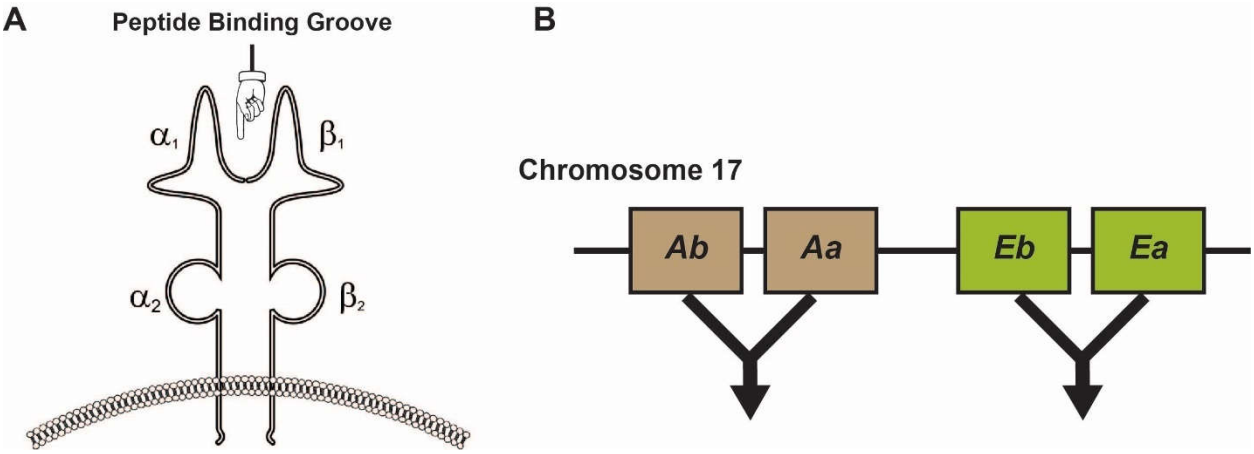

Figure 1.5: Murine MHC II. (A) The MHC II molecule is a heterodimer composed of the α1-α2 peptide dimerized with the β1-β2 peptide, forming a peptide binding groove to present peptide fragments to the adaptive immune system. **(B)** The MHC II molecule is expressed from two functional α loci (Aa and Ea) and two functional β loci (Ab and Eb). Gene products from either Aa and Ab or Ea and Eb combine to form the MHC II molecule.

Newly synthesized MHC II molecules from the endoplasmic reticulum are attached with CD74, also known as the invariant chain (158, 190). The attachment of the invariant chain serves two major purposes: 1) to occupy the peptide binding groove on MHC II molecules and prevent inappropriate peptide binding, and 2) to guide newly synthesized MHC II to low-pH endosome compartments (158, 190). As MHC II molecules mature, the invariant chain is degraded until only a small peptide, the class II-associated invariant-chain peptide (CLIP), remains bound to the MHC II peptide binding groove. It is not until the fusion with phagolysosomes that CLIP molecules are displaced from the peptide binding groove via the activity of the H2-M enzyme (158), and MHC II molecules are free to load antigens (Fig.1.6). Loaded MHC II molecules are transported to the cell surface where it can then, in conjunction with other co-stimulatory molecules, bind and activate T-lymphocytes. Although peptide loading onto MHC II molecules are generally derived from extracellular antigens that have been phagocytosed and processed, atypical loading of MHC II molecules can also occur through the loading of intracellular peptides via the autophagy pathway (37, 187). Despite loading intracellular antigens onto MHC II molecules, autophagy-induced peptide loading onto MHC II can not only avoid the development of auto-immunity but can also elicit an anti-inflammatory effect by limiting T-lymphocyte activation (212).

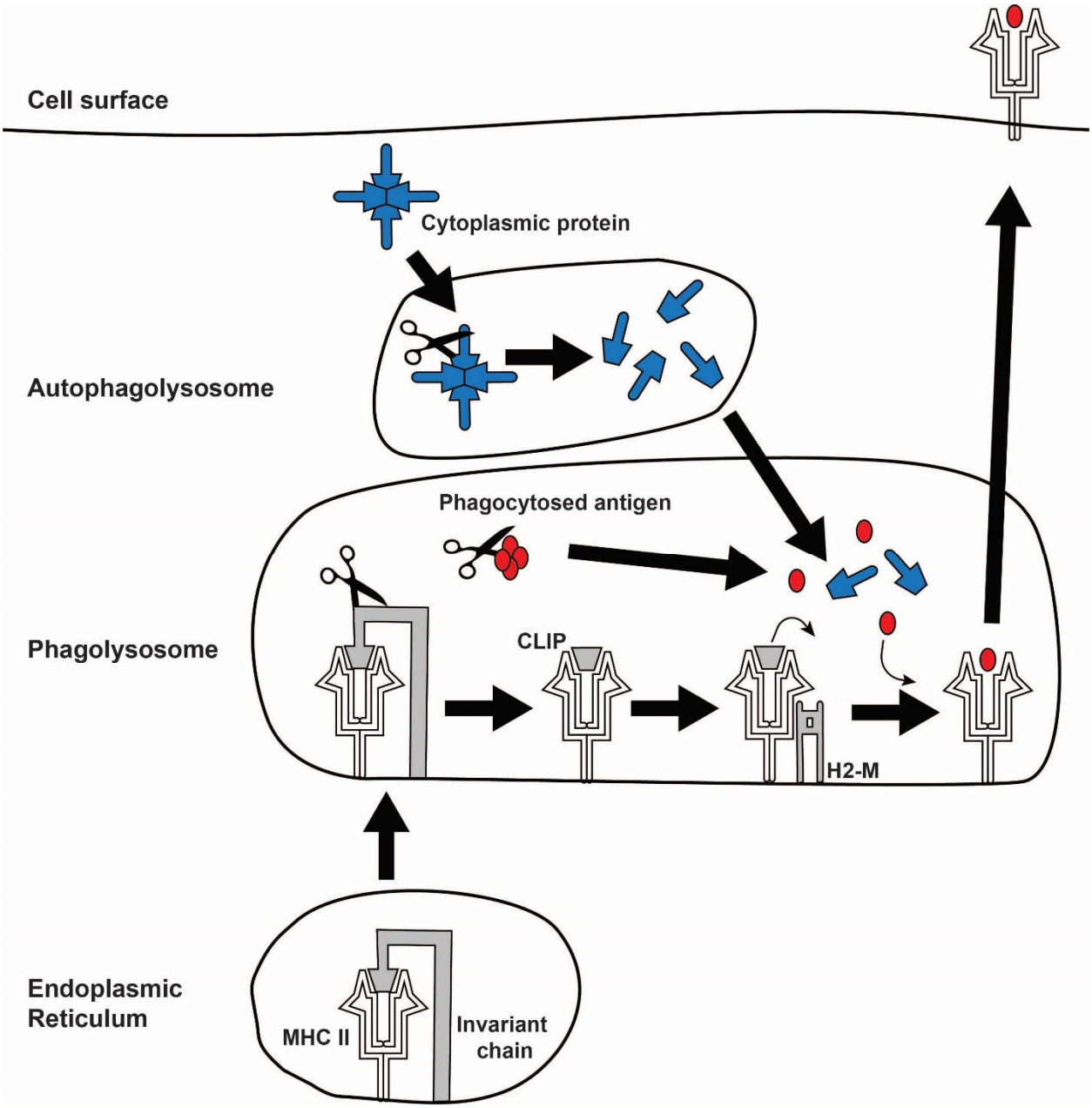

Figure 1.6: Maturation of MHC II molecules. Newly synthesized MHC II molecules are bound with the invariant chain peptide and transported into phagolysosomes, where proteolytic cleavage processes the bound invariant chain into the CLIP peptide while maintaining MHC II binding. The association of MHC II with the H2-M molecule (the murine equivalent to the human DM molecule) allows for the release of the CLIP

Figure 1.6 (cont'd):

peptide from the MHC II peptide binding groove in order to load endocytosed peptides for presentation on the cell surface. Processing of cytoplasmic proteins in autophagolysosomes can also merge into this pathway and load cytoplasmic peptides onto MHC II molecules for presentation.

The autophagy pathway is important during development, but it also represents a method for cells to degrade and recycle cellular components during times of stress or starvation (102, 146). The autophagy pathway also plays a role in pathogen defense by playing a role in the degradation and presentation of intracellular bacterial and viral pathogens (103, 131). Furthermore, in a normal resting state, basal activation of autophagy and loading of autophagy-derived intracellular peptides contributes to immune tolerance (156). In the context of GI homeostasis, autophagic pathways are important in the maintenance of proper microbiota composition (200, 217), as autophagy dysfunction is associated with intestinal dysbiosis (98).

The regulation of the autophagy pathways is an important method of maintaining homeostasis, and defective autophagy pathways have far reaching effects in the body. For example, dysfunction in the autophagy pathway is associated with genetic instability and tumorigenesis (101, 117), neurodegenerative diseases such as Huntington's disease and Parkinson's disease (115, 156, 213), GI diseases such as IBD and liver dysfunction (79, 147), and myopathies in both cardiac and skeletal muscle (140, 196). In the context of antigen presentation, while basal activation of autophagy-mediated MHC II loading is beneficial, a dysregulated autophagy pathway could theoretically lead to the presentation of inappropriate antigens to the adaptive immune system, but this has yet to be established (34, 172, 187, 205).

The Adaptive Immune System

The adaptive immune system demonstrates fairly different characteristics compared to the innate immune system. While innate immunity occurs quickly after binding pattern recognition receptors, the activation of the adaptive immune system is

much slower and generally involves cellular proliferation of specific adaptive immune cells after activation (215). This represents a second ‘phase’ of immunity after the innate immune system. A factor contributing to response time is the fact that innate immune cells are positioned in areas near potential infection sites. However, the adaptive immune system is generally positioned in different lymphoid organs and require recruitment (145). As described above, the adaptive immune system also requires contributions from the adaptive immune system in the form of antigen presentation cells. The presentation of antigens on MHC II molecules is crucial in order to direct the adaptive immune response (145, 183). In contrast, the activation of the innate immune system by binding of pattern recognition receptors elicits a consistent innate response, and not a ‘tailored’ adaptive response to specific antigens. Finally, unlike the innate immune system which provides the same response at each exposure to a given antigen, the adaptive immune system provides a lasting ‘memory’ for a quicker and more robust reaction at subsequent exposures to the same antigen.

The adaptive immune system is comprised of two major cell types: T-lymphocytes and B-lymphocytes. While B-lymphocytes are associated with humoral immunity from antibodies secreted by activated B-lymphocytes, T-lymphocytes are associated with cell-mediated immunity via direct cell-to-cell interactions. These two cell types do not act independently from each other, as specific T-lymphocyte subsets are involved in the activation of B-lymphocytes as well (12, 145). Contributions from both B and T-lymphocytes leads to robust adaptive immune responses.

T-lymphocytes

T-lymphocytes form in the thymus, and through rigorous selection processes, they develop into either CD4-expressing or CD8-expressing T-lymphocytes (54). Once developed, but prior to encountering an antigen or stimulation, they are in a naïve and inactive state stored in lymphoid organs (145). However, antigen presentation and stimulation from antigen presenting cells causes the activation and proliferation of T-lymphocytes. The activation state of a T-lymphocyte can be determined not only by the cytokines it expresses and proliferation rate, but also by the cell surface markers that are present. For example, acute markers for T-lymphocyte activation include CD69, which becomes detectable on the cell surface within several hours, and CD25, which becomes detectable within several days (31, 178). Once activated, T-lymphocytes take on effector roles to bring about immune responses (145).

CD8-expressing T-lymphocytes are also known as cytotoxic T-lymphocytes, and they carry out their cytotoxic effector functions via cell-to-cell contact and binding of ‘non-self’ MHC I molecules (145). These T-lymphocytes are especially important in the detection of intracellular pathogens such as viruses and modified ‘self’, such as in cancer (159). CD8+ T-lymphocytes rely on the recognition and binding of cell surface receptors and perform cytotoxic effects by perforating the target cell wall with perforin. The subsequent introduction of granzyme molecules into the target cell cytoplasm activates the caspase cascade and triggers apoptosis in the target cell (145).

Unlike their CD8-expressing counterparts, CD4+ T-lymphocytes do not generally carry out their effector functions via cell receptor binding. Instead, their effector functions are carried out by releasing soluble molecules and cytokines (145). CD4+ T-

lymphocytes are also referred to as helper T-lymphocytes due to their function to aid in the activation of other T-lymphocyte subtypes or other immune cells. CD4+ T-lymphocytes can be further subdivided into different cell types based on the primary cytokine or transcription factor expressed by each CD4+ T-lymphocyte subtype. T-lymphocyte subtypes that are of interest in this dissertation include T_h1, T_h2, T_h17, and T_{reg} T-lymphocytes.

Each T-lymphocyte subtype expresses a unique combination of cytokines and can drive different forms of immune reactions. For example, T_h1 T-lymphocytes are identified by their expression of IFNγ, IL-2, and TNF-β to activate macrophages, B cells, and cell mediated immunity with a predominantly phagocytic-dependent inflammation (161). Crohn's disease has traditionally been thought of as a T_h1-mediated disease (161), but recent studies demonstrate a joint role for T_h1 and T_h17 in its pathogenesis (133). In contrast, ulcerative colitis is regarded as a T_h2-mediated disease with distinct cytokine profiles, expressing IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (133, 160, 161, 188). T_h2 T-lymphocytes can also inhibit T_h1 effects such as phagocyte-dependent inflammation, while inducing an antibody-mediated response (161). Depending on the surrounding environment, activated T_h17 T-lymphocytes express multiple cytokines including IL-9, IL-10, IL-17A, IL-17F, IL-21, IL-22, granulocyte-macrophage colony stimulating factor, and IFNγ (9, 116). The physiologic role for T_h17 T-lymphocytes is to regulate neutrophil recruitment and provide immunity against bacterial and fungal pathogens via cytokine and chemokine release (9). However, improper T_h17 regulation contributes to IBD pathogenesis and is associated with auto-immune diseases such as type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus and multiple

sclerosis (9, 100, 197). Finally, the T_{reg} subtype of T-lymphocytes act in an anti-inflammatory manner and limits proliferation in the other T-lymphocyte subtypes to promote immune tolerance, limit auto-immunity, and maintain tissue homeostasis and wound repair (86, 167, 221). They can be identified through their expression of the FoxP3 transcription factor, and they carry out immunosuppressive activities through the release of anti-inflammatory cytokines IL-10 and transforming growth factor beta (TGF-β), sequestering pro-inflammatory cytokines, and inducing the anergy and apoptosis of other pro-inflammatory T-lymphocyte subtypes (86, 100, 167).

Current models of intestinal inflammation

A variety of animal models have been developed in order to model intestinal inflammation and inflammatory bowel diseases. These models are primarily chemically induced, genetically induced, or cell-transfer induced colitis models. While many different genetic models have been developed that develop intestinal inflammation, these models generally also trigger inflammatory responses or pathology in other organ systems as well. One of the more widely used animal knockouts are IL-10 knockout animals that demonstrate a spontaneous development of colitis after roughly 3 months of age (125, 126). Other knockout models include IL-2 knockout, TGFβ knockout, Toll-Like Receptor 5 knockout, signal transducer and activator of transcription 3 knockout, T-lymphocyte receptor α knockout, among others (125, 126). Adoptive transfer of CD4+ or CD8+ T-lymphocytes is another technique to model intestinal inflammation. Naïve CD4+ T-lymphocytes transferred from the spleen of wildtype animals to immune-deficient mice cause a T_h1-mediated response to elicit transmural colitis when in the absence of regulatory T-lymphocytes (125). Similarly, transfer of naïve CD8+ T-lymphocytes to

recombination activating gene-2 knockout animals elicits colitis via activated CD8+ T-lymphocytes expressing IL-17. In addition to the transfer of naïve T-lymphocytes, the transfer of CD8+ T-lymphocytes that are specific for heat shock protein 60 or ovalbumin (in the presence of administered ovalbumin) also elicits intestinal inflammation (125). Despite the effectiveness of genetic models or adoptive transfer of T-lymphocytes in generating intestinal inflammation, the most common type of model utilized for IBD comes in the form of chemically induced intestinal inflammation and IBD, due to the low cost and the ease of these models (155). These chemicals include dextran sulfate sodium (DSS), oxazolone, dinitrobenzene sulfonic acid, trinitrobenzene sulfonic acid, and acetic acid. These models are beneficial in that it does not require specific genetic strains of animals and can be administered to form discrete regions of intestinal inflammation. For example, DSS-induced colitis utilizes 1-5% of the polymer DSS in drinking water to induce acute phase colitis, characterized by increased levels of TNF-α (125, 155). Since colitis is triggered by intestinal epithelial damage due to osmotic stress from DSS and not due to the activation of adaptive immune cells, colitis can be elicited in animals lacking T- and B- lymphocytes (39). In contrast, oxazolone-induced colitis utilizes an intrarectal administration of haptenizing agent oxazolone with ethanol to elicit an acute development of colonic inflammation. As oxazolone is a haptenizing agent, it requires an intact adaptive immune system, specifically working via natural killer T cells (68). Similarly, the administration of haptenizing agents dinitrobenzene sulfonic acid and trinitrobenzene sulfonic acid by enema with ethanol allows for the development of regional intestinal inflammation and favors a T_h1-mediated inflammatory response (125, 155). Finally, acetic acid enema causes intestinal epithelial damage and

is characterized by neutrophil recruitment and ulceration and necrosis in the mucosal and submucosal layers (155).

This dissertation aims to examine the contributions of enteric glia on the development of intestinal inflammation. Given the severity of inflammation in the developed IBD models described above, experiments in this dissertation utilize a different form of inflammatory stimuli. Based on findings from Zhang et al. (223), we utilize intraperitoneal (IP) injections of IFNγ and LPS as inflammatory stimuli. This is to ensure that inflammatory stress does not overwhelm our experimental model and to prevent inflammatory responses being dominated by other immune cell types, thereby masking contributions from enteric glia. Details of our model and injection of inflammatory stimuli are described in Chapter 3.

Summary and Aims of Dissertation

The ENS is composed of enteric neurons and enteric glia, and it is the primary regulator of GI function (48, 194). Inflammatory damage to the ENS can lead to the loss of enteric neurons and glia, contributing to secretomotor dysfunctions seen in the GI diseases (56). Therefore, immune modulation in the GI tract is extremely important in maintaining ENS health and function. Neuroimmune interactions are becoming increasingly recognized as important aspects to immune modulation. As a part of the ENS, enteric glia demonstrate similar characteristics to antigen presenting cells. This suggests that enteric glia may play a similar role as antigen presenting cells and interact with the adaptive immune system, but their exact role in immune modulation has yet to be studied.

During homeostasis, enteric glia play important roles in the maintenance of enteric neuronal health and can modulate neuronal activity to maintain normal GI secretomotor functioning (32, 65, 135). However, during inflammation, enteric glia can contribute to neuronal death and express MHC II and T-lymphocyte co-stimulatory molecules (4, 18, 53, 201). Co-culture experiments demonstrate the ability for enteric glia to also modulate T-lymphocyte activity (90).

The objective of this dissertation is to understand the role enteric glia play *in vivo* in modulating immune activity during homeostasis and in pro-inflammatory environments. Chapter 2 takes a further look into recent studies that demonstrate the potential for enteric glia to function as an immunomodulator. Chapter 3 investigates the capabilities of enteric glia to act as an antigen presenting cell, their effects on T-lymphocyte activation, and the contributions autophagy has on glial MHC II expression. Together, these chapters aim to improve the understanding of how enteric glia interact with T-lymphocytes, and their role in immunomodulation.

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CHAPTER 2:

Potential roles of enteric glia in bridging neuroimmune communication in the gut

Work presented in this chapter has been published as:

Chow AK, Gulbransen BD. Potential roles of enteric glia in bridging neuroimmune communication in the gut. Am J Physiol Gastrointest Liver Physiol. 2017 Feb 1;312(2):G145-G152.

Abstract

The enteric nervous system (ENS) is a network of neurons and glia that controls ongoing gastrointestinal (GI) functions. Damage or injury to the ENS can lead to functional GI disorders. Current data support the conclusion that many functional GI disorders are caused by an imbalance between gut microbes and the immune system but how the ENS is involved in these interactions is less understood. Due to the proximity of the ENS to bacteria and other foreign antigens present in the GI tract, it is important to prevent the passage of these antigens through the GI epithelium. If any foreign compounds manage to pass through, an immune response is triggered to prevent injury to the ENS and underlying structures. However, careful modulation of the inflammatory response is required to allow for adequate elimination of foreign antigens while avoiding inappropriate over-activation of the immune system as in autoimmune disorders. Enteric neurons and glial cells are capable of performing these immunomodulatory functions to provide adequate protection to the ENS. Here, we review recent studies examining the interactions between the ENS and the immune system, specifically focusing on enteric glial cells and their ability to modulate inflammation in the ENS.

Introduction

The enteric nervous system (ENS) is the largest division of the autonomic nervous system and it is housed entirely within the walls of the gastrointestinal (GI) tract. Neural reflexes mediated through the ENS are responsible for coordinating most ongoing activities of the GI tract such as patterns of motility, secretions and local blood flow. Like the brain, the ENS is entirely composed of neurons and glial cells that are housed within ganglia in two ganglionated plexuses. The neural circuitry in the submucosal plexus is responsible for coordinating gut secretions, absorption and local blood flow while the myenteric plexus is primarily responsible for coordinating contractions and relaxations of gut smooth muscle involved in patterns of gut motility. Proper orchestration of GI functions requires that both plexuses are intact and maintain healthy interactions with target cells. Injuries inflicted on the ENS during local inflammation disrupt the functional connectivity of enteric neural networks and create long–lasting changes in ENS function (13). These changes in the local control of gut reflexes contribute to a broad range of common functional GI disorders such as irritable bowel syndrome (IBS) and functional constipation and contribute to persistent intestinal dysmotility during remission in inflammatory bowel disease (IBD).

Clearly, cells within the innate and adaptive arms of the immune system are the main drivers of gut inflammation. Generally speaking, inflammation is a protective response to invading pathogens that evade the first line of defense posed by the intestinal epithelial barrier and defects in the balanced immune response to intestinal bacteria is a major contributing factor to gut disease. The acute inflammatory response predominantly involves the recruitment and activation of the innate arm of the immune system (30) while

chronic inflammation involves both innate and adaptive components. Likewise, an overly aggressive adaptive immune response can attack native antigens in the gut and contribute to the development of inflammation (28). Current animal models used to study bowel inflammation are based on these principles and act to either increase epithelial permeability, such as the dextran sodium sulfate colitis model, or to enhance T-cell stimulation, such as the trinitrobenzene sulfonic acid (TNBS), dinitrobenzene sulfonic acid, oxazolone, T-cell adoptive transfer, and interleukin (IL)-10 knockout models (38). The understanding of the immune response in colitis is becoming increasingly refined and has led to many important breakthroughs in therapies. However, how inflammation produces long–lasting changes in gut function through effects on the ENS is still poorly understood. Importantly, new data suggest that bi-directional interactions between cells in the ENS and immune cells may actively participate in the modulation of inflammatory responses themselves. Here, we provide a short review of recent studies that highlight potential roles for the ENS in the regulation of gut inflammation and particularly focus on the key roles of enteric glial cells.

Contribution of enteric neurons to inflammation

Many immunomodulatory effects of the parasympathetic and sympathetic branches of the autonomic nervous system have emerged in recent years and the general picture is that vagal parasympathetic innervation is anti-inflammatory while α-adrenergic sympathetic innervation is pro-inflammatory (6, 10). However, far less is known about how intrinsic enteric neurons influence immune responses in the gut. The current evidence to date strongly suggests that enteric neurons have major immunomodulatory capabilities. The relative contributions of direct neuron-immune cell communication and

indirect communication via intermediary cells like enteric glia is still largely unknown. It is likely that many neuro-immune interactions at the ganglionic interface are mediated through enteric glia because enteric glia delineate the boundaries of enteric ganglia and largely surround enteric neuron cell bodies. However, neuronal processes extending outside the ganglia have a high potential for direct interactions with various immune cells. The capacity of neurons to influence immune responses through both direct and indirect pathways likely accounts for observation that enteric neuron number and density influences intestinal inflammation (48). Margolis et al. found that transgenic mice with a hyperplastic ENS and higher density of enteric neurons developed greater colonic inflammation when exposed to TNBS as compared to wildtype animals (48). The fact that this study used a transgenic animal model where high neuronal density is present prior to the administration of TNBS strongly suggests that neuronal hyperplasia is not merely a response to induced colonic inflammation but could, in fact, be a risk factor for the development of colitis. These findings are particularly interesting in light of the discovery that enteric glia kill a significant portion of neurons during acute inflammation (14). Given the pro-inflammatory effect of enteric neurons, it is tempting to speculate that glial-driven neurodegeneration functions to protect against the neuronal potentiation of inflammation but this concept is still unproven at this point.

How higher numbers of enteric neurons translates to worsening inflammation is not immediately clear but likely occurs through the neuronal release of cytokines and neurotransmitters/neuromodulators. For example, enteric neurons from patients with Crohn’s disease demonstrate high expression of pro-inflammatory prostaglandin D2 (44). Enteric neurons are also capable of secreting leukocyte chemoattractive factors that

include tumor necrosis factor-α (TNF-α), IL-6, the monocyte chemoattractant protein-1 and possibly IL-8 when challenged with pathogenic bacteria (15, 60) (Fig.2.1). Interestingly, enteric neurons do not increase their secretion of these chemoattractive factors in response to Gram-positive bacteria despite the ability of both Gram-positive and Gram-negative bacteria to activate NF-κβ signal transduction pathways in neurons (15). This may be achieved through the effects of concurrent Wnt signaling within enteric neurons because the stimulation of the Wnt pathway in the presence of LPS confers an anti-inflammatory phenotype in enteric neurons in vitro (41) (Fig.2.1). These findings suggest that neurons are capable of differentiating between beneficial and harmful bacteria and participate in defensive responses to clear potentially pathogenic species. However, the inherent ability of neurons to recruit leukocytes could be potentially detrimental when additional neurons are present and may contribute to enteric ganglionitis. Although untested at this point, aberrant neuronal chemoattractant secretion under these conditions could be an important mechanism that contributes to a pro-inflammatory environment and a predisposition to gastrointestinal disease.

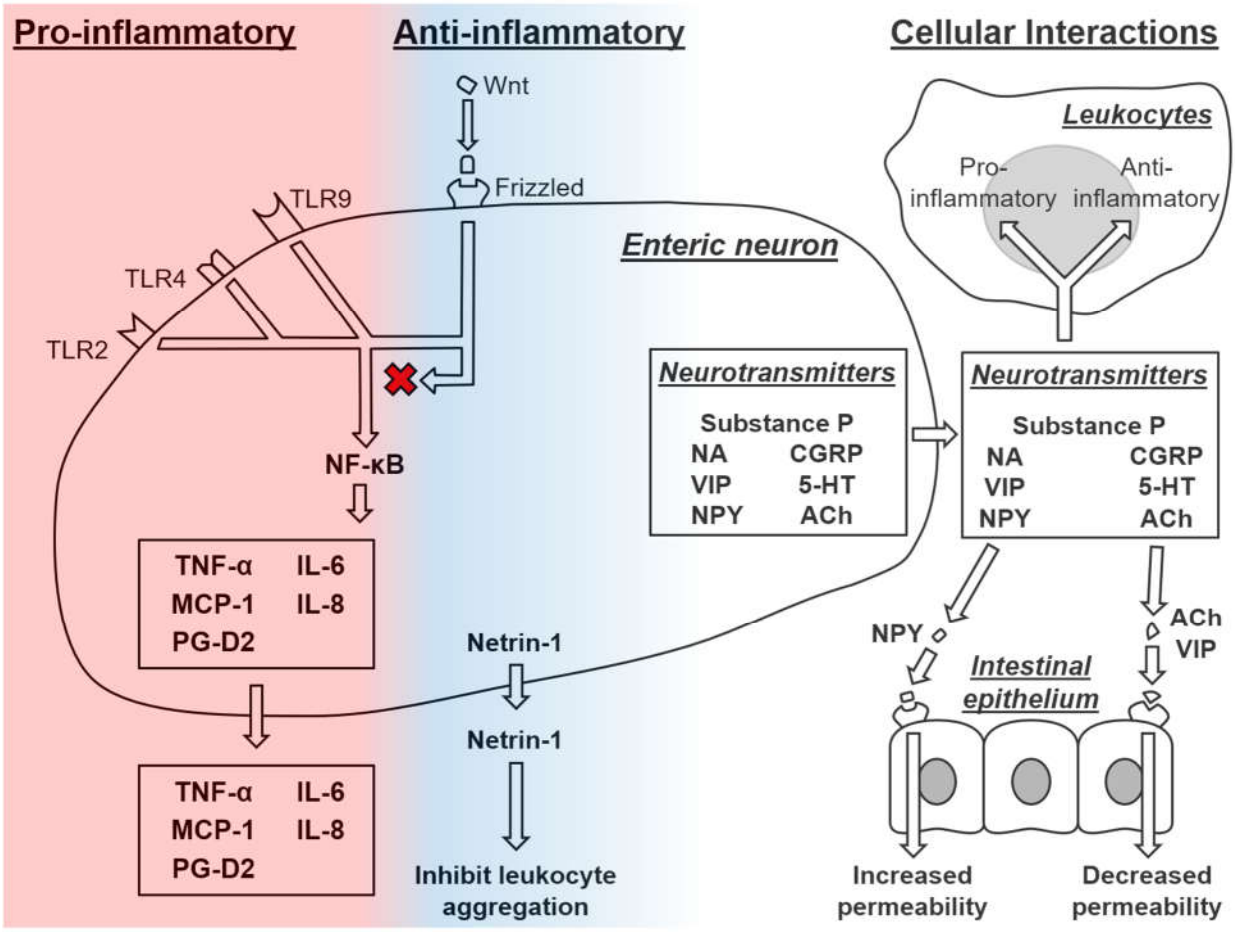

Figure 2.1: Overview of enteric neuron responses to select signals. Pathways highlighted in red denote pro-inflammatory pathways while those highlighted in blue lead to anti-inflammatory effects. Enteric neuron interactions with other cell types are shown with a white background.

In addition to their pro-inflammatory potential, enteric neurons may also contribute to anti-inflammatory effects via the synthesis and secretion of factors like netrin-1. Netrin-1 is typically known for its role in neuronal development but its expression is also induced during hypoxia-induced tissue damage (59) and the secretion of this molecule can induce angiogenesis, the activation of tissue stem cells and bind neutrophil cell surface receptors such as A2B adenosine receptors (3, 36). The main effect of netrin-1 on neutrophils is to inhibit their migration and accumulation and may induce an egress away from the source of netrin-1 (3). As such, enteric neurons could theoretically synthesize and secrete netrin-1 as a response to inflammatory damage for neuronal survival and promote regeneration, and a loss in netrin-1 secretion could theoretically contribute to inappropriate leukocyte recruitment (4) (Fig.2.1). However, other major sources of netrin-1 in the GI system include the colonic epithelium; therefore further studies are required to elucidate the extent to which the ENS contributes to netrin-induced anti-inflammatory effects.

Although activation of the Wnt pathway and secretion of prostaglandin D2 can induce inflammation, they can also inhibit the progression of inflammation. As described earlier, activation of the Wnt pathway with a concurrent LPS costimulatory signal on enteric neurons cause anti-inflammatory effects via the release of IL-10 (41). Neuronal prostaglandin D2 can be metabolized into 15-deoxy-$\Delta^{12\text{-}14}$-prostaglandin J2, an anti-inflammatory compound (44). The dual functioning of the Wnt pathway and prostaglandin release suggests that enteric neurons may be important for the selective activation and ‘fine-tuning’ of inflammatory responses in the ENS. In addition to cytokines and chemokines, neurotransmitters present in the ENS such as ATP, acetylcholine, noradrenaline, vasoactive intestinal peptide (VIP), neuropeptide Y, calcitonin gene-

related peptide, substance P, and serotonin can modulate the activities of intestinal leukocytes (1, 29, 34, 49) (Fig.2.1). These neurotransmitters yield both pro- and anti-inflammatory effects and likely have a major influence on inflammatory processes in the ENS. Margolis and Gershon (2016) recently reviewed the roles of several neurotransmitters in the regulation of intestinal inflammation and readers are referred to this excellent summary for further details (47). To summarize, the ability for immune cells to interact with neurotransmitters suggests a close relationship and frequent communication between enteric neurons and the immune system.

In addition to acting directly on immune cells, these neurotransmitters can act on intestinal mucosa and alter epithelial barrier permeability. For example, in vitro experiments demonstrate that neuropeptide Y increases the epithelial barrier permeability and exposes the ENS to potential pathogens (17), whereas cholinergic and VIPergic signaling maintains barrier integrity and decreases permeability (18, 39, 55) (Fig.2.1). The control of intestinal epithelial barrier function and the exposure of the ENS to potential pathogens can modulate the inflammatory state of the ENS. This may represent an indirect means by which enteric neurons exert their influence on the immune system, further reinforcing the involvement of the ENS in immunomodulation.

Glia in the healthy intestine

Enteric glial cells are historically regarded as a network of cells that establish the homeostatic environment within enteric ganglia and function to provide both structural and nutritional support for enteric neurons. They are found primarily in both the submucosal and myenteric plexuses of the ENS, but may also be present outside the ENS, such as in the mucosal lamina propria. They are a heterogeneous group of cells

that can be subdivided into several types based on morphology and expression profiling (8, 56). These glial cell subtypes respond differently to purinergic signaling, suggesting different glial subtypes activate in varying situations. However, specific roles for the distinct glial populations have yet to be elucidated. Beyond structural and nutritional support, subsets of enteric glial cells also exert influence over other nearby cells of the GI tract and have local immunomodulatory effects. These functions largely resemble those of astrocytes in the central nervous system (CNS), and in this way, it is thought that certain groups of enteric glia fulfill astrocytic-like roles in the ENS.

The relationship between enteric neurons and enteric glial cells begins early and new data show that glial cells are necessary for the proper maturation of neurons in the ENS(7). Glial cell guidance of neuronal development occurs through purinergic signaling between the two cell types and impaired signaling can lead to decreased enteric neuron density and complexity (7). Purinergic signaling between enteric glia and neurons continues into adulthood and plays an important role in neural circuits that coordinate GI motility (33, 50, 51). These exciting discoveries suggest that alterations to purinergic intercellular signaling between neurons and glia could contribute to gut dysfunction by impairing the development and maturation of enteric neurons or by altering neuronal activity. In agreement, impairing glial purinergic signaling in adult mice hinders coordinated contractions of the GI tract and slows colonic transit time (51).

Mucosal enteric glial cells also form interactions with non-neuronal cells such as enterocytes and enteroendocrine cells that are important in the maintenance of GI homeostasis. Glial cells influence the development and maturation of enterocytes and these roles contribute to the maintenance of an intact GI epithelial barrier (52). Less is

known about mucosal glia-enteroendocrine interactions but specialized connections between the two cell types suggest that glia may have the potential to influence gastrointestinal hormone release (9). The fundamental roles of barrier regulation and hormone release in GI physiology suggest that defective interactions between enteric glia and enterocytes or enteroendocrine cells could contribute to GI inflammation and possibly even metabolic diseases such as diabetes and obesity (9, 65).

Glial cells are activated by cytokines and immunomodulatory signals

The ability of enteric glial cells to respond to immunomodulatory signals such as cytokines, bacteria and neurotransmitters in the extracellular milieu suggests that glia mediate a significant amount of the cross talk between the ENS and the immune system. For example, glial cells can detect local levels of the pro-inflammatory cytokine IL-1, IL-4, and TNF-α (12) (Fig.2.2). Binding of these cytokines can elicit glial cell activation similar to reactive gliosis in the CNS and contribute to inflammation and GI dysfunction (61). Likewise, the activation of glial toll-like receptors 2 and 4 by LPS and other bacterial components drives pro-inflammatory pathways that are important in the protective response against bacteria that successfully bypass the gut epithelial barrier (58, 62). Eposito et al. demonstrated that LPS-activated glial cells occupy a crucial role in the creation of a robust inflammatory response in the local ENS environment and that the inhibition of the NF-κβ pathway in the glia can effectively ameliorate colonic inflammation (24). However, LPS-activated cultured human glia can also increase transcription of growth factors and several anti-inflammatory genes (42). This paradoxical elevation in the transcription of growth factors and anti-inflammatory genes with simultaneous expression of pro-inflammatory genes hints at a complex regulation of expression that likely involves

negative feedback pathways. Interestingly, Turco et al. showed that cultured human enteric glial cells are able to distinguish pathogenic from probiotic bacteria and modulate their expression of toll-like receptors accordingly (62). Similarly, Liddo et al. showed that exposing mixed cultures of rat enteric neurons and glia to a combination of LPS and Wnt3a induces the secretion of anti-inflammatory agents and inhibits NF-κβ activity in vitro (41). These findings suggest that the glial response to bacterial components in the ENS and the decision of whether to undertake pro- or anti-inflammatory pathways depends on the identity of the bacteria and which signaling molecules are being co-expressed.

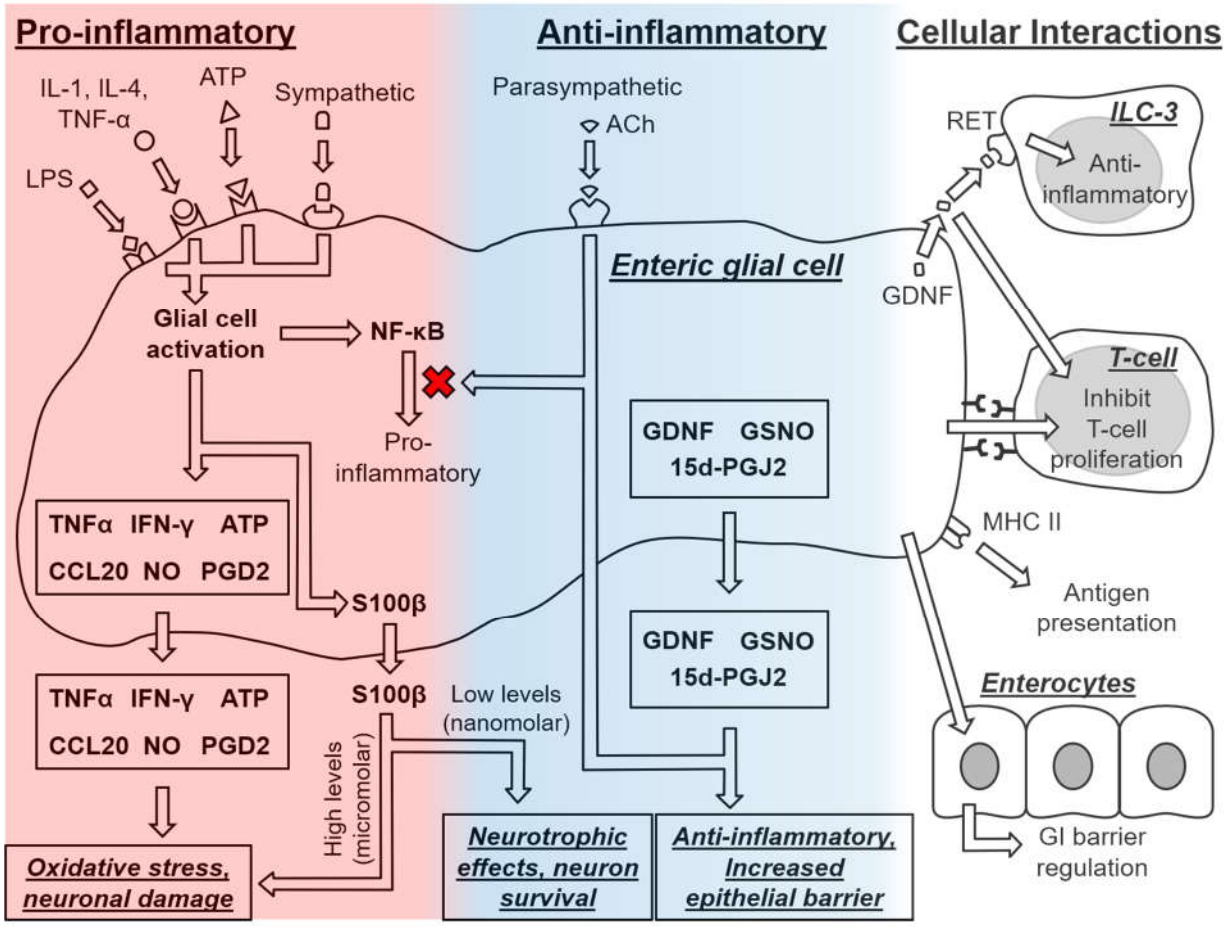

Figure 2.2: Overview of enteric glial cell responses to select signals. Pathways highlighted with a red background are pro-inflammatory, while those highlighted in blue yield anti-inflammatory effects. Glial cell interactions with other cell types are shown with a white background. Note that glial response to certain signals such as ATP and LPS depend on the context by which the glial cell is stimulated and may yield varying responses.

Purines are a major component of the “inflammatory soup” generated during tissue injury and purines contribute to the pathophysiology of many inflammatory diseases of the intestine. Enteric glia are highly responsive to purines and express receptors for ATP and derivatives such as ADP and adenosine. The glial response to ADP through the activation of P2Y1 receptors is the most well characterized and is involved in the coordination of neural circuits and the promotion of neuronal maturation under normal conditions (7, 50, 51). However, glial P2Y1 receptors also play an important role in the glial response to neuronal danger cues released during inflammation and the activation of glia in this context causes neurodegeneration (14). The cause of the vastly different glial responses to P2Y1 receptor activation appears to primarily involve the influence of pro-inflammatory mediators, such as nitric oxide, on downstream effectors like connexin-43 hemichannels (14). Therefore, the response of glia to purinergic agonists depends heavily on the context in which they are activated. How the activation of glia by other purines influences their response to inflammation is much less clear. For example, cultured human enteric glia express adenosine receptors that primarily couple to intracellular signal transduction pathways that utilize cAMP (42). Given the potent anti-inflammatory effect of adenosine in the gut, it is likely that adenosine has similar anti-inflammatory actions on glia but how the activation of glia by adenosine influences gut physiology and pathophysiology is currently unknown.

Finally, enteric glial cells are innervated by both sympathetic and parasympathetic neurons of the autonomic nervous system (32, 63) (Fig.2.2). Stimulation of glial cells via parasympathetic vagal innervation confers an anti-inflammatory and neuroprotective effect, similar to the effects of parasympathetic vagal stimulation on intestinal

macrophages (49). Specifically, the activation of glial cholinergic receptors in vitro promotes increased epithelial barrier function and inhibits the transcription of the pro-inflammatory factor NF-κβ (18). These findings support previous data showing that the beneficial effects of vagal nerve stimulation on the intestinal epithelium following burn injuries is mediated through effects on enteric glia (22). Conversely, sympathetic-mediated glia activation occurs through purinergic (32) and noradrenergic (54) signaling. As noted above, the activation of glia by ATP may contribute to a pro-inflammatory phenotype but how the activation of glia by norepinephrine influences glia is not known.

In summary, glia respond to a diverse array of immunomodulatory mediators that include pro-inflammatory cytokines, bacterial compounds, purines and transmitters from the autonomic nervous system. Activated glial cells exhibit some characteristics similar to astrocytes undergoing reactive astrogliosis in the CNS such as increased calcium influx and signaling and an increase in glial acidic fibrillary protein or S100β expression (12, 18, 42). However, glial activation is context specific and the outcome response of glia depends on integrative processing of all cues available at that particular time.

Glia express and secrete cytokines and pro-inflammatory signals

Mounting evidence in recent years suggests that glial cells are not idle bystanders during injury and inflammation in the ENS. What this evidence shows is that when activated, glia can contribute to a pro-inflammatory environment by secreting cytokines and other immunomodulatory signals. For example, one response of glia that are activated by inflammatory cues in the gut includes alterations to their morphology and expression and/or secretion of key proteins such as S100β and glial fibrillary acidic protein, similar to the process of reactive astrogliosis in the CNS (57). Increased secretion

of S100β by glia contributes to inflammation by activating receptors for advanced glycation end-products. This in turn leads to the production of nitric oxide by inducible nitric oxide synthase, the formation of reactive oxygen species, increased oxidative stress in the local environment and neuronal damage in ex vivo and in vitro studies (20, 23, 42, 46, 62). It is important to note that extracellular concentrations of S100β in the micromolar range are needed for pro-inflammatory effects and that basal S100β secretion in the nanomolar range yields neuroprotective effects (23) (Fig.2.2). Interestingly, despite an increase in local S100β during inflammation of the GI tract, Celikbilek et al. show a counterintuitive decrease in serum levels of S100β in patients with ulcerative colitis (16), possibly due to damaged enteric glial cells secreting reduced S100β as a result of exposure to prolonged inflammatory stress. Serum S100β levels over time in IBD may show fluctuations depending on glial cell viability and may prove useful in determining chronicity of inflammation. The stimuli for glial S100β release are incompletely understood but do include toll-like receptor agonists (62) and also likely includes purinergic signaling. Glial activation by purines during inflammation involves similar pathways as S100β such as inducible nitric oxide synthase and the release of ATP from activated glial cells (14). Glial S100β and ATP release both have neurotoxic effects on enteric neurons and these mediators are key drivers of ENS damage during acute inflammation in vivo (14, 23) (Fig.2.2).

The glial secretion of cytokines and chemokines is of specific interest among the repertoire of compounds secreted by enteric glia because of their direct immunologic effects. Specifically, recent in vivo and in vitro studies demonstrate that glia secrete a number of cytokines and chemokines that include interferon gamma (IFN-γ), chemokine

ligand 20, TNF-α and prostaglandin D2 (26, 44, 58) (Fig.2.2). However, conflicting data both support and refute the concept that enteric glia secrete TNF-α. For example, Coquenlorge et al. showed that glial cell cultures are unable to secrete TNF-α even when stimulated with LPS (21). Based on this data, they concluded that TNF-α in the ENS originates from enteric neurons and not from glia. In contrast, Guedia et al. found that enteric glial cell lines are capable of secreting TNF-α when stimulated with LPS and that the effect is further enhanced when exposed to Tat protein derived from HIV (31). These conflicting results might suggest that multiple pathways can converge to induce inflammation and that there are various regulatory systems in place to restrict inappropriate inflammatory activation. Once activated, the glial secretion of cytokines and chemokines likely play an important role in the activation and recruitment of immune cells and modulates inflammation. Beyond IFN-γ, chemokine ligand 20, and TNF-α, numerous other pro-inflammatory cytokines and chemokines undergo transcriptional changes in activated enteric glia in culture. However, detectable protein secretion of these compounds has not been measured from activated glia (42, 58).

Glial cells act as antigen presenting cells

The many similarities between enteric glial cells and astrocytes hints that enteric glia, like astrocytes, may function as antigen presenting cells. New data show that enteric glia have phagocytic capabilities because glia transplanted from the ENS into the brain are capable of degrading pre-formed Aβ amyloid plaques in a similar manner as resident astrocytes (25, 64). In vivo and in vitro studies demonstrate that human enteric glial cells from both the myenteric and submucosal plexuses are also capable of expressing major histocompatibility complex II molecules, especially after exposure to bacteria or parasites

(5, 27, 62). In addition, enteric glia in patients with megacolon due to Chagas disease express the T cell co-stimulatory molecules cluster of differentiation (CD)80 and CD86 on their cell surface (5). Taken together, these data show that enteric glia have the ability to engulf and subsequently, present antigens to innate and adaptive immune cells and hints that enteric glial antigen presentation may play an important role at the neuro-immune interface. The role of glial antigen presentation in the gut is not currently understood but it could theoretically function to prime the adaptive immune system to bacteria in the ENS. By the same token, it would be possible for enteric glia to present neuronal debris to the adaptive immune system, thereby triggering enteric ganglionitis and an auto-reactive immune response against ENS neurons similar to what is seen in multiple sclerosis (45).

Enteric glia exert immunosuppressive and anti-inflammatory effects

In line with the thought that glial cells play a neuroprotective role to maintain homeostasis, there is increasing evidence that glial cells are capable of immunosuppression and anti-inflammatory actions. These anti-inflammatory effects are controlled, in part, by the release of soluble glial compounds. Of specific interest are glia-related peptides and lipids that include glial derived neurotrophic factor (GDNF), glial derived S-nitrosoglutathione (GSNO) and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 because these compounds have beneficial effects on intestinal epithelial permeability, inflammation and neuron survival (2, 19, 35, 65) (Fig.2.2). GDNF signals through RET and GFRα receptors present on innate immune cells and enterocytes of the gut to decrease expression of pro-inflammatory cytokines and help maintain and strengthen the intestinal epithelial barrier, respectively (35, 52, 65). Specifically, Ibiza et al. demonstrated via in vivo and in vitro mouse studies that ligands from the GDNF family act on type 3

innate lymphoid cells, leading to a release of anti-inflammatory IL-22 and increased expression of repair genes in the gut epithelium (35). In addition, RET and GFRα expression has been demonstrated in B lymphocytes, CD4+ and CD8+ T lymphocytes (53) but the direct effects of GDNF on these cells have yet to be described. Recent studies with cultured human enteric glial cells derived from the myenteric plexus suggest that GDNF contributes to the inhibition of proliferation by previously activated CD8- and CD8+ T lymphocytes. However, it seems that direct cell-to-cell interactions, as opposed to secreted compounds, play a larger role in preventing T lymphocyte proliferation (37). Furthermore, Kermarrec et al. showed that enteric glia from patients with Crohn's disease exerted a greater immunosuppressant effect than control glia, suggesting that activated glial cells play an important role in immunomodulation. Similarly, von Boyen et al. describe increased activated mucosal glial cells expressing increased GFAP and GDNF in patients with Crohn's disease. They further show that pro-inflammatory cytokines are an effective stimulus for GDNF secretion in vitro (11). Taken together, effects of GDNF contribute to an anti-inflammatory phenotype to reduce epithelial damage, prevent neuronal loss, suppress activated T lymphocytes and improve gut function, potentially as a negative feedback mechanism to limit the damage due to inflammation and pro-inflammatory cytokines (11, 37, 43) (Fig.2.2). Similar to the actions of GDNF, in vitro and in vivo studies show that GSNO acts to promote a robust permeability barrier in the intestinal epithelium. This is achieved by inducing increased expression of zonula occludens-1 and occludin protein to form more tight junctions in the epithelium (19, 40). In addition, GSNO provides anti-inflammatory effects via the inhibition of NF-κβ pathways that promote the expression of pro-inflammatory agents (40). Finally, secretion of 15-deoxy-$\Delta^{12,14}$-prostaglandin J2

from cultured rat myenteric glia elicits neuroprotective effects through the transcription factor NF-E2 related factor 2 pathway leading to neuronal synthesis of glutathione in vitro (2). Therefore, enteric glia can effectively modulate and control the extent of local inflammation and shift the local ENS environment back towards homeostasis.

Conclusions

Enteric glial cells have gained increasing attention because of their diverse emerging roles in the modulation of gut health and disease. Increasing evidence supports the idea of cross-talk between glial cells and many cells including enterocytes, neurons and immune cells. Specifically, activated glial cells play a substantial role in both the progression and inhibition of inflammation in the ENS. The seemingly contradictory actions may be due to the amount of secretion and extracellular concentration of glial products, as seen in S100β secretion, or the signaling pathway by which glial cells are activated, as demonstrated by cholinergic-induced versus cytokine-induced activation. Regulation of glial activation has not been well studied but may give insights into the specific contexts by which certain activation pathways are chosen to yield either pro- or anti-inflammatory effects in the ENS. Furthermore, various pro- and anti- inflammatory genes are transcribed in parallel, though many upregulated genes never reach translation and expression. This suggests potentially complex expression regulation and that enteric glial cells may self-regulate, to an extent, via negative feedback.

The possible phagocytic ability of enteric glia, along with their capability to induce oxidative stress in their local environment and secretion of cytokines likens enteric glial cells to that of astrocytes and the innate immune system. Their ability to express major

histocompatibility complex II and T-cell costimulatory molecules suggests that glia play an important role in antigen presentation to T-cells. Further, the ability of enteric glial cells to modulate inflammation and immune activation shows that glia are an important mediator of neuro-immune interactions. Understanding the specific contribution of glia towards ENS injury and neuronal viability will offer important new insight into the pathogenesis of inflammatory and functional GI disorders.

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CHAPTER 3:

Enteric glial cells induce T_h17 and T_{reg} activation via autophagy-mediated MHC II expression

Abstract

Background and Aims

Enteric glial cells express type II major histocompatibility complex (MHC II) molecules in Crohn's disease and Chagas disease, but it is unclear whether the expressed molecules are functional. We examined the capabilities of enteric glia to act as an antigen presenting cell *in vivo* and have immunomodulatory effects.

Methods

We used a tamoxifen-inducible glial-specific MHC II knockout (*Sox10*^{CreERT2};*IAB*$^{fl/fl}$) to isolate effects of enteric glial antigen presentation. We tested potential peptide sources to be loaded onto glial MHC II by measuring phagocytic activity and autophagy activation. We also determined enteric glial immunomodulatory capabilities by measuring T-lymphocyte activation and serum and colonic tissue cytokine levels.

Results

Enteric glia express MHC II molecules in response to low dose IFNγ and LPS exposure in the absence of intestinal tissue damage or inflammation. Glial MHC II expression increases activation in CD4+ T-lymphocytes, particularly in the T_h17 and T_{reg} subtypes, but not in T_h1 or T_h2 subtypes. Enteric glial MHC II does not influence serum or regional colonic tissue cytokine levels but may influence local cytokine levels. Autophagy activation is necessary, but insufficient to induce enteric glial MHC II expression.

Conclusions

Enteric glial MHC II expression is induced by pro-inflammatory stimuli IFNγ and LPS and is dependent on autophagy activation. The expression of MHC II modulates T-lymphocyte activation, particularly in the T_h17 and T_{reg} subpopulations, but does not lead to changes in serum or colonic tissue cytokine levels.

Keywords: enteric glial cell, autophagy, major histocompatibility complex type II, T-lymphocyte

Introduction

Antigen presentation is a crucial process that maintains immune homeostasis by dictating T-lymphocyte activation at the transition between the innate and adaptive arms of the immune system. Dysfunction in antigen presentation can lead to the under- or over-activation of the immune system, or immune activation against inappropriate targets(8, 74). As a consequence, presenting the correct antigens and directing immune activity against the proper targets is of prime importance in the gastrointestinal (GI) tract where bacteria, viruses, parasites, and other foreign particles are constant challenges. Dysfunction in antigen presentation is a major contributing factor to immune dysregulation in diseases such as systemic lupus erythematosus, multiple sclerosis, and inflammatory bowel disease (IBD) (8, 48, 53, 74, 80).

Professional antigen presenting cells are the primary cell types responsible for the process of antigen presentation and utilize major histocompatibility complex (MHC) II molecules to present specific peptides to T-lymphocytes. The identity of presented peptides activates specific T-lymphocytes, and therefore modulates immune activation. Since this process is sensitive to slight changes in peptides presented on MHC II molecules (4, 28), CD74 molecules are utilized to block peptide loading onto newly synthesized MHC II until their fusion with phagolysosomes (60). Although phagocytosis of extracellular foreign particles is the primary source of peptides for presentation MHC II molecules, autophagolysosomes can also contribute intracellular and ‘self’ peptides for loading onto MHC II molecules (65, 67). Peptide-loaded MHC II molecules are then targeted to the cell surface for presentation to T-lymphocytes.

In addition to professional antigen presenting cells expressing MHC II molecules, a set of 'atypical' MHC II-expressing cells, such as endothelial cells, epithelial cells, mast cells, and glial cells demonstrate variable levels of MHC II expression (35). Among these, enteric glia have gained considerable interest based on their ability to affect both T-lymphocyte activation and neuronal signaling (17, 24, 36). Enteric glial cells express MHC II molecules in an inducible manner, and share similar characteristics with antigen presenting cells by expressing T-lymphocyte costimulatory molecules CD80 and CD86 (3, 23, 70). Enteric glia are capable of interacting with T-lymphocytes when expressing neoantigens (15), and co-culture studies suggest that enteric glia modulate T-lymphocyte activation and regulate the activity of innate lymphoid type 3 cells (31, 36). Enteric glia also express both pro-inflammatory (20, 46, 62) and anti-inflammatory (1, 10, 31, 79) molecules *in vitro*, and may exhibit immune-modulating functions similar to those of related glial populations in the central nervous system (13). Although glial-T-lymphocyte interactions have been alluded to by *in vitro* studies, whether enteric glia affect play a significant role as antigen presenting cells *in vivo* is not known.

The goal of this study was to test the hypothesis that glial antigen presentation modulates immune homeostasis in the intestine through interactions with CD4+ T-lymphocytes. We tested our hypothesis using a mild proinflammatory stimulus composed of IFNγ and LPS to perturb immune homeostasis without driving overt inflammation and tested the role of glial MHC II by generating mice with a conditional ablation of MHC II in enteric glia. The data show that enteric glia express MHC II in response to IFNγ and LPS and that glial MHC II expression is necessary for adequate CD4+ T-lymphocyte activation. Ablating glial antigen presentation impaired the

activation of T-lymphocyte subsets expressing IL-17 and FoxP3, suggesting that glial antigen presentation influences T_h17 and T_{reg} activation. Glial antigen presentation was independent of an increase in phagocytic activity and required the activation of autophagy. Taken together, these results show that glial autophagy triggered in response to proinflammatory stimuli induces glial antigen presentation, which functions to modulate the activation of T-lymphocyte subsets involved in tolerance.

Materials and Methods

Animals

All protocols involving animals were approved by the Michigan State University Institutional Animal Care and Use Committee. Male and female C57BL/6 mice between 14 to 16-week-old were used unless stated otherwise. Animals were housed in ventilated cages with a 12-hour light-dark cycle, and access to food and water *ad libitum*. Mice with a tamoxifen-inducible conditional deletion of major histocompatibility complex II (MHC II) in enteric glia (*Sox10^{CreERT2};IAB$^{fl/fl}$*) were generated in-house by breeding *Sox10$^{CreERT2+/-}$* mice ((42), gift from Dr. Vassilis Pachnis, The Francis Crick Institute, London, England) with floxed IAB mice (B6.129X1-H2-Ab1^{tm1Koni}/J, RRID:IMSR_JAX:013181, The Jackson Laboratory, Bar Harbor, ME). RiboTag mice (*Rpl22$^{tm1.1Psam}$*/J) expressing hemagglutinin on ribosomal protein L22 (Rpl22; 011029;RRID:IMSR_JAX:011029; The Jackson Laboratory, Bar Harbor, ME) were bred with the glial *Sox10^{CreERT2}* driver line to generate *Sox10$^{CreERT2+/-}$;Rpl22$^{tm1.1Psam}$/J* mice for experiments assessing glial transcriptional profiles as described previously (16). Enteric

glial reporter mice (*Sox10$^{CreERT2+/-}$;tdTomato*) were generated by breeding *Sox10$^{CreERT2+/-}$* mice with Ai14 mice (B6.Cg-*Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$*/J; RRID: IMSR_JAX:007914; The Jackson Laboratory, Bar Harbor, ME). CreER^{T2} activity was activated by feeding animals a 400mg/kg tamoxifen citrate diet for 2 weeks, and used for experiments immediately after the 2-week tamoxifen diet.

Induction of glial MHC II

Mice were injected with mouse recombinant interferon-gamma (IFNγ) (1µg/mouse, intraperitoneal (IP), cat# BMS326, eBioscience, Grand Island, NY), followed by an IP injection of E.coli O111:B4 lipopolysaccharide (LPS) (300µg/kg, cat# L4391, Millipore Sigma, St. Louis, MO) 2 hours afterwards to induce MHC II expression in enteric glia. Animals were scored for sickness severity and weights were measured at injection, and prior to sacrifice. Scoring for sickness severity was adapted from Shrum et al. (66). Briefly, animals were scored from 0-4 on each of the following categories: appearance, level of consciousness, activity, response to stimulus, eyes appearance, respiration rate, and respiration quality. Animals that had a sickness severity score of 21 or greater or lost >15% of body weight were euthanized and not included in analysis. Animals were euthanized and colons harvested at stated timepoints following LPS injection.

Phagocytosis assay

Phagocytic activity was assessed in longitudinal muscle-myenteric plexus (LMMP) whole mount preparations in vitro. Colons were isolated from transgenic mice expressing tdTomato in glia (*Sox10$^{CreERT2+/-}$;tdTomato)* and whole mounts of myenteric plexus were prepared by removing the mucosa, submucosa, and circular muscle layers

by microdissection. Dissected tissues were incubated in Dulbecco's Modified Eagle Medium (DMEM) containing IFNγ (10ng/mL, cat# BMS326, eBioscience, Grand Island, NY), LPS (5µg/mL, cat# L4391, Millipore Sigma, St. Louis, MO), and AlexaFluor488 conjugated mouse anti-CD45.2 antibody (cat#109816, BioLegend, San Diego, CA) and phagocytic substrates. Substrates were green pHrodo conjugated E. coli BioParticles (1mg/mL, cat# P35366, Thermo Fisher Scientific, Waltham, MA) AlexaFluor 488 labeled LPS (cat#L23351, Thermo Fisher Scientific, Waltham, MA) or FluoSpheres (1.0µm, cat#F8803, Thermo Fisher Scientific, Waltham, MA) for 2 hours at 37^{o}C. pHrodo conjugated E. coli BioParticles fluoresce only in low pH environments such as within phagolysosomes. Fluorescence was visualized by confocal or epifluorescence and brightfield microscopy.

Whole-Mount Immunofluorescence

Colons were harvested from mice, fecal pellets flushed with phosphate buffered saline (PBS), and tissues fixed with Zamboni's fixative at 4^{o}C overnight. Fixed tissues were micro-dissected to remove the mucosa, submucosal plexus, and circular muscle layers. The remaining longitudinal muscle-myenteric plexus preparation were processed via immunofluorescence as described in prior work (24). Briefly, tissue preparations were washed three times at room temperature with Triton X-100 (0.1%, Sigma-Aldrich, St. Louis, MO) in PBS for 10 minutes each. Tissue were then incubated in blocking solution containing 4% normal goat serum, 0.4% Triton X-100, and 1% bovine serum albumin in PBS for 45 minutes at room temperature. Following blocking, samples were incubated with primary antibodies for 72 hours at 4^{o}C. Tissues underwent three 10-minute washes in PBS prior to a 2-hour incubation with secondary antibodies at room temperature.

Following two 10-minute washes in PBS, labeled tissues were washed once in 0.1M phosphate buffer for 10 minutes and mounted onto microscope slides. Antibodies and dilutions used are listed in Table 3.1. Labeled tissues were visualized by epifluorescence microscopy with a 40X objective (0.75 numerical aperture; Plan Fluor, Nikon, Melville, NY) on a Nikon Eclipse Ni upright microscope (Melville, NY) using a QImaging Retiga 2000R camera (Teledyne Photometrics, Tucson, AZ), or by confocal microscopy using an Olympus Fluoview FV1000 microscope (Olympus, Center Valley, PA) with a 60x Plan-Apochromat oil immersion objective (1.42 numerical aperture). Immunofluorescence was quantified using Fiji software (https://imagej.nih.gov/ij/). Control experiments were performed prior to immunofluorescent labeling of samples by omitting primary antibodies in labeling protocols in order to control for non-specific binding and autofluorescence.

H&E Staining

Colon tissues were fixed overnight in Zamboni's fixative at 4°C and washed with PBS. Tissues were embedded in paraffin and tissue sections (4-5µm thickness) were stained with hematoxylin and eosin by the MSU Investigative HistoPathology Laboratory.

Frozen section Immunofluorescence

Mesenteric lymph nodes and spleen were harvested from animals and fixed in Zamboni's fixative at 4°C overnight. Fixed samples were washed with PBS and incubated in a 30% sucrose solution in 0.1M phosphate buffer at 4°C for 72 hours. Samples were submitted to the Michigan State University Histology Core for cryo-sectioning. Microscope slides with 6µm thick tissue sections were first warmed to room

temperature, rehydrated in distilled water, and warmed to 60°C. Following a 10-minute incubation in PBS at room temperature, tissue sections were labeled in a similar fashion as described above. Briefly, slides were incubated in PBS for 10 minutes at room temperature followed by two incubations of Triton X-100 (0.1%, Sigma-Aldrich, St. Louis, MO) in PBS for 10 minutes at room temperature. Slides were incubated with blocking solution containing 4% normal goat serum, 0.4% Triton X-100, and 1% bovine serum albumin in PBS for 45 minutes at room temperature. Following blocking, samples were incubated with primary antibodies for 72 hours at 4°C in a humidified slide chamber. Slides underwent three 10-minute washes in PBS prior to a 2-hour incubation with secondary antibodies at room temperature. Following two 10-minute washes in PBS, labeled slides were washed once in 0.1M phosphate buffer for 10 minutes, and mounted onto microscope slides. Antibodies and dilutions used are listed in Table 3.1.

Flow cytometry

Mouse mesenteric lymph nodes were harvested, and cells were isolated by mechanical dissociation and kept in FACS buffer consisting of PBS with 1% bovine serum albumin. Cells were passed through a 40μm cell strainer (Corning Life Sciences, Tewksbury MA) and centrifuged at 350g for 5 minutes at 4°C. Supernatant was removed and the cell pellet is washed once and resuspended in 1mL of ice-cold FACS buffer. To label cell surface markers, cells were incubated with conjugated antibodies for 30min at 4°C in the dark. Labeled cells were washed once more in FACS buffer before analysis with a BD LSR II flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ). To label intracellular targets for flow cytometric analysis, mesenteric lymph node cells are first processed for cell surface labelling. Surface-labeled cells were fixed and

permeabilized with FoxP3 Fixation / Permeabilization buffer (eBioscience 00-5523-00, Grand Island, NY) overnight at 4°C in the dark. Fixed cells were washed with permeabilization buffer prior to incubation with intracellular-specific antibodies for 30min at 4°C in the dark. Cells were washed once more with permeabilization buffer prior to resuspension in ice-cold FACS buffer and analyzed with a BD LSR II flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ). Flow cytometry data was analyzed with FCS Express 7 Research Edition (De Novo Software, Pasadena, CA). Antibodies and dilutions used are listed in Table 3.1. We eliminated doublets from flow cytometric analysis by plotting cells of interest on a forward scatter area vs height plot and gating for singlets. Resultant cells were plotted on a side scatter width vs height plot to ensure elimination of doublets. To properly control flow cytometric analysis of mesenteric lymph nodes, we incorporated unstained samples consisting of mesenteric lymph node cells incubated with FACS buffer without labeled antibodies to determine autofluorescence of mesenteric lymph node cells in each channel. Single stained controls consisted of UltraComp eBeads (Thermo Fisher Scientific, Waltham, WA) incubated with a single labeled antibody of each antigen of interest to calculate fluorescent compensation between each fluorophore. To identify the boundary between positive and negative fluorescence for each antigen of interest, we incorporated a panel of fluorescence minus one controls by labeling mesenteric lymph node cells with all fluorophores except the one being measured.

Gene expression

Sox10-RiboTag mice were used to quantify gene expression levels in enteric glial cells. Mice were treated with a 400mg/kg tamoxifen citrate diet for 2 weeks to induce glial-

specific ribosomal HA-tag expression. Colons were harvested, fecal pellets flushed with PBS, and tissue snap frozen on dry ice and stored at -80^{o}C. The RNA extraction protocol was performed as described by Delvalle et al. with minor modifications (16). Briefly, homogenization buffer was made by mixing 7.38mL RNAse-free water with 1mL Nonident P-40 (cat#: 21-3277, Millipore Sigma, St. Louis, MO), 1mL of 1M potassium chloride, 500µL of pH 7.4 Tris 1.0, 120µL of 1M magnesium chloride, and 100mg of sodium deoxycholate. Frozen colon tissues were placed in 1mL of ice-cold supplemented homogenization buffer containing 949µL homogenization buffer, 40µL cycloheximide (Millipore Sigma, St. Louis, MO), 40µL protease inhibitor (cat#: P8340, Millipore Sigma, St. Louis, MO), 20µL RNAse inhibitor (cat#: AM2684, Thermo Fisher Scientific, Waltham, WA), 2µL dithiothreitol (cat#: 646563, Millipore Sigma, St. Louis, MO), and 3mg/mL heparin (cat#: H3149, Millipore Sigma, St. Louis, MO). Frozen tissues were homogenized using a GentleMACS Dissociator (Miltenyi Biotech, Auburn, CA) in M-tubes running the program RNA_02. Tissue homogenates were centrifuged at 10,000g at 4^{o}C for 10min, supernatants collected, and incubated with 5µL of monoclonal anti-hemagglutinin (HA) antibody (16B12, BioLegend, San Diego, CA) at 4^{o}C for 4 hours on a tube rotator. During antibody incubation, magnetic protein A/G beads (Thermo Fisher Scientific, Waltham, WA) were washed twice in a magnet with 500µL homogenization buffer. HA-labeled tissue homogenate supernatants were then incubated with the washed magnetic beads on a tube rotator at 4^{o}C overnight. Following incubation, samples were washed three times in a magnet with 800µL of high-salt buffer. 350µL of RLT lysis buffer (Qiagen, Germantown, MD) were added to samples, and RNA released from magnetic beads by vortexing for 30 seconds. RNA was

collected by placing sample on a magnet and collecting the supernatant. Total RNA was purified using a RNeasy Micro kit (Qiagen, Germantown, MD) according to manufacturer's instructions. Purified RNA was flash frozen and submitted to the UCLA Center for Systems Biomedicine core facility (UCLA, Los Angeles, CA) for analysis via a custom NanoString nCounter multiplex panel (NanoString, Seattle, WA). Gene expression levels were quantified by based on counts of fluorescent barcodes specific to individual genes.

Cytokine quantification

Serum and homogenized colon tissue samples were processed for cytokine quantification as described in prior work by Hernandez et al. with minor modifications (27). Briefly, serum samples were prepared by first harvesting whole blood from mice by cardiac puncture and allowed to clot by incubating at room temperature undisturbed for 15min in a covered test tube. Tubes were then centrifuged at 1500g at 4^{o}C for 10 minutes. Serum was collected from the supernatant, diluted 2-fold with PBS to a final volume of 80µL, frozen at -80^{o}C, and submitted to Eve Technologies (Calgary, AB, Canada) on dry ice for analysis. For tissue cytokine analysis, colon tissues were collected, and fecal pellets flushed prior to snap freezing on dry ice and storage at -80^{o}C. Frozen tissue samples are weighed and placed in 10% w/v of tris-buffered saline containing 0.05% tween-20, 25mM Tris-HCl, 130mM sodium chloride, 2.7mM potassium chloride, and SIGMAFAST protease inhibitor cocktail (1 tablet, cat#: S8820, Millipore Sigma, St. Louis, MO). Tissues are homogenized using a GentleMACS Dissociator (Miltenyi Biotech, Auburn, CA) in M tubes running the program Protein_01. Tissue homogenates were centrifuged at 4,000g at 4^{o}C for 5min, and supernatants collected.

Supernatants were diluted with homogenization buffer to a protein concentration of 4mg/mL in a final volume of 75µL. Samples were frozen at -80°C prior to submission to Eve Technologies (Calgary, AB, Canada) for analysis via the mouse 31-plex multiplex cytokine array.

Autophagy induction and detection

Live whole mount preparations of myenteric plexus were prepared as described above and in prior work (24). Autophagy was induced by incubating samples with DMEM containing Rapamycin (50nM,cat#13346, Cayman Chemical, Ann Arbor, MI) for 16 hours at 37°C. Autophagy was inhibited by incubating samples with DMEM containing 3-MA (5mM, cat#13242, Cayman Chemical, Ann Arbor, MI) for 16 hours at 37°C. Autophagy was detected in enteric neurons and glia by measuring P62 immunofluorescence and LC3B puncta per cellular area. LC3B puncta was detected using FIJI by first thresholding the LC3B channel using the auto setting of the Intermodes algorithm in the 'Threshold' function of FIJI. LC3B puncta in enteric glia and neurons was measured in FIJI using the 'Analyze Particles' function, setting particle size of interest from 0 to Infinity and circularity from 0 to 1.

Data Analysis

Confocal immunofluorescent z-stack images were imported to FIJI software (https://imagej.nih.gove/ij/) as a Hyperstack using the Bio-Formats plugin (Open Microscopy Environment, University of Dundee, UK). To measure fluorescent intensity of a specific antigen within enteric glia or neurons, we first created a region of interest outlining GFAP-positive cells in each ganglion to isolate enteric glial expression. The

GFAP-positive region of interest was constructed in FIJI using the auto setting of the Otsu algorithm in the ‘Threshold’ function of FIJI. The resultant selection was converted into a region of interest in FIJI using the ‘Create Selection’ function. Total ganglionic expression of a given labeled antigen was measured by creating a ganglionic region of interest by utilizing the GFAP channel to outline the total ganglionic area. Glial fluorescent intensity or particle number was subtracted from total fluorescent intensity or particle number within the ganglion to calculate levels within enteric neurons. To calculate Mander’s overlap coefficient between two fluorophores, we utilized the FIJI plugin ‘Just Another Colocalisation Plugin’ (6), and adjusted thresholding parameters to include GFAP+ glial processes without incorporating background noise, or MHC II and CD74 signal without incorporating background noise. Additionally, using Metamorph software, we performed line-scans across enteric ganglion confocal z-projection images. Confocal z-stack images were processed by first converting z-stacks into maximum projection images. Background subtraction was applied in each channel to remove noise. Maximum gray values of labeled antigens were measured along the length of a line drawn within enteric ganglia to visualize co-presence of fluorophores. We used the 3D Viewer plugin in FIJI to create a 3-dimensional reconstruction of GFAP-positive ganglia and MHC II expression. 3-dimensional reconstruction is displayed as a volume with a resampling factor of 2. We analyzed flow cytometric data using FCS Express 7 Research Edition (De Novo Software, Pasadena, CA) and determined gating cutoffs for CD4, CD69, IL4, IL17, IFNγ, and FoxP3 markers using compensation and fluorescence minus one controls as described above. We determined the activated fraction of each T-lymphocyte subtype by calculating the proportion of T-lymphocyte

subtype marker and CD69 double positive cells within the total number of subtype marker positive cells. We used IFNγ as a marker for Th_1, IL4 for Th_2, IL17 for Th_{17}, and FoxP3 for T_{reg} subtypes. Nanostring gene expression data was processed by nSolver v4.0 software (NanoString Technologies, Seattle, WA). Data was curated to remove entries flagged by nSolver quality control or entries that have fewer reads than negative controls. Data is represented as fold change compared to the mean number of reads in saline control condition. For cytokine quantification, a four-parameter logistic regression was utilized to calculate the concentration of each analyte, and samples below or above the logistic regression were designated as 0 or the highest standard curve value, respectively.

Statistics

All data are represented as mean ± SEM and were analyzed by GraphPad Prism 7 (GraphPad Software, San Diego, CA). Immunofluorescence, flow cytometry, and quantified cytokine data were compared between genotype and treatment conditions using unpaired two-way ANOVA with Tukey-corrected multiple comparisons. Gene expression data retrieved from RNAseq databases are expressed as transcripts per million (tpm) and were compared by one-way ANOVA with Tukey-corrected multiple comparisons. A level of $P < .05$ was set as the cutoff for statistical significance. *P* values are denoted as follows: < .05 (*), < .005 (**), < .0005 (***), <.0001 (****). Outliers were detected in GraphPad Prism 7 using the ROUT method with Q=1%.

Antigen Target	Host Species	Dilution	Conjugate	Manufacturer	Product no.
GFAP	Chicken	1:1000	n/a (primary Ab)	Abcam	ab4674
MHC II	Rat	1:200	n/a (primary Ab)	Novus	NBP2-21789
CD74	Rabbit	1:200	n/a (primary Ab)	Bioss	BS-2518R
P62	Rabbit	1:200	n/a (primary Ab)	Abcam	ab109012
LC3B	Rabbit	1:200	n/a (primary Ab)	Abcam	ab192890
Anti-chicken IgY	Goat	1:400	Dylight405	Jackson	103-475-155
Anti-rat IgG	Goat	1:400	AlexaFluor 594	Jackson	112-585-003
Anti-rabbit IgG	Goat	1:400	AlexaFluor 488	Invitrogen	A-11034
CD45.2	Mouse	1:200	AlexaFluor488	BioLegend	109816

Table 3.1: Antibodies used in Chapter 3.

Table 3.1 (cont'd)

CD4	Rat	1:200	FITC	BioLegend	100509
CD8	Rat	1:200	APC/Cy7	BioLegend	100714
CD69	Armenian Hamster	1:200	PE/Cy7	BioLegend	104512
IFNg	Rat	1:100	PE	BioLegend	505808
IL-4	Rat	1:100	APC	BioLegend	504105
IL-17	Rat	1:20	Brilliant Violet 510	BioLegend	506933
FoxP3	Rat	1:100	Pacific Blue	BioLegend	126410

Results

Conditional ablation of glial MHC II in Sox10^{CreERT2};IAB$^{fl/fl}$ mice and IFNγ + LPS challenge

Enteric glia express MHC II in response to proinflammatory stimuli such as LPS and IFNγ (Fig.3.1a, d-f), similar to findings by Turco et al. (70). These stimuli also elicit antigen presentation by professional immune cells which can mask the contribution of glia. Therefore, we used a minimal pro-inflammatory stimulus (IFNγ 1µg / mouse; LPS 0.3mg/kg; i.p.) to study glial effects *in vivo* without an overwhelming inflammatory response from other immune cells (Fig.3.1c). This challenge is sufficient to induce glial MHC II (Fig.3.1d-f) and causes minor weight loss (Fig.3.2a) but does not increase sickness behavior (Fig.3.2b), alter tissue architecture (Fig.3.2c), or cause significant histological damage (Fig.3.2d).

Murine MHC II molecules are composed of gene products from the I-A or I-E loci. C57BL/6 mice have a H-2b MHC haplotype due to a deficient I-E loci and rely solely on the I-A loci for MHC II expression (29, 30, 39). We isolated the specific role of enteric glia by creating a tamoxifen-inducible, glial-specific MHC II knockout (KO) mouse model (*Sox10^{CreERT2};IAB$^{fl/fl}$*) where the IAB gene, a crucial component in the MHC II I-A loci, is deleted in Sox10+ enteric glia (Fig.3.1b,c).

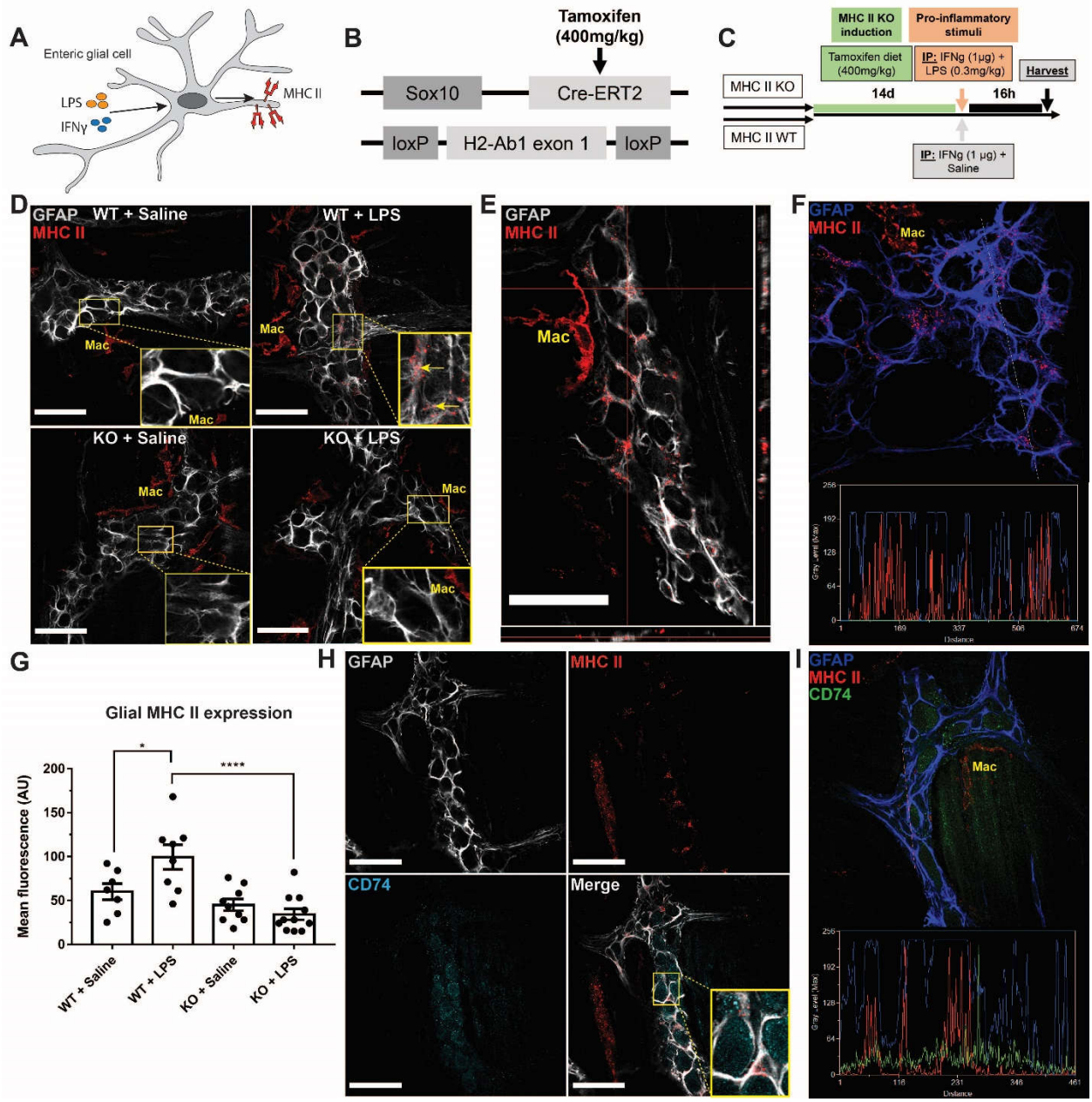

Figure 3.1: Enteric glial cells express MHC II when stimulated with IFNγ and LPS. **(A)** Schematic depicting enteric glial expressing MHC II molecules as a response to exposure to 1μg of IFNγ and 0.3mg/kg LPS. **(B)** Schematic depicting gene targeting strategy in *Sox10*^{CreERT2}*;IAB*$^{fl/fl}$ mice. Tamoxifen-sensitive CreERT2 expression is confined to glia by the Sox10 promoter and excises the floxed H2-Ab1 exon 1 loci. **(C)** Schematic depicting exposure protocol. Wildtype and MHC II KO mice are fed

Figure 3.1 (cont'd):

400mg/kg tamoxifen in chow for 14 days to induce Cre recombinase and MHC II ablation. Animals are injected via IP with pro-inflammatory stimuli consisting of 1µg of IFNγ followed by 0.3mg/kg LPS 2 hours afterwards or 1µg of IFNγ and volume-matched saline as controls. Tissues were harvested 16 hours post-injection. **(D)** Representative confocal images of GFAP (grey) and MHC II (red) immunofluorescence labeling in enteric ganglia. Enteric glial cells express MHC II when stimulated with IFNγ and LPS. Muscularis macrophages (Mac) bordering enteric ganglia show constitutive MHC II expression in all treatment conditions (scale bar: 50µm for all images). **(E)** Orthogonal views of confocal z-stacks confirm MHC II (red) labeling in GFAP-expressing (grey) enteric glia (scale bar: 50µm). **(F)** Line-scans through enteric ganglia demonstrate co-expression of MHC II (red) and GFAP (blue) labeling. **(G)** Quantified mean fluorescent intensity of MHC II labeling on GFAP-expressing enteric glia in the myenteric plexus (n= 7-11 mice; 2-way analysis of variance with Tukey-corrected multiple comparisons; *$P \leq .05$, ****$P \leq .0001$). **(H)** Representative confocal images of GFAP (grey), MHC II (red) and CD74 (cyan) immunolabeling in wildtype animals treated with IFNγ and LPS show a neuronal and glial distribution of CD74 (scale bar: 50µm for all images). **(I)** Line-scans through enteric ganglia show minimal overlap between MHC II (red) and CD74 (green) in GFAP-expressing (blue) enteric glia.

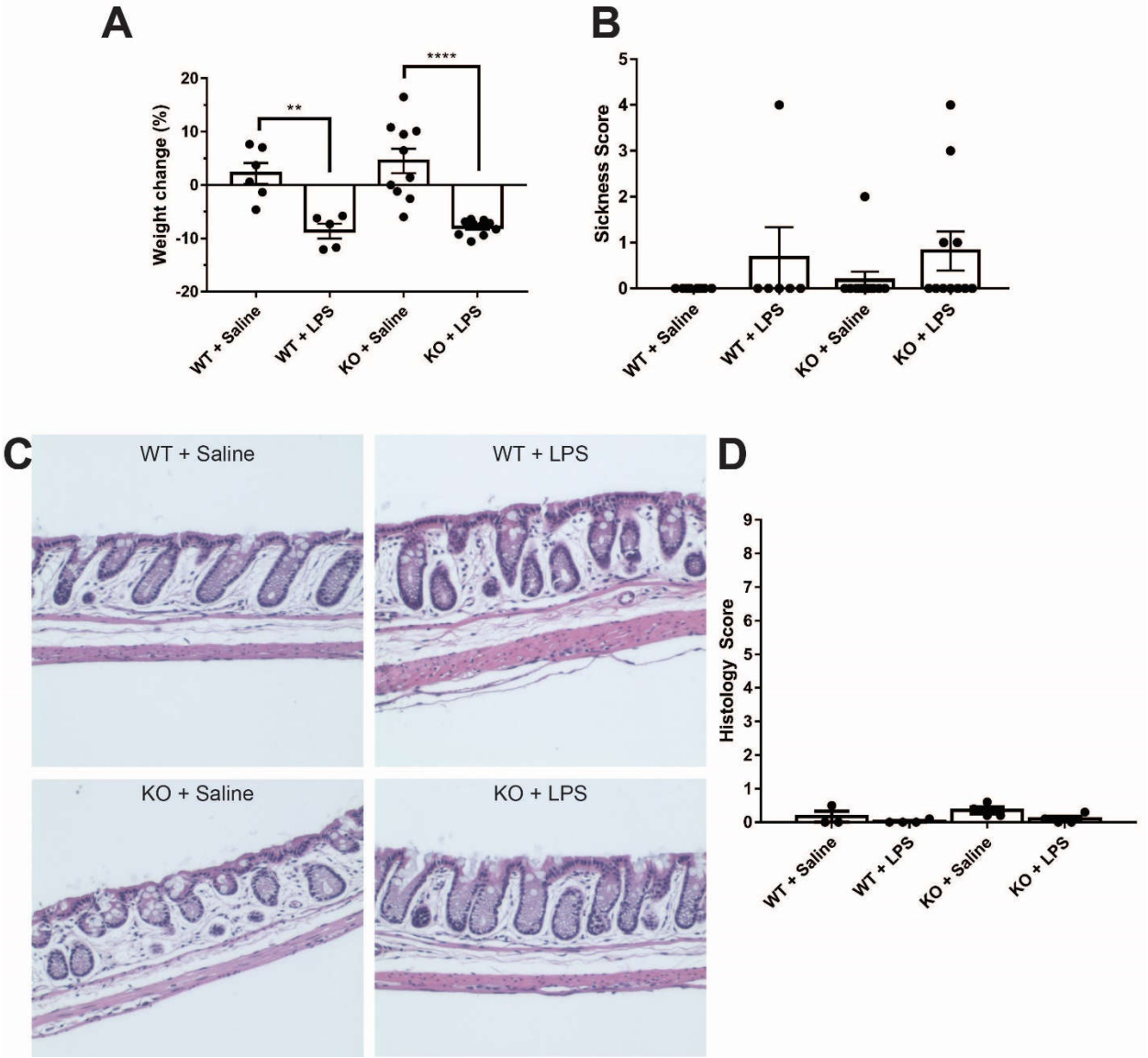

Figure 3.2: Exposure to IFNγ and LPS causes weight loss but does not cause significant tissue damage or observable inflammation. (A) Animals show weight loss when treated with IFNγ + LPS in wildtype (P = .0054) and knockout (P < .0001) animals (n=5-10 mice; 2-way analysis of variance with Tukey-corrected multiple comparisons) **(B)** Sickness scores were not significantly different between IFNγ + LPS challenged animals compared to controls. **(C)** No tissue architecture changes were

Figure 3.2 (cont'd):

observed in any experimental group. **(D)** No elevation in tissue histology scores was present in any condition.

Enteric glia express mature MHC II in response to IFNγ + LPS

Enteric glia increased MHC II expression in animals challenged with IFNγ and LPS despite no overt inflammation or tissue architecture changes (Fig.3.1d; Fig.3.2). Within enteric ganglia, MHC II expression is limited to glia and no neuronal expression was observed (Fig.3.1d). Confocal image analyses confirm that MHC II primarily colocalizes with GFAP-labeled enteric glia in enteric ganglia (Mander's overlap coefficient of 90%; Fig.3.1e-f). Treatment with IFNγ alone was not sufficient to induce glial MHC II expression (Fig.3.1d). Quantification of MHC II immunolabeling shows that enteric glia significantly (P = .0370) upregulate MHC II expression in response to IFNγ and LPS in wildtype animals, and that this effect is absent in animals that lack glial MHC II ($Sox10^{CreERT2};IAB^{fl/fl}$) (Fig.3.1g). Muscularis macrophages at the level of the myenteric plexus demonstrate constitutive expression of MHC II (54), and exposure to IFNγ and LPS stimuli did not alter expression level or pattern in wildtype or glial MHC II KO animals (Fig.3.3a,b). These observations are consistent with prior data showing that LPS stimulates enteric glial MHC II expression through TLR4 receptor pathways (62, 70) and that enteric neurons lack expression of TLR4 and MHC II (19). Importantly, these results show that the MHC II KO mouse model effectively ablates MHC II expression specifically in enteric glia and not within immune cells, thereby isolating any changes in immune activation to enteric glia.

In antigen presenting cells, immature MHC II molecules contain CD74 bound within their peptide-binding groove until fusion with phagolysosomes when the CD74 dissociates to allow loading of processed peptides (58, 60). We tested whether the MHC II molecules expressed by enteric glia are mature by labeling for CD74. CD74

immunoreactivity was present in both enteric neurons and glia (Fig.3.1h) and this is consistent with the pattern of CD74 gene expression in enteric neurons and glia (ADD REFS for databases here). However, no co-labeling of enteric glial MHC II and CD74 was detected (Fig.3.1h). Furthermore, line-scans through enteric ganglia show little colocalization between CD74 and MHC II (Mander's overlap coefficient of 2%; Fig.3.1i). Together, these results show that enteric glia are sensitive to pro-inflammatory stimuli and respond to low doses of IFNγ and LPS by expressing mature MHC II molecules.

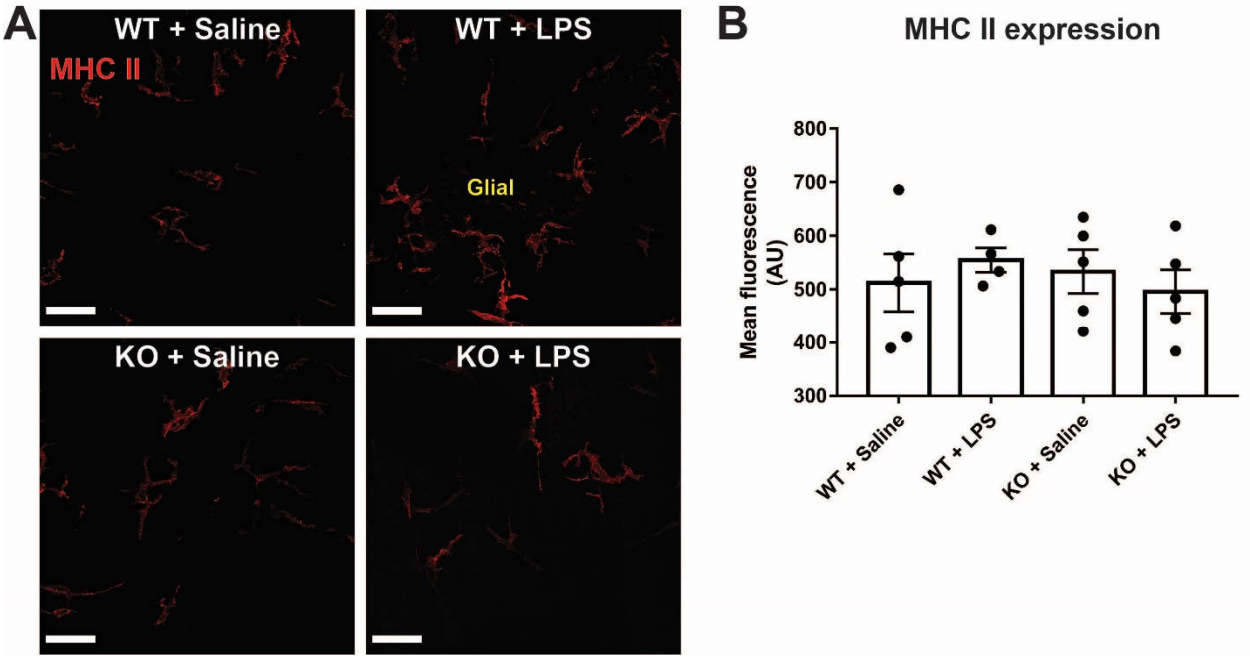

Figure 3.3: Pattern and level of MHC II expression in macrophages does not change when treated with IFNγ and LPS. (A) Representative confocal images of MHC II (red) immunofluorescent labeling in enteric ganglion (scale bar: 50μm for all images). **(B)** Quantified mean fluorescent intensity of MHC II labeling in muscularis macrophages bordering enteric ganglion (n = 4-5 mice; 2-way analysis of variance with Tukey-corrected multiple comparisons).

Enteric glial MHC II expression contributes to CD4+ T-cell activation in mesenteric lymph nodes

MHC II molecules expressed by professional antigen presenting cells play an important role in interactions that activate T-lymphocytes. Therefore, we tested whether glial MHC II molecules are functionally relevant in antigen presentation to T-lymphocytes. We began by using immunolabeling to examine whether enteric glia and T-lymphocytes are within sufficient proximity for potential interactions. In support, CD4 + T-lymphocytes were observed in close proximity to enteric glia at the level of the myenteric plexus (Fig.3.4). Next, we tested whether glial MHC II mediates functional interactions with T-lymphocytes that influence their activation state by measuring CD69 expression in T-lymphocytes in control and glial MHC II KO animals. The majority of CD4+ cells are in an inactive state in wildtype control animals and only 38% of CD4+ cells expressed the acute activation marker CD69 (Fig.3.5b,c). Exposure to IFNγ and LPS expanded the population of activated CD4+ cells by 29% and increased the percentage of CD4+ cells expressing CD69 to 67% (P = .0010). Ablating glial MHC II significantly (P = .0309) blunted the effect of IFNγ and LPS and decreased the proportion of CD4+ cells expressing CD69 to 46%, which is similar to the proportion of active CD4+ cells observed in saline treated control animals (Fig.3.5c). Glial MHC II ablation had no effect on the percentage of activated CD4+ cells in saline treated control animals (38%)(Fig.3.5b,c), and this is expected given that glial MHC II expression is only observed in animals challenged with LPS (Fig.3.1) . Enteric glial MHC II expression also affected CD8+ T-lymphocyte activation in mesenteric lymph nodes (Fig.3.6a). In healthy wildtype control animals, 61% of CD8+ T-lymphocytes

expressed CD69 and this proportion significantly increased to 87% in wildtype animals challenged with IFNγ and LPS (P = .0132) (Fig.3.6b). However, the IFNγ and LPS challenge did not affect CD8+ T-lymphocyte activation in KO animals (Fig.3.6b). Together, these results show that enteric glial antigen presentation mediated by MHC II plays an important role in regulating immune activity through effects on CD4+ and CD8+ T-lymphocytes.

Since enteric glial MHC II ablation influences the activation state of CD4+ T-lymphocytes, we examined whether a specific T-lymphocyte subtype is preferentially affected by glial MHC II. The baseline activated proportions of T-lymphocyte subtypes expressing CD69 in wildtype control animals were 66% of IFNγ+ cells, 48% of IL4+ cells, 46% of IL17+ cells, and 43% of FoxP3+ cells (Fig.3.5d, Fig.3.7). Challenge with IFNγ and LPS in wildtype animals did not change the activated portion of IFNγ+ or IL4+ cells, but significantly elevated the activated portions of IL17+ (P = .0025) and FoxP3+ (P = .0121) cells to 83% and 77%, respectively (Fig.3.5d, Fig.3.7). Glial MHC II ablation in stimulated animals did not significantly alter the activated portions of IFNγ+ or IL4+ cells, but significantly decreased the activated portions of IL17+ (P = .0021) and FoxP3+ (P = .0057) cells to levels comparable to healthy wildtype controls. Glial MHC II ablation in control animals had no effect on T-lymphocyte subtype activation. Taken together, these results show that enteric glial MHC II primarily affects the activation state of T_h17 and T_{reg} subtypes within the CD4+ population of T-lymphocytes in mesenteric lymph nodes.

Given the major effect of glial MHC II ablation on T-lymphocyte activation in mesenteric lymph nodes, we wondered if local Sox10+ cells might exist within

mesenteric lymph nodes that would be susceptible to MHC II ablation and mediate the observed effects. We assessed this possibility by using *Sox10$^{CreERT2+/-}$;tdTomato* reporter mice to screen for potential Sox10+ cells in the mesenteric lymph nodes. We did not detect any tdTomato-expressing Sox10+ cells within mesenteric lymph nodes in either resting conditions or when stimulated with IFNγ and LPS (data not shown). The absence of Sox10+ cells in mesenteric lymph nodes suggests that changes in T-lymphocyte activation state are due to changes in enteric glial MHC II expression in the intestine and not other cell types residing in the mesenteric lymph node.

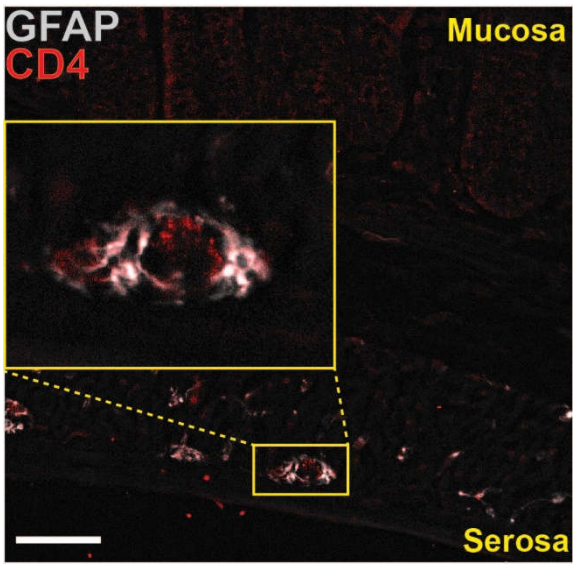

Figure 3.4: CD4 expressing T-lymphocytes are in close proximity with GFAP expressing enteric glia in the myenteric plexus. Representative image of GFAP (grey) and CD4 (red) immunofluorescent labeling in a colon cross section. Enlarged image emphasizes the presence of CD4+ T-lymphocytes within the myenteric plexus. The top region of image represents mucosal surface and bottom represents serosal surface (scale bar: 50μm)

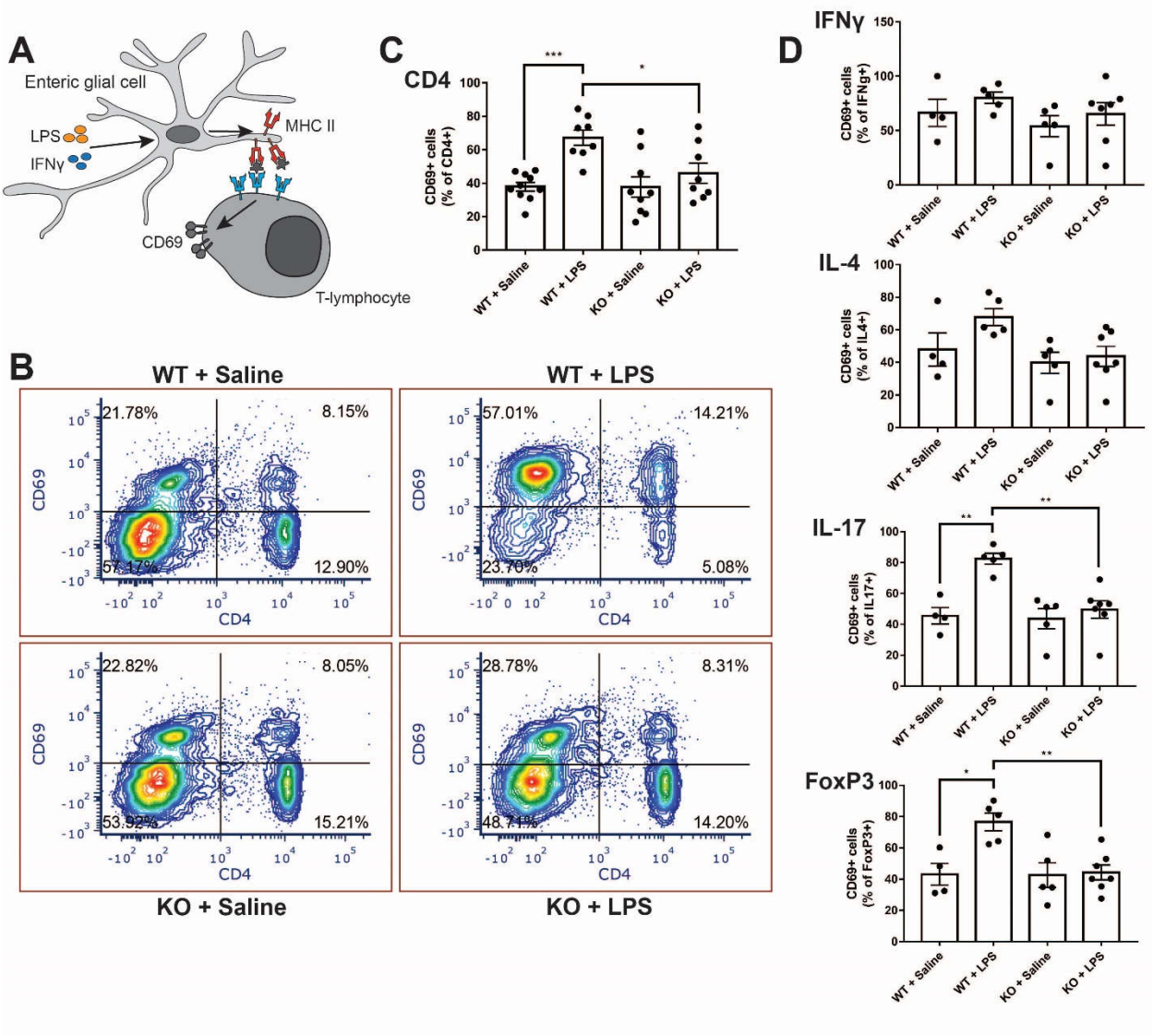

Figure 3.5: Enteric glial MHC II expression affects T-lymphocyte activation. (A) Schematic depicting enteric glial cells expressing MHC II when stimulated with IFNγ and LPS. MHC II molecules interact with T-lymphocytes and cause changes in activation state as measured by CD69 expression. **(B)** Representative flow cytometric contour plots of mesenteric lymph nodes labeled with CD4 and CD69. Proportion of total gated cells in each quadrant expressed as percentage. **(C)** Levels of activated CD4+ cells quantified as a percentage of total CD4+ cells that are CD4+CD69+ (n=8-10 mice; 2-

Figure 3.5 (cont'd):

way analysis of variance with Tukey-corrected multiple comparisons; *$P \leq .05$, ***$P \leq .001$). **(D)** Levels of activated IFNγ+, IL-4+, IL-17+, or FoxP3+ cells quantified as a percentage of total subtype-marker expressing cells that are also expressing CD69 (n=4-7 mice; 2-way analysis of variance with Tukey-corrected multiple comparisons; *$P \leq .05$, **$P \leq .005$).

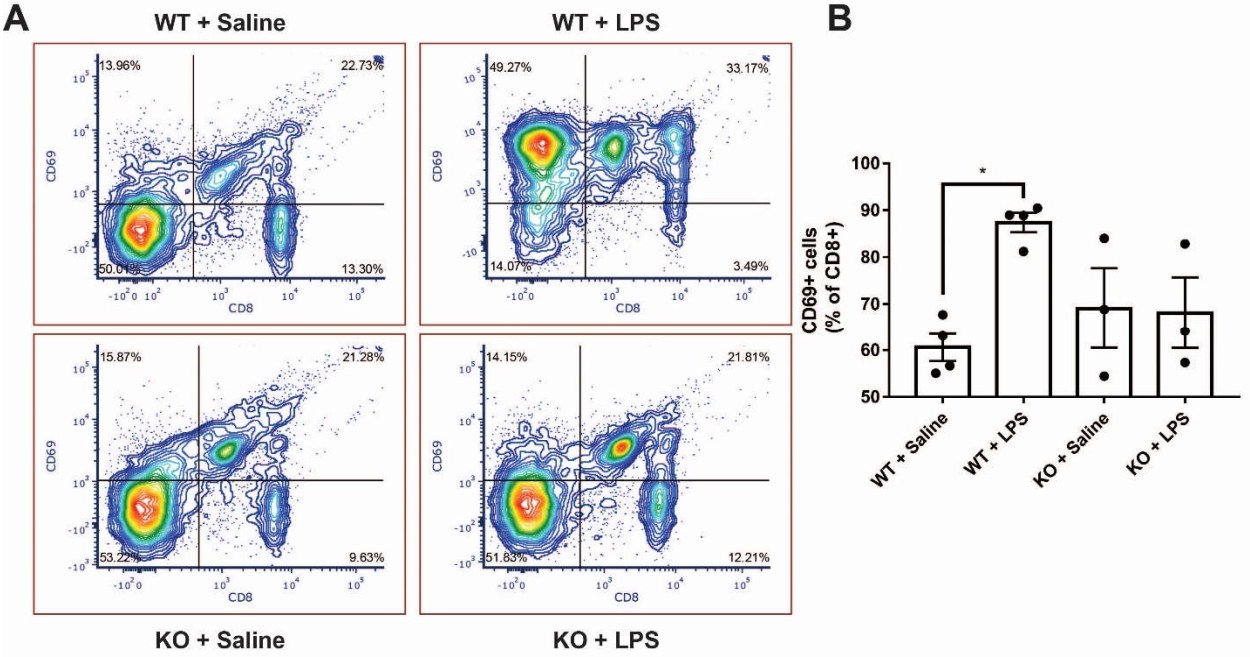

Figure 3.6: Activation of CD8+ T-lymphocytes decreased when MHC II ablated in enteric glia. (A) Representative contour plots of mesenteric lymph nodes labeled with CD8 and CD69. Proportion of gated cells in each quadrant expressed as percentage. **(B)** Proportion of total CD8+ T-lymphocytes expressing CD69 quantified in each treatment condition (n=3-4 mice; 2-way analysis of variance with Tukey-corrected multiple comparisons; * $P \leq .05$).

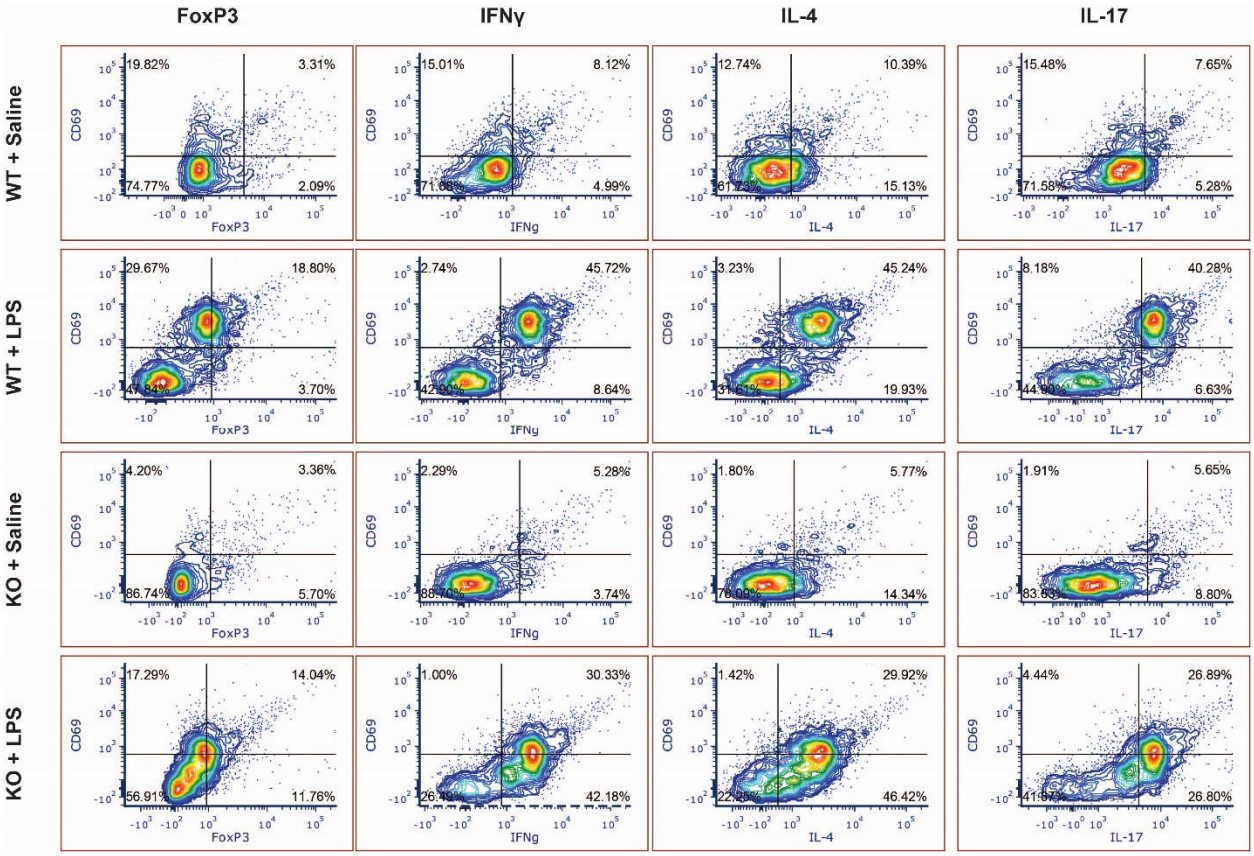

Figure 3.7: Enteric glial MHC II expression affect FoxP3+ and IL-17+ cell populations to a greater degree than IFNγ+ and IL-4+ cell populations.

Representative contour plots of mesenteric lymph nodes labeled with FoxP3, IFNγ, IL-4, IL-17, and CD69. Proportion of total gated cells in each quadrant expressed as percentage.

Enteric glia affect local, but not serum or colonic cytokine levels

Enteric glia express pro-inflammatory cytokines when stimulated with IFNγ and LPS *in vitro* (45). We tested whether IFNγ and LPS stimulation in wildtype animals *in vivo* causes enteric glial gene expression of select cytokines. Gene expression in majority of cytokines examined were not significantly affected by IFNγ and LPS challenge. However, a significant (P = .0001) increase in IL-1 gene expression and a trend towards increased tumor necrosis factor (P = .0595) was observed (Fig.3.8). Our data suggests that enteric glia respond to *in vivo* IFNγ and LPS challenge by altering gene expression to select cytokines and may alter cytokine levels in the local microenvironment.

Activation of T-lymphocytes during inflammation has profound effects on tissue and serum cytokine levels (64, 82). Since enteric glial MHC II expression alters T-lymphocyte activation, we examined whether enteric glial MHC II expression alters cytokine levels as a downstream effect. The majority of molecules examined in a panel of 31 cytokines and chemokines were not significantly affected by the IFNγ and LPS stimulus (Fig.3.9b,c). However, significant increases were observed in colonic tissue levels of leukemia inhibitory factor and CCL5 (Fig.3.10), and serum levels of granulocyte colony stimulating factor, macrophage colony stimulating factor, IL-6, CXCL-1, and CCL4 (Fig.3.11). Ablating enteric glial MHC II had little effect on colonic tissue and serum cytokine levels and expression in MHC II KO animals did not differ significantly from wildtype animals (Fig.3.10, 3.11). Therefore, while glial antigen presentation does affect the activation profiles of immune cells in mesenteric lymph nodes, serum and regional cytokine profiles are mainly driven by other immune cells in this model.

It is possible that the lack of effects on serum cytokines in *Sox10^{CreERT2};IAB$^{fl/fl}$* mice is complicated by also ablating MHC II in Sox10+ glia in the spleen (73). In agreement with prior work, we observed that Sox10+ spleen glia do express MHC II (Fig.3.12). However, Sox10 expressing cells in the spleen were rare and their calculated density is only 1.5 cells / mm^2 in splenic tissue sections (data not shown). Therefore, it is unlikely that MHC II ablation in Sox10+ cells of the spleen has a major impact that would confound our cytokine measurements in the serum.

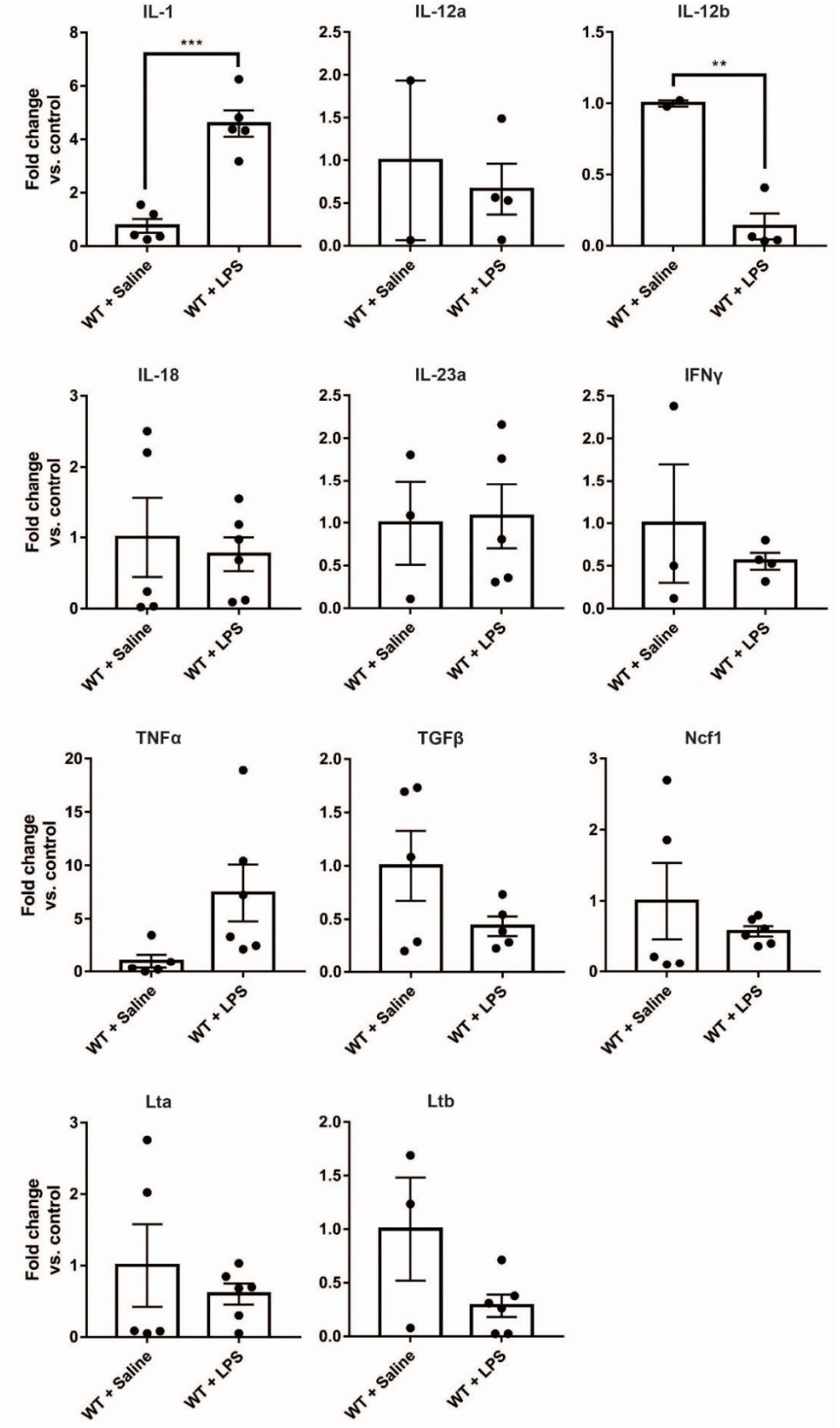

Figure 3.8: Enteric glial gene expression. Gene expression of select cytokines /

Figure 3.8 (cont'd):

chemokines in RiboTag animals stimulated with IFNγ + LPS or controls (n = 2-6 mice; unpaired student's t-test; **P ≤ .005, ***P ≤ .0005).

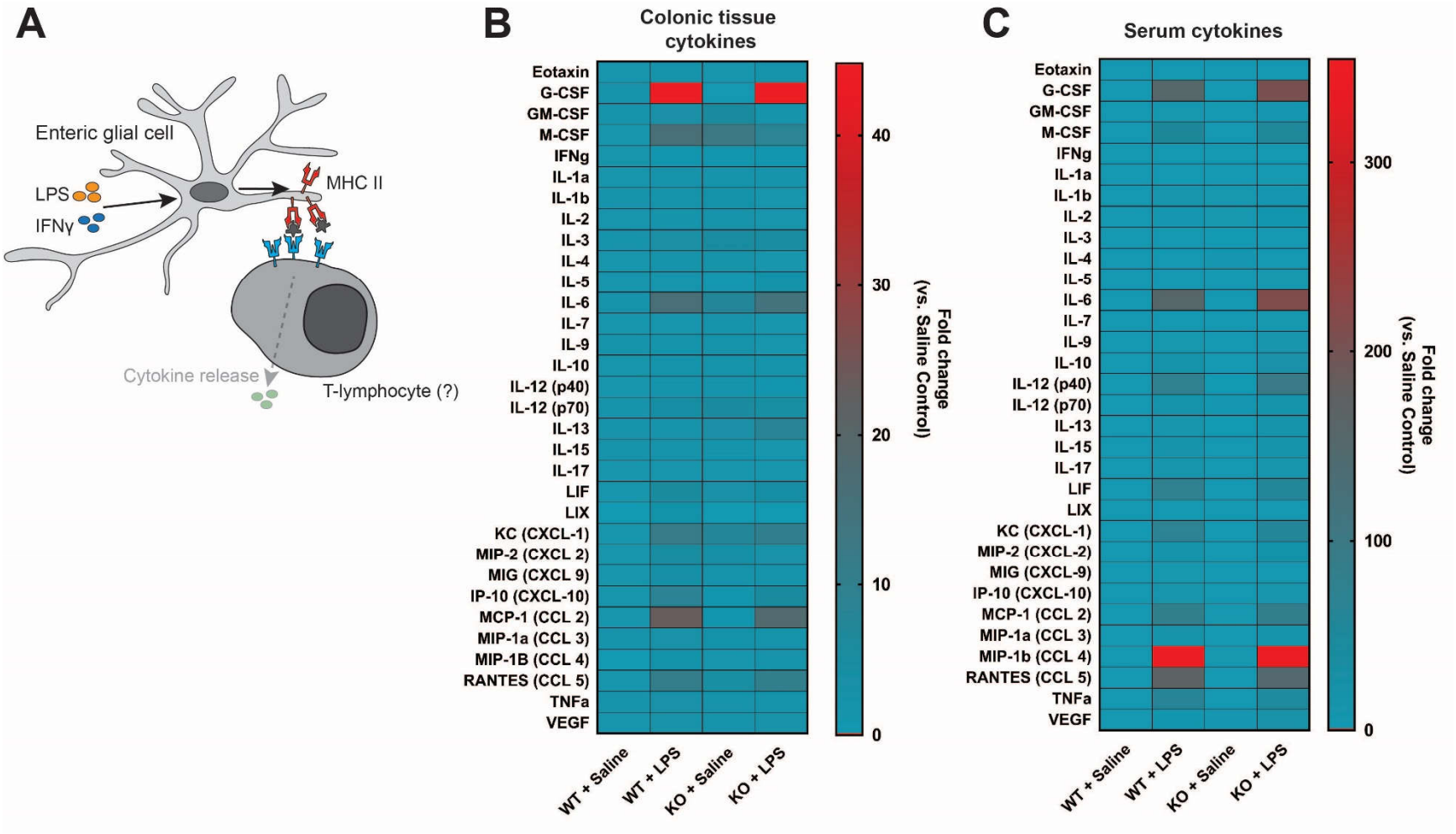

Figure 3.9: Enteric glial MHC II expression has limited effects on serum and colonic tissue cytokine levels. (A) Schematic depicting potential source of cytokines release from T-lymphocytes after activation via binding enteric glial MHC II. **(B)** Heat map depicting fold change compared to saline controls in quantified cytokines and chemokines in

Figure 3.9 (cont'd):

colonic tissue homogenates of male mice (n=4-10 mice). **(C)** Heat map depicting fold change compared to saline controls in quantified cytokines and chemokines in serum samples of male mice (n=4-8 mice).

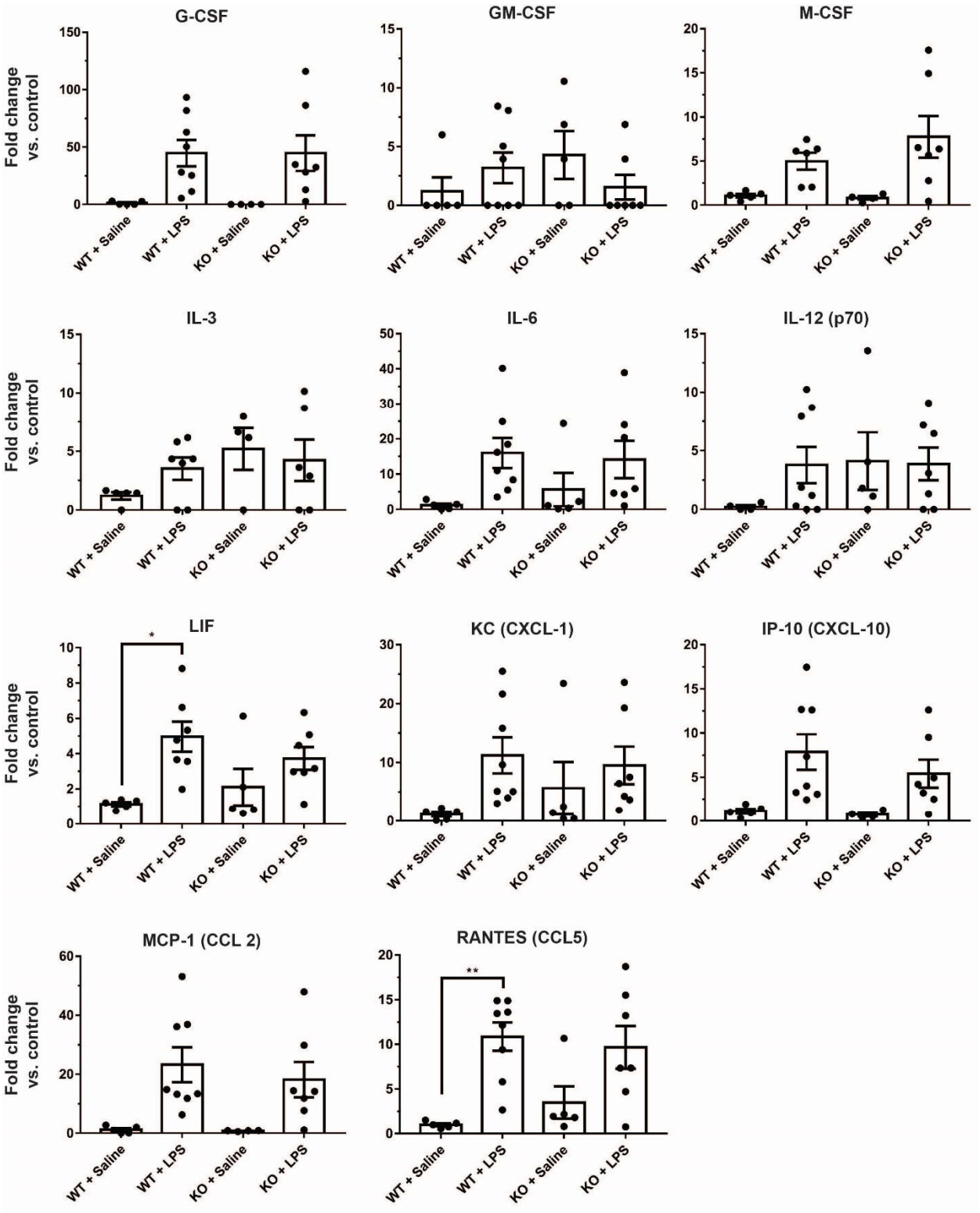

Figure 3.10: MHC II ablation in enteric glial cells do not show changes in colonic tissue level of select cytokines. Enteric glial MHC II KO animals challenged with IFNγ

Figure 3.10 (cont'd):

+ LPS do not display significantly lower levels of regional colonic cytokines compared to wildtype counterparts challenged with IFNγ + LPS.

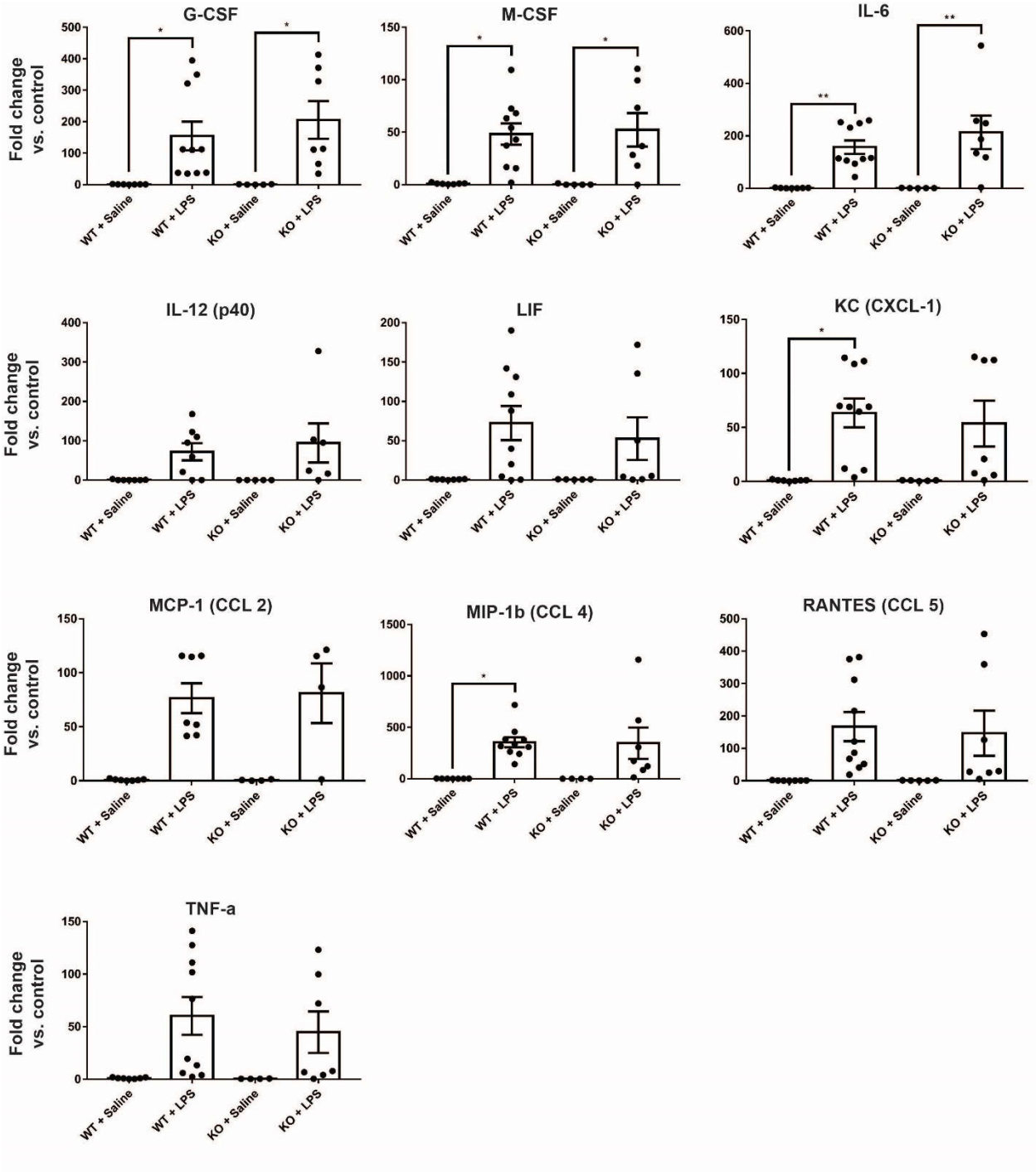

Figure 3.11: MHC II ablation in enteric glial cells do not show changes in serum level of select cytokines. Enteric glial MHC II KO animals challenged with IFNγ + LPS

Figure 3.11 (cont'd):

do not display significantly lower levels of serum cytokines compared to wildtype counterparts challenged with IFNγ + LPS.

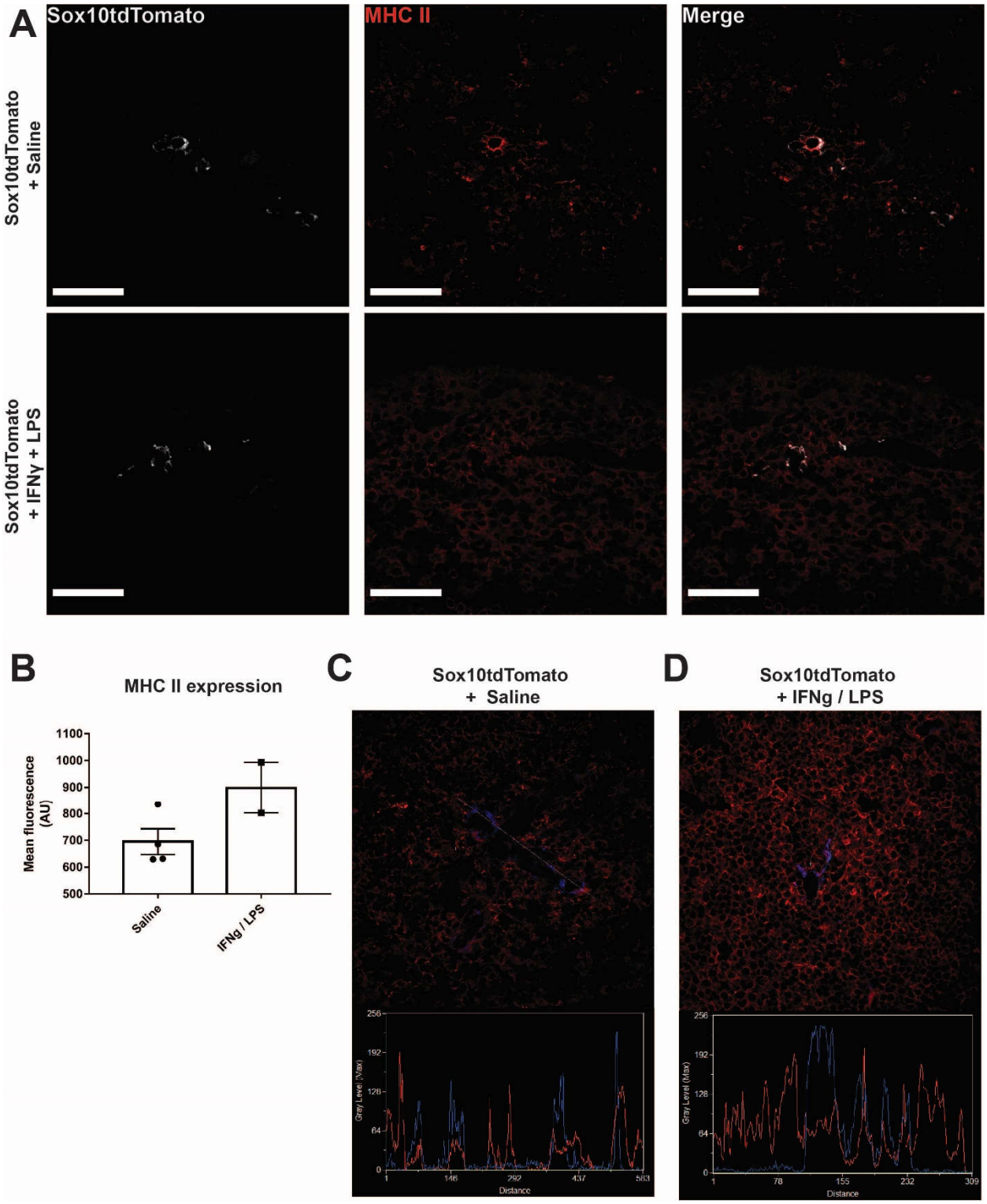

Figure 3.12: Sox10 expressing cells are present in sparse numbers in spleen tissue and express MHC II. (A) Representative confocal images of tdTomato (white)

Figure 3.12 (cont'd):

and MHC II (red) immunofluorescent labeling in spleen sections in IFNγ + LPS treated and control animals (scale bar: 50μm for all images). **(B)** Quantified MHC II mean fluorescent intensity in Sox10tdTomato expressing cells **(C-D)** Line-scan of MHC II (red) and Sox10tdTomato (blue) labeled spleen tissue from **(C)** control or **(D)** IFNγ + LPS treated mice.

Pro-inflammatory stimuli IFNγ and LPS do not trigger phagocytosis in enteric glia

The effects of glial MHC II on T-lymphocyte activation state suggest that glia MHC II molecules are not empty and are loaded with peptides(63, 75). In professional antigen presenting cells, peptides loaded onto MHC II are typically derived from the phagocytosis and processing of foreign antigens (60). We tested whether enteric glia present phagocytosed antigens onto MHC II molecules by first examining the phagocytic potential of enteric glia. Exposure to pHrodo BioParticles elicited phagocytosis in CD45+ immune cells neighboring enteric ganglia (Fig.3.13c), but phagocytosis or internalization of labeled E.coli particles was never observed in tdTomato+ enteric glia in 18 separate experiments (Fig.3.13b,c). We reasoned that this lack of effect might be due to using pHrodo labeled E.coli particles as the phagocytic substrate and that glia might engulf material through other mechanisms such as endocytosis. Therefore, we used labeled LPS molecules and 1.0μm fluorescent polystyrene microspheres to test whether TLR4-mediated endocytosis or size are contributing factors to glial phagocytosis. Incubation with fluorescently labeled LPS or fluorescent polystyrene microspheres did not elicit any uptake within enteric glial cells (data not shown), suggesting that enteric glia are either incapable of phagocytosis or that there is insufficient stimulation to trigger phagocytic behavior in enteric glia. Taken together, our results demonstrate that expression of MHC II molecules on enteric glia when stimulated with low dose IFNγ and LPS is not due to the phagocytosis of foreign antigens but is derived from other pathways.

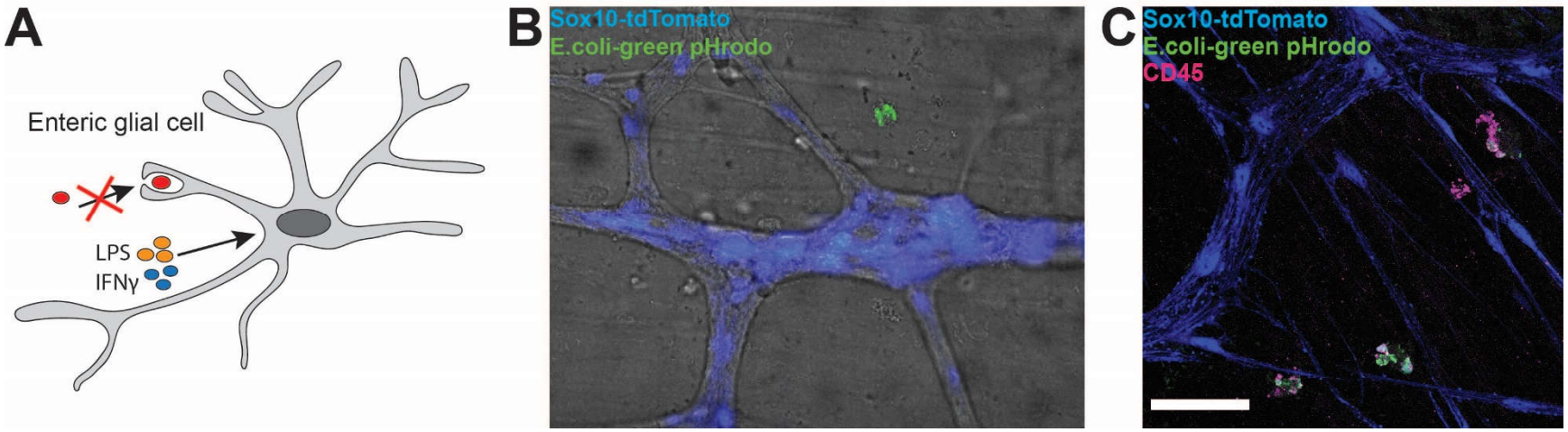

Figure 3.13: Enteric glia do not exhibit phagocytic activity when stimulated with IFNγ and LPS. (A) Schematic depicting that enteric glial cells do not exhibit phagocytic activity when stimulated with IFNγ and LPS. **(B)** Representative brightfield and epifluorescent image of tdTomato-expressing Sox10+ cells (blue) and pHrodo labeled E.coli BioParticles (green). **(C)** Representative confocal image of tdTomato-expressing Sox10+ cells (blue), pHrodo labeled E.coli BioParticles (green), and CD45 immunolabeling (magenta) (scale bar: 50μm).

Glial autophagy regulates MHC II expression

Our data show that glial MHC II expression occurs independently of phagocytosis. Therefore, we examined alternative pathways for MHC II induction. In antigen presenting cells such as B cells, a deficiency in autophagic protein Atg5 decreases MHC II expression levels, suggesting that autophagy regulates MHC II expression levels in antigen presenting cells (78). We tested whether a similar autophagy mechanism controls enteric glial MHC II expression by measuring levels of the autophagy proteins p62 and LC3 over a 16-hour time-course. As described above, MHC II expression was detected in enteric glia by 4 hours post injection (Fig.3.14b). Expression of autophagic proteins LC3 and p62 was not limited to enteric glia and is distributed in both enteric neurons and glia. The punctate nature of LC3 labeling shows a greater density in enteric glia, while p62 labeling is more prominent in neurons compared to glia, consistent with findings in single-cell RNA seq databases (19) (Fig.3.14b,c). Glial MHC II expression significantly (P = .0021) increased by 8 hours post-injection with IFNγ and LPS and further increased by 16 hours (P < .0001) (Fig. 3.14b,c,d). LC3+ puncta density in both enteric glia (Fig.3.14f) and enteric neurons (Fig.3.14i) trended towards increased levels over 16 hours, while p62 expression was significantly elevated in enteric glia (P = 0.0060) and neurons (P = .0270) by 4 hours post-injection and subsequently decreased to pre-injection levels by 16 hours (Fig.3.14e,h). The spike and subsequent decrease in p62 labeling, with the concurrent trend in increasing LC3 puncta over 16 hours suggests that autophagy is active in both enteric glia and neurons (55). The timing of MHC II upregulation in enteric glia coincides with autophagy-mediated consumption of p62 molecules and accumulation of LC3

deposits, showing that enteric glial MHC II expression occurs concurrently with autophagy pathways.

Since autophagy can lead to the presentation of intracellular antigens on MHC II molecules (18), we tested whether our findings are indicative of autophagy-induced glial MHC II expression. We used a combination of IFNγ and LPS, the autophagy stimulator rapamycin, and the autophagy inhibitor 3-methyladenine (3-MA) to test the regulation of glial MHC II expression by autophagy. A 16-hour incubation with IFNγ and LPS significantly (P = 0.0015) increased MHC II levels in enteric glia, similar to our *in vivo* findings (Fig.3.14g). Combining IFNγ and LPS with rapamycin further increased glial MHC II expression level compared to control (P < .0001), but interestingly, incubation with rapamycin alone did not increase MHC II levels compared to control (P = .8354) (Fig.3.14g). However, tissues incubated with a combination of IFNγ, LPS, and 3-MA, or IFNγ, LPS, rapamycin, and 3-MA had significantly decreased levels of glial MHC II expression compared to non-3-MA treated counterparts (P = .0047 and P < .0001, respectively) and reduced glial MHC II expression to levels comparable to untreated controls (Fig.3.14g). Taken together, our data show that inhibition of glial autophagy effectively eliminates MHC II labeling, suggesting that glial MHC II expression requires autophagy activation. However, the inability for rapamycin alone to induce glial MHC II suggests that the activation of autophagy alone is insufficient to trigger glial MHC II expression.

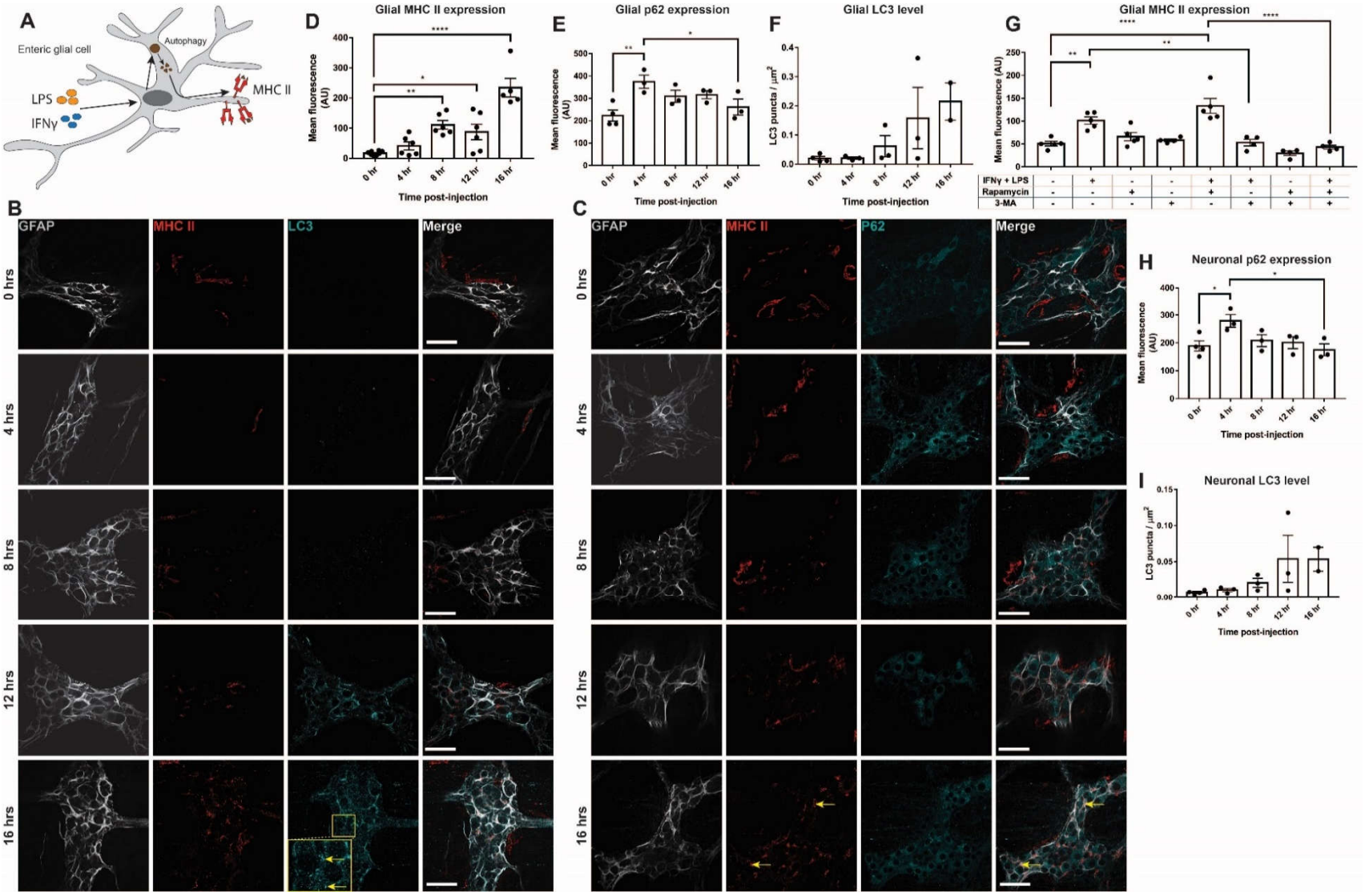

Figure 3.14: Enteric glia express MHC II via autophagy pathways. (A) Schematic depicting enteric glial MHC II loading

Figure 3.14 (cont'd):

and expression of autophagy-derived peptides when stimulated with IFNγ and LPS. **(B)** Representative confocal images of GFAP (grey), MHC II (red), and LC3 (cyan) immunofluorescent labeling in enteric ganglia in wildtype animals injected with IFNγ and LPS over a 16-hour time-course. LC3 show punctate labeling and is distributed in a neuronal and glial pattern (scale bar: 50μm for all images). **(C)** Representative confocal images of GFAP (grey), MHC II (red), and P62 (cyan) immunofluorescent labeling in enteric ganglia in wildtype animals injected with IFNγ and LPS over a 16-hour time-course. P62 labeling is prominent in enteric neurons but is distributed in both neurons and glia (scale bar: 50μm for all images). (D,E) Quantified mean fluorescent intensity of MHC II and p62 labeling on GFAP-expressing enteric glia in the myenteric plexus. Wildtype animals were injected with IFNγ and LPS and colons harvested over a 16-hour time-course ((D) n=5-8 mice and (E) n=3-4 mice; 1-way analysis of variance with Sidak-corrected multiple comparisons; *P ≤ .05, **P ≤ .005, ****P ≤ .0001). **(F)** Quantified density of LC3 puncta labeling over the area of GFAP-expressing enteric glia in the myenteric plexus. Wildtype animals were injected with IFNγ and LPS and colons harvested over a 16-hour time-course (n=2-3 mice; 1-way analysis of variance with Sidak-corrected multiple comparisons). **(G)** Quantified mean fluorescent intensity of MHC II labeling on GFAP-expressing enteric glia in the myenteric plexus. LMMP tissue preparations from wildtype animals were incubated with a combination of IFNγ and LPS, Rapamycin, and 3-MA for 16 hours prior to fixation and immunolabeling. (n=4-5 mice; 1-way analysis of variance with Sidak-corrected multiple comparisons; **P ≤ .005, ****P ≤ .0001). **(H)** Quantified mean fluorescent intensity of p62 labeling within enteric ganglion

Figure 3.14 (cont'd):

outside of GFAP-expressing enteric glia in the myenteric plexus. Wildtype animals were injected with IFNγ and LPS and colons harvested over a 16-hour time-course (n=3-4 mice; 1-way analysis of variance with Sidak-corrected multiple comparisons). **(I)** Quantified density of LC3 puncta labeling within enteric ganglion outside of GFAP-expressing enteric glia in the myenteric plexus. Wildtype animals were injected with IFNγ and LPS and colons harvested over a 16-hour time-course (n=2-3 mice; 1-way analysis of variance with Sidak-corrected multiple comparisons).

Discussion

The notion that enteric glia function as antigen presenting cells stems from observations in Crohn's and Chagas disease where glia increase MHC II expression (REFS). The functional significance of these observations has remained in question ever since. Here, we show that antigen presentation by enteric glia functions to modulate immune responses through interactions with T-lymphocytes. Enteric glia are sensitive to inflammatory stimuli and respond to low dose IFNγ and LPS by expressing MHC II molecules. Enteric glial MHC II molecules are functional and contribute to CD4+ T-lymphocyte activation, particularly in the Th_{17} and T_{reg} subpopulations. These effects do not involve large shifts in proinflammatory mediators. Glial MHC II expression is not phagocytosis-mediated and is instead, dependent on autophagy activation. However, autophagy activation alone is not sufficient to elicit glial MHC II expression.

MHC II is primarily expressed by professional antigen presenting cells and plays an important role in their interactions with T-lymphocytes. We show that enteric glia express MHC II in an inducible manner when exposed to proinflammatory stimuli, consistent with prior *in vitro* and *in vivo* human studies which show that glial MHC II expression is induced by enteroinvasive E.coli or inflammation (3, 23, 70). Furthermore, the punctate distribution of MHC II along glial processes is consistent with findings in Chagas disease (3). In our study, enteric glia expressed MHC II in the absence of inflammation or observable tissue damage. This suggests that glial antigen presentation may act as an early responder to inflammatory stress. This conclusion is consistent with observations in Crohn's disease showing that enteric glia express MHC II in diseased areas and macroscopically uninvolved areas (23). Interestingly, occasional glial MHC II

labeling is also observed in samples from healthy individuals (3, 23). We did not observe glial MHC II expression in healthy animals and it is not observed in cultured human enteric glia *in vitro* (70). This discrepancy in baseline glial MHC II expression may be due to the lack of a microbiome *in vitro*, and potentially due to murine tolerance to LPS and bacterial components (14). However, baseline glial expression of MHC II in humans suggests that enteric glia constantly monitor the ENS and modulate immune activation in response to the constant presence of microbiome-derived antigens. The baseline MHC II expression also suggests that enteric glial MHC II may have anti-inflammatory effects. Anti-inflammatory actions of MHC II may contribute to homeostasis by preventing repeated immune activation and inflammation against the normal microbiome. Similar anti-inflammatory effects of MHC II expression by intestinal epithelial cells limit T_h1 cell infiltration and modulate T-lymphocyte subtype distribution in the intestine (69).

The presence of enteric glial MHC II expression *in vivo* and *in vitro* (3, 23, 70) suggests potential interactions with T-lymphocytes. Enteric glial interactions with T-lymphocytes *in vivo* when expressing neoantigens (15) suggest that T-lymphocytes can recognize enteric glia. Moreover, enteric glia inhibit T-lymphocyte activation in co-cultures (36). However, the functional role of antigen presentation by enteric glia and their potential to influence T-lymphocytes *in vivo* is not understood. Here, we show that enteric glial MHC II expression plays an important role in the activation of CD4+ T-lymphocytes in mesenteric lymph nodes. We also noted a surprisingly large population of CD4- cells expressing CD69 in stimulated animals. A portion of those cells responding to glial MHC II represent CD69-expressing CD8+ T-lymphocytes (51, 57,

59). The decreased CD8+ T-lymphocyte activation may contribute to the lack of histological tissue damage. Additional cell types may include natural killer cells (11, 33) and dendritic cells (2, 40), but further study is warranted to further elucidate this cell population. Our data show that glial MHC II expression has a propensity towards affecting T_h17 and T_{reg} subpopulations moreso than T_h1 and T_h2 subpopulations. This apparent conflict in immune regulation by activating both pro- and anti-inflammatory T-lymphocyte subpopulations suggest a feedback inhibition to limit T_h17 activity (9). Alternatively, the T_h17 subpopulation may exhibit anti-inflammatory actions by secreting anti-inflammatory cytokines (22, 52, 56, 84). Furthermore, enteric glia may contribute to an anti-inflammatory phenotype by destabilizing interactions with certain T-lymphocyte subtypes to inhibit proliferation, similar to effects seen in dendritic cells (76). Despite enteric glial MHC II expression having no apparent effect on regional tissue cytokine levels, gene expression changes suggest that enteric glia may have a mixed role in the local ENS microenvironment by expressing IL-1 and tumor necrosis factor, while decreasing IL-12b. The apparent expression of certain pro-inflammatory cytokines despite decreasing others may be due to the concurrent activation of separate pathways. While IL-1 and tumor necrosis factor gene expression may be driven by direct binding of LPS molecules onto TLR4 receptors on enteric glia, a decrease in IL-12b may be due to the activation of other separate signaling pathways. In support, enteric glia show strong immunosuppressive effects that inhibit T-lymphocyte proliferation in co-culture models and this effect is strengthened in enteric glia derived from Crohn's patients (36). In contrast, enteric glia can contribute to neuronal cell death (7), and cultured human enteric glia challenged with IFNγ and LPS increase gene

expression of various pro-inflammatory cytokines (45). This suggests that the dynamic immunomodulatory capabilities of enteric glia depend on the surrounding environment.

Our current data focuses on the glial MHC II KO (*Sox10*^{CreERT2};*IAB*$^{fl/fl}$) model and its effects on T-lymphocytes of the mesenteric lymph nodes. Since we did not detect any Sox10+ cells that would be susceptible to the MHC II KO in mesenteric lymph nodes, how enteric glia in the ENS affect T-lymphocyte activation while in separate anatomical locations remains to be understood. Given the proximity of local CD4+ T-lymphocytes to enteric glia, it is possible that enteric glia influence the activation of local T-lymphocytes via cell interactions or secreted compounds, and the migration of activated lymphocytes could subsequently have a wider reaching effect in regional lymph nodes (36). Similarly, enteric glia may potentially influence nearby dendritic cells to bring about lymph node effects. Enteric glial expression of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (1) can conceivably influence dendritic cells to stimulate activation of type 1 regulatory T-lymphocytes (72), but this interaction has yet to be described.

Phagocytosis is an important pathway for professional antigen presenting cells to uptake foreign antigens for processing and presentation. In our study, we did not find evidence that enteric glia uptake labeled E.coli, labeled LPS, or fluorescent latex beads. The lack of phagocytosis in our study, however, may be due to the strength of the proinflammatory stimuli used, since others have successfully demonstrated phagocytic potential in glia in other organ systems (49) and in other related glial populations in the CNS (21, 34, 38, 71). Therefore, stronger stimuli or the dysfunction and death of nearby cells may be necessary for enteric glia to demonstrate phagocytic activity. Given their shared characteristics with astrocytes (12), their proximity to enteric neurons (5) and

their ability to contribute to neuronal demise (7), it is conceivable that enteric glia also uptake neuronal fragments during neuronal injury or death. The presentation of neuronal fragments to the adaptive immune system could lead to the generation of autoreactive antibodies and may be a mechanism contributing to the production of anti-HuC/D antibodies in patients with irritable bowel syndrome (77). The expression of enteric glial MHC II in situations of inflammation and neuron loss, such as in Chagasic megacolon or Crohn's disease (3, 23), suggest that enteric glia play a role in these disease states. Whether enteric glia contribute to neuronal death and inflammation through MHC II expression or promote anti-inflammatory pathways to maintain homeostasis remains to be studied.

The ability of enteric glia to express MHC II molecules in the absence of phagocytosis suggests that glia, like B-lymphoma cell lines, use alternative pathways such as autophagy promote the loading of intracellular and self-antigens onto MHC II for presentation (18, 67). Enteric glial MHC II expression is dependent on autophagy activation and the timing of MHC II expression coincides with autophagy activation, which raises the possibility that enteric glia present autophagy-derived peptides on their MHC II molecules. Although the presentation of autophagy-derived self-antigens on MHC II molecules suggest the potential development of autoimmune reactions, our findings suggest otherwise, as no pro-inflammatory cytokine levels were changed when glial MHC II expression status changed and no inflammatory cell recruitment was observed in colonic tissue. These data are consistent with findings by other groups demonstrating anti-inflammatory effects from autophagy and autophagy-induced MHC II

expression (76, 81). However, mass spectrometry analysis of glial MHC II molecules is necessary to conclusively determine the identity of presented peptides.

The autophagic response to IFNγ and LPS differed between enteric neurons and glia in their expression of LC3 and p62, with more prominent labeling of p62 in enteric neurons and greater LC3 puncta in enteric glia. Although unclear, the more prominent p62 labeling may indicate that enteric neurons exhibit a higher sensitivity to inflammatory stimuli than enteric glia. The observed elevation in density of glial LC3 puncta may in fact be related to glial phagocytosis and antigen presentation in the form of LC3-associated phagocytosis via TLR4 activation (25, 26, 61). However, as we did not detect any phagocytic activity, a more detailed examination specifically into LC3-associated phagocytosis is needed to test this hypothesis (50). Taken in the context of IBD, which is considered having dysfunctional autophagy pathways (32, 41), the activation of autophagy in enteric glia and neurons may in fact be a compensatory mechanism to limit potential inflammatory damage to the ENS. This potential mechanism is indeed supported by findings from numerous groups demonstrating the activation of autophagy pathways can inhibit intestinal inflammation and reduce colitis (37, 47, 81).

Our data show that autophagy activation is required for enteric glial MHC II expression, agreeing with prior studies demonstrating that dendritic cells require autophagy machinery to express peptides on MHC II molecules (43). Furthermore, our data show that autophagy activation by rapamycin alone is insufficient to induce glial MHC II expression. This may be due to the nature of rapamycin, as other groups have shown *in vitro* that rapamycin has varying effectiveness in eliciting autophagy in

different glial cell lines (68). Alternatively, enteric glia may simply require both a stimulus with a concomitant activation in autophagy to express MHC II molecules. However, LPS as a stimulus may in fact contribute to autophagy inhibition (44, 83). Taken together with the upregulation of pro-inflammatory cytokine gene expression, our data suggest that two separate pathways may be concurrently activated by IFNγ and LPS challenge. Direct LPS binding onto enteric glial TLR4 receptors may contribute to a pro-inflammatory phenotype in enteric glia, while the activation of a separate pathway induces autophagy and an anti-inflammatory phenotype.

In conclusion, our results show that enteric glia respond to inflammatory stimuli by expressing functional MHC II molecules to modulate T-lymphocyte activation (Fig.3.15). Furthermore, we show that autophagy activation is necessary, but insufficient, to induce enteric glial MHC II expression. Together, these observations provide evidence for enteric glia being an important immune regulator during inflammatory stress and suggest that they may play an anti-inflammatory role during intestinal inflammation and IBD in an attempt to maintain homeostasis.

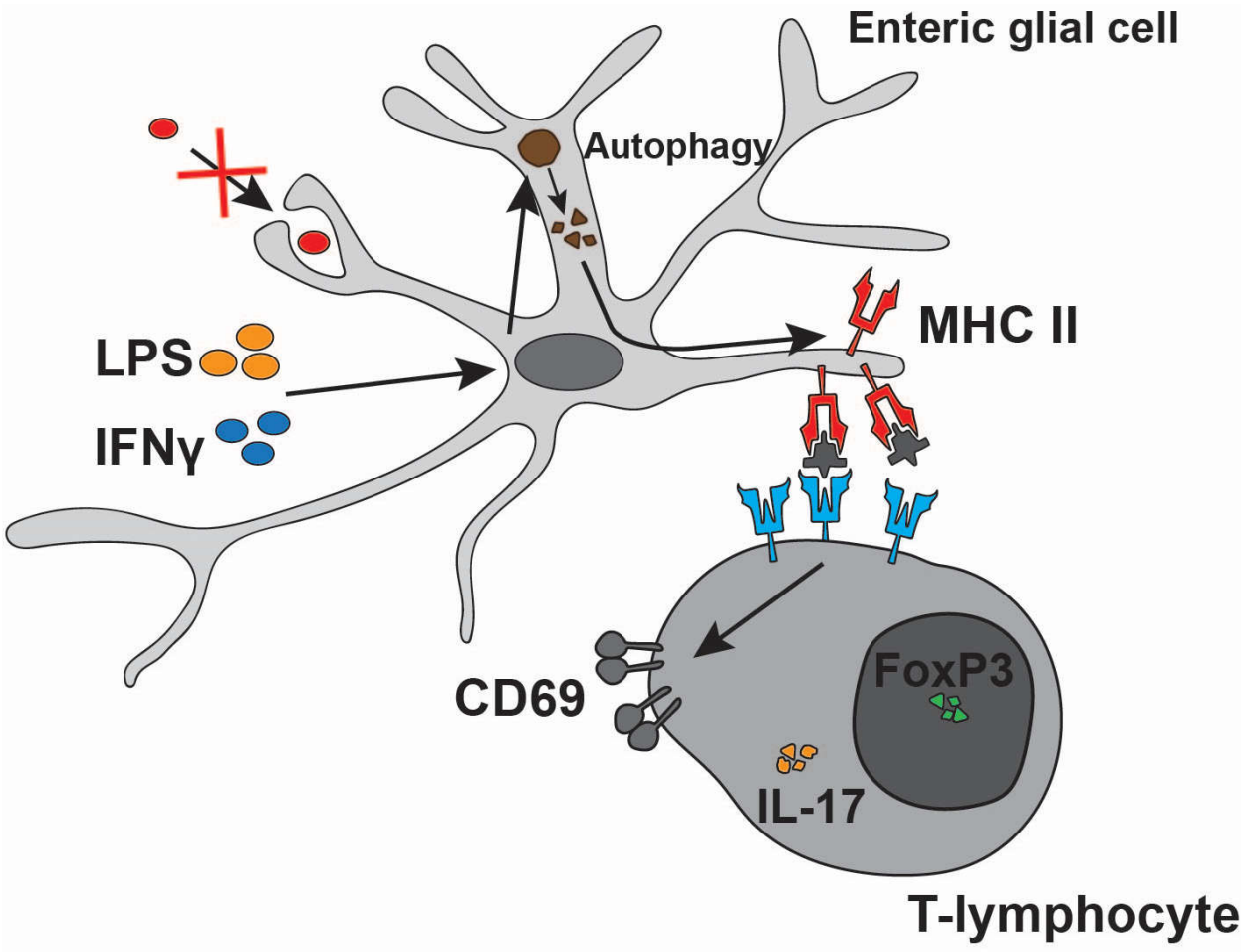

Figure 3.15: Schematic mechanism of enteric glial MHC II expression. Enteric glia exposed to pro-inflammatory stimuli IFNγ and LPS upregulate expression of MHC II molecules on their cell surface. Enteric glial MHC II is not derived from phagocytosed foreign antigens, but rather derived from products of autophagy. Enteric glial MHC II are functional molecules that can interact with CD4+ T-lymphocytes and modulate CD69 expression levels primarily in IL-17 and FoxP3 expressing CD4+ T-lymphocytes.

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CHAPTER 4:

Summary, Key Findings and Future Directions

Summary

Enteric glia, as part of the ENS, play important roles in the maintenance of ENS health and modulation of enteric neuronal activity. Enteric glial cells express and release both pro- and anti-inflammatory mediators (1, 7, 12, 17, 18, 28), and behave similarly to atypical antigen presenting cells by expressing MHC II molecules in select conditions (2, 9). However, it has not been shown whether the expressed MHC II molecules are functional or whether enteric glial cells can carry out other antigen presenting cell functions in an *in vivo* system. Our study here examines enteric glial behavior in the context of an *in vivo* murine system with low inflammatory stress. We isolate glial contributions to overall immune and inflammatory responses by using a glial-specific inducible MHC II KO (*Sox10*^{CreERT2}*;IAB*$^{fl/fl}$). Overall, our findings suggest that the enteric glia may be responding to a low dose or initial inflammatory challenge by inducing anti-inflammatory effects via autophagy-mediated MHC II expression to maintain homeostasis.

Key Findings

Enteric glial cells express MHC II in response to low dose IFNγ and LPS

Animals challenged with a low dose of IFNγ and LPS was sufficient in eliciting an increase in MHC II expression in enteric glia. We found that the glial MHC II expression was similar in morphology to glial MHC II expressed in human Chagas disease patients (2), and this MHC II expression was in the absence of macroscopic or microscopic intestinal inflammation or tissue damage. Our results suggest that enteric glia are sensitive to the local microenvironment and may represent an ‘early responder’ to

inflammatory stress. Together with the findings that normal non-inflamed patients occasionally show enteric glial MHC II expression, these results suggest that the early expression of MHC II as a response to inflammatory challenge may have anti-inflammatory effects in order to maintain homeostasis. Compared to previous studies that examined enteric glial cells in the context of Chagas disease and Crohn's disease patients, our mouse model did not experience overt activation of the inflammatory pathway and phenotypes dominated by the activation of other immune cells (2, 9). Therefore, we are able to isolate glial responses inflammatory stress and not their response to cytokine signaling from activated immune cells.

Enteric glial MHC II alter T-lymphocyte activation

Previously, the expression of enteric glial MHC II has not been demonstrated to be a functional molecule in modulating T-lymphocyte activation *in vivo*. Kermarrec et al. have shown that enteric glial MHC II have an inhibitory effect on T-lymphocyte proliferation, but the study was performed *in vitro* with primary cultured human enteric glial cells co-cultured with activated T-lymphocytes (16). Our results in a mouse *in vivo* system demonstrate that glial MHC II expression are functional, and influence T-lymphocyte levels as measured in regional mesenteric lymph nodes. In addition, we show that the T_h17 and T_{reg} subpopulations are the primary subtypes affected by enteric glial MHC II expression. Our data suggests that enteric glial MHC II may be involved in the pro-inflammatory and subsequent T_{reg}-mediated inhibition of T-lymphocyte activation. Alternatively, MHC II expression on enteric glia may induce an anti-inflammatory phenotype in T_h17 T-lymphocytes to contribute alongside T_{reg} activities and maintain homeostasis (21, 24). Despite influencing the activation state of T-

lymphocytes, our data show that cytokine levels at the tissue or serum level remain unchanged, suggesting that enteric glial have more of an impact on the local microenvironment around the ENS than at the regional or whole-body level.

Enteric glial MHC II expression is derived from autophagy pathways

MHC II molecules are responsible for loading and presenting peptide antigens that were generally derived from the phagocytosis of extracellular molecules (26). Our results show that, despite enteric glial cells expression of MHC II molecules, they do not phagocytose extracellular antigens when challenged with IFNγ and LPS. Furthermore, our data shows that the activation of autophagy is necessary, but not sufficient to induce MHC II expression in enteric glia. Our findings suggest that enteric glia present intracellular antigens, and potentially self-antigens, for presentation onto MHC II molecules. Given that cells maintain a basal level of autophagic presentation of self-peptides onto MHC II while maintaining immune tolerance and the stimulation of autophagy ameliorates inflammation (19, 30), our data suggest that autophagy activation in enteric glia may have an anti-inflammatory effect when challenged with IFNγ and LPS. Together with our other findings, this paints a picture that enteric glia are actively attempting to maintain homeostasis in the local ENS microenvironment when faced with initial pro-inflammatory stimuli. This may help maintain proper ENS functioning prior to overt activation of pro-inflammatory immune responses that can lead to ENS damage and GI dysfunction.

Study Limitations

Pharmacological modulators

We used Rapamycin and 3-methyladenine as chemicals to stimulate or inhibit the autophagy pathway. Our findings and conclusions from experiments assume that these molecules are specific in their target activity and do not have other side effects that may confound findings.

Sex of experimental animals

Cytokine quantification experiments were performed in only male mice. As sex is an important variable in cytokine levels, the changes in cytokine levels observed in our studies may not be applicable to a wider population.

MHC II knockout animals

Our MHC II KO mouse strain is driven by the Sox10 promoter to target enteric glia in our experiments. Any cells actively utilizing the Sox10 promoter would be ablated in MHC II. We examined mesenteric lymph nodes and spleens and did not see evidence of Sox10+ cells. However, whether other Sox10+ cells in the periphery influence our findings is not known.

Future Directions

Determination of antigens presented on enteric glial MHC II

Our findings showed that the expression of enteric glial MHC II is functional in modulating T-lymphocyte activation and is mediated by the autophagy pathway. This suggests the possibility that the peptides presented on glial MHC II are intracellular ‘self’

peptides. Since the presentation of peptides on MHC II molecules can dictate the target of immune responses, it would be interesting to see what peptides enteric glial cells are presenting on their MHC II molecules. In order to determine the identity of peptides expressed to T-lymphocytes, mass spectrometry on peptides eluted from enteric glial MHC II would be beneficial.

Enteric glial autophagy effects in modulating T-lymphocyte activation

As discussed earlier, our results show that enteric glial MHC II expression is dependent on the activation of the autophagy pathway. Given that our data also show that enteric glial MHC II expression can influence T-lymphocyte activation, the next step would be to study the direct effects of autophagy modulation on T-lymphocyte activation, and whether a similar level of T-lymphocyte activation is seen in animals with inhibited autophagy compared to MHC II KO animals.

Glial MHC II contributions in overt inflammation

Our studies here were based on using a low dose of IFNγ and LPS as an inflammatory stimulus. While this inflammatory challenge is beneficial in isolating contributions from enteric glia and not from other immune cells, it would be interesting to study whether enteric glial MHC II expression have noticeable effects on the inflammatory pathway during stronger inflammatory challenge and overt inflammation. Utilizing a stronger inflammatory challenge would be beneficial in studying whether enteric glial behavior changes based on inflammation intensity. Furthermore, examining the impact of glial MHC II in stronger inflammatory stress and established inflammation

would give relevant findings to the pathogenesis of inflammatory bowel disease Crohn's and ulcerative colitis.

Sympathetic effects on enteric glial-mediated T-lymphocyte activation

The sympathetic and parasympathetic nervous systems innervate and modulate immune activation and ENS activity (23, 25). Furthermore, the autonomic nervous system innervates enteric glial cells in the ENS (6, 10). Therefore, a future set of experiments could examine the effects autonomic activity has on enteric glial autophagy activation, expression of MHC II, and their ability to modulate T-lymphocyte activation. We performed pilot studies examining MHC II expression in the wildtype control animals after surgical sympathectomy (removal of the celiac and inferior mesenteric ganglia), chemical sympathectomy (single subcutaneous injection of 100mg/kg 6-hydroxydopamine (6-OHDA)), or norepinephrine depletion (single subcutaneous injection of 0.5mg/kg reserpine). Tissues harvested 7 days after treatments demonstrate a trend in increasing enteric glial MHC II expression in surgical sympathectomy and reserpine-treated conditions, but not in 6-OHDA-mediated chemically sympathectomized animals (Fig.4.1a,b). Since these results were observed in wildtype tissues without inflammatory challenge, changes in autonomic innervation to enteric glia in tissues and animals challenged with inflammatory stimuli may show more significant results. As mentioned above, it would be beneficial to examine how autonomic activity affects autophagy activation in enteric glia, and, by performing sympathectomy treatments to MHC II KO animals, we can elucidate the effects of autonomic activity on enteric glial activation of T-lymphocytes.

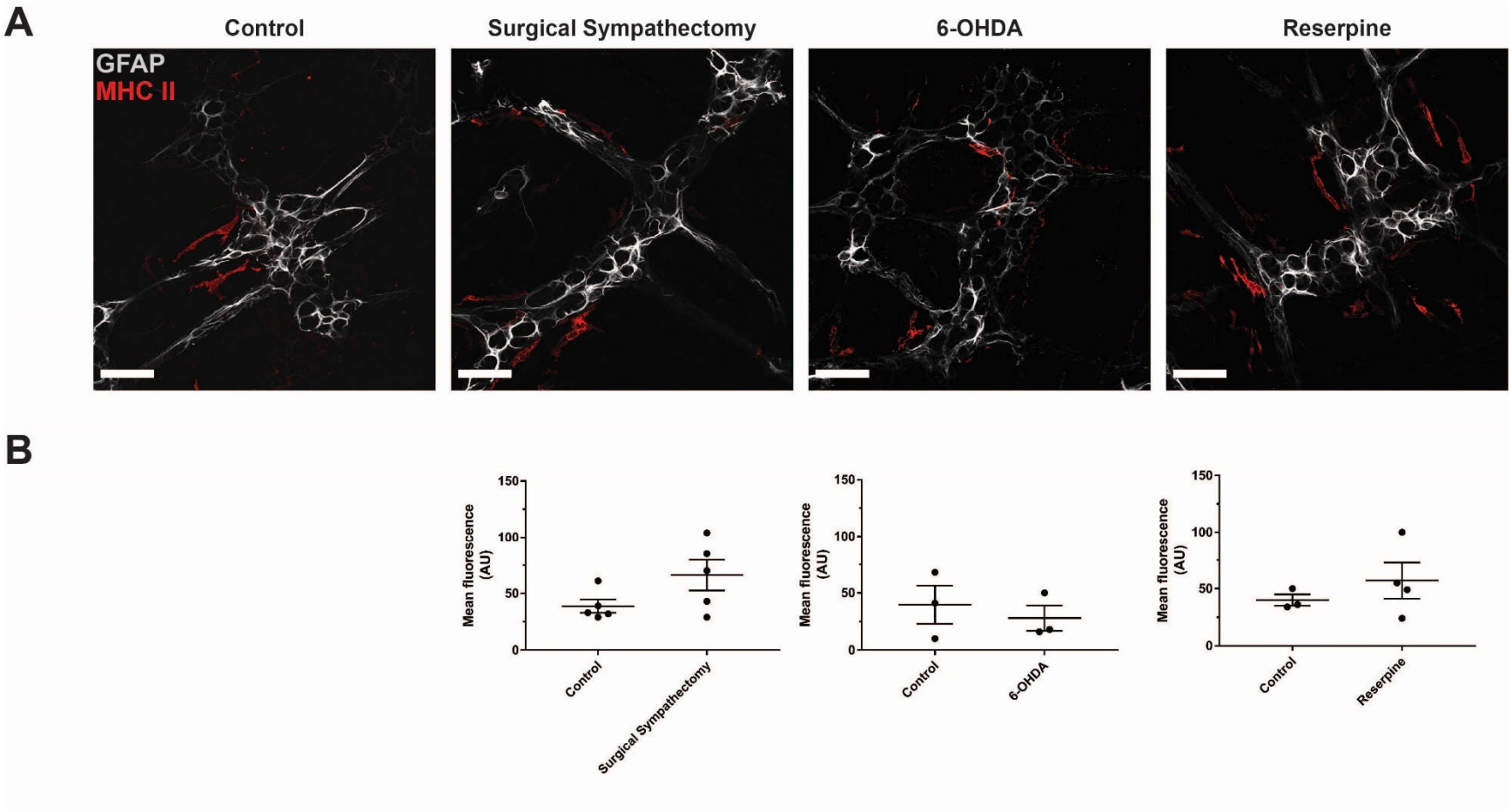

Figure 4.1: Autonomic innervation onto enteric glia may modulate MHC II expression. (A) Representative images of GFAP (grey) and MHC II (red) immunofluorescent labeling in enteric glia (scale bar: 50μm for all images) **(B)** Quantified mean fluorescent intensity of MHC II labeling on GFAP-expressing enteric glia in the myenteric plexus (n=3-5 mice; student's unpaired t-test).

Local renin angiotensin effects

Finally, the local renin angiotensin system within the GI tract may contribute to intestinal inflammation. Studies within the GI tract and CNS demonstrate that the binding of the prorenin receptor by prorenin or renin, along with the binding of angiotensin receptor 1 by angiotensin II can elicit pro-inflammatory effects, oxidative stress, and neuronal damage (3, 8, 31, 33) (Fig.4.2). In contrast, the binding of angiotensin receptor 2 by angiotensin II and the mas receptor by angiotensin (1-7) leads to anti-inflammatory effects, decreased oxidative stress, and increased neuronal survival (4, 11, 15, 20, 27, 29) (Fig.4.2). We performed pilot studies to examine the myenteric plexus and the expression of compounds associated with the renin-angiotensin pathway. In this collaborative pilot study with Dr. Yumei Feng Earley (University of Nevada), we examined the myenteric plexus of mice fed with a high fat diet compared to controls. Since a high fat diet in mice can lead to intestinal inflammation (5), we examined the expression of angiotensin converting enzyme-2 within the myenteric plexus as a measure of potential anti-inflammatory behavior in enteric glia. Our preliminary results show that the expression of angiotensin converting enzyme-2 is in a punctate morphology in enteric glia, and expression is increased in the high fat diet condition compared to control animals (P = .0417) (Fig.4.3a,b). These preliminary results suggest that enteric glia play a role in shifting the local renin-angiotensin pathway towards a more anti-inflammatory phenotype in the context of a high fat diet to potentially negate inflammatory effects due to elevated dietary fat. Furthermore, we will examine the role enteric neurons play in renin-induced inflammation with the use of transgenic animals with a neuron-specific KO in the

prorenin receptor, developed by Dr. Yumei Feng Earley (University of Nevada). These studies will help further elucidate the contributions of the local renin-angiotensin system in inflammatory stress in the GI tract. Although our preliminary study is lacking in power, our findings, like others (13, 14, 22, 32), suggest that the local renin-angiotensin system is at play in the GI tract and is involved in regulating intestinal inflammation. However, the role the ENS plays in this pathway is largely unknown and warrants further study.

Taken together, the data presented in this dissertation unveils a novel role that enteric glia play as an immune modulator by interacting and controlling T-lymphocyte activation. Together with proposed future experiments, the role enteric glia play as an antigen presenting cell can be further elucidated in the context of intestinal inflammation, leading to potentially new therapeutic targets for inflammatory bowel diseases.

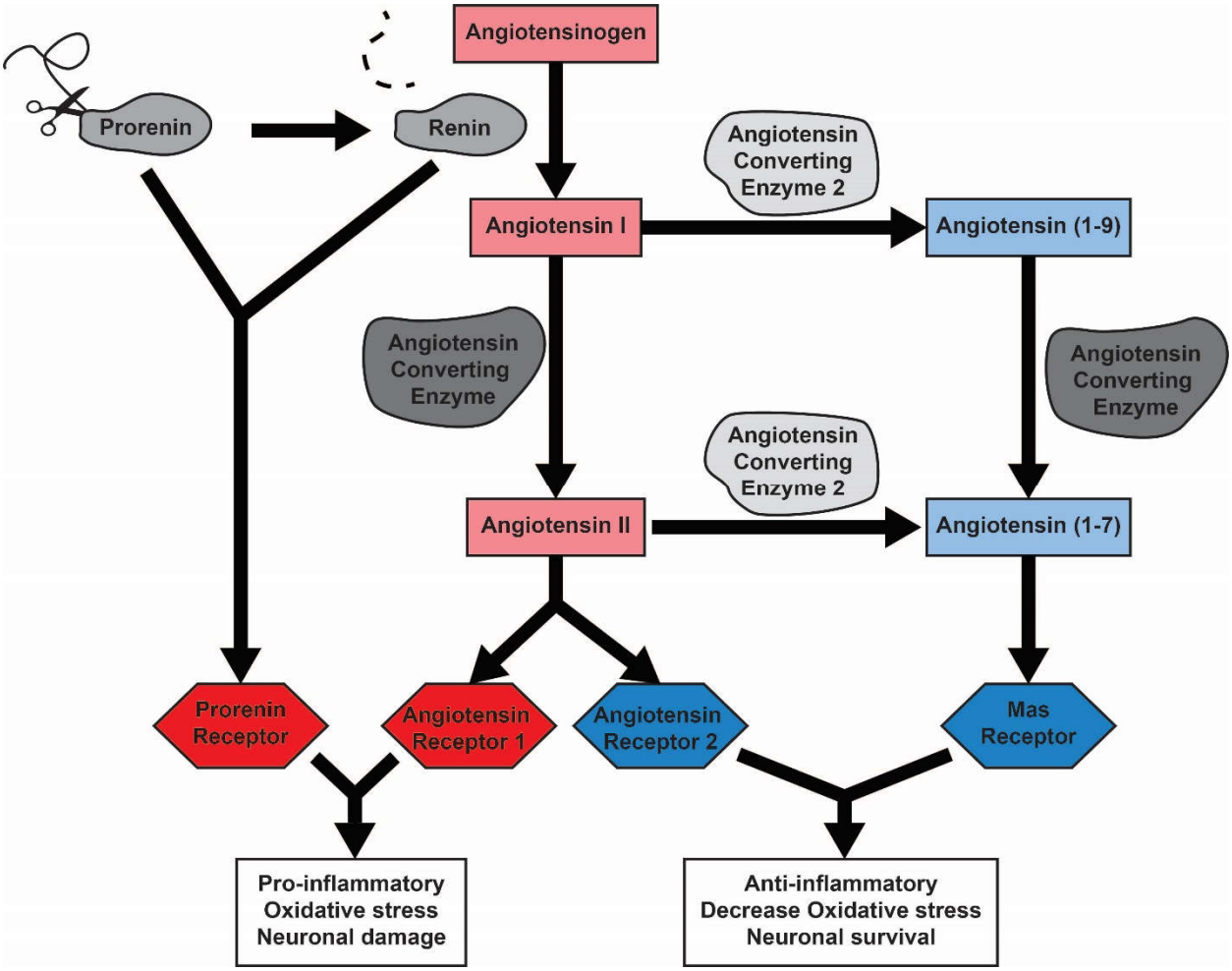

Figure 4.2: The renin-angiotensin pathway neuronal damage and inflammation.

The renin-angiotensin pathway works through several receptors (prorenin receptor, angiotensin receptor 1, angiotensin receptor 2, and mas receptor) to bring about pro- and anti-inflammatory effects. Angiotensin II can bind both angiotensin receptor 1 and angiotensin receptor 2 to bring about opposing effects, but the angiotensin II molecule is generally regarded as pro-inflammatory. Angiotensin converting enzyme 2 shifts the renin-angiotensin pathway away from a pro-inflammatory phenotype to a more anti-inflammatory phenotype by increasing production of angiotensin (1-7) and binding of mas receptor.

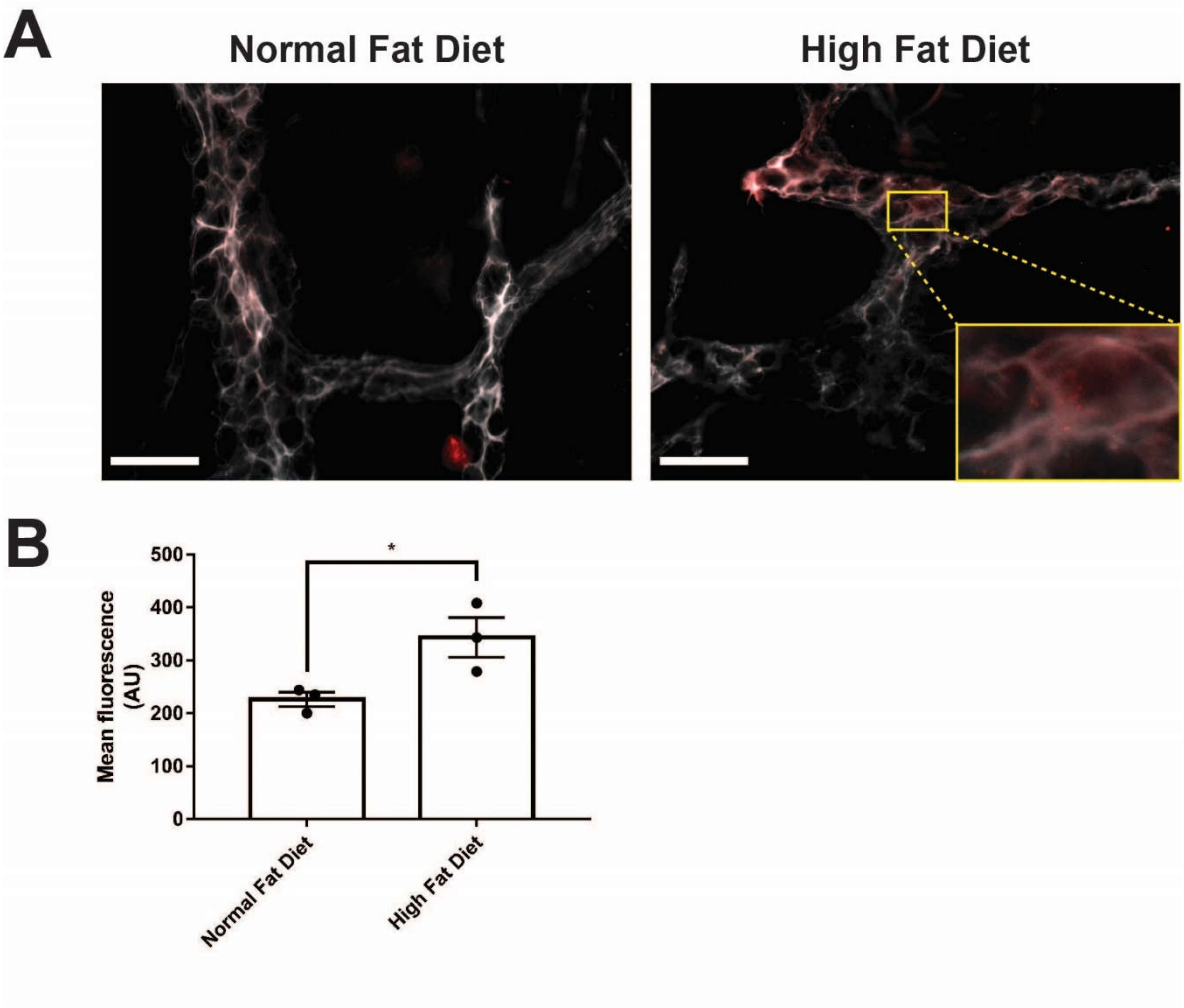

Figure 4.3: Enteric glia increase expression of ACE2 during high fat diet conditions. (A) Representative Images of GFAP (grey) and ACE2 (red) immunofluorescent labeling in enteric glia. **(B)** Quantified mean fluorescent intensity of ACE2 labeling on GFAP-expressing enteric glia in the myenteric plexus (n=3 mice; student's unpaired t-test; *P < .05)

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