Interleukin 33 in the Initiation of CNS Inflammation Following Traumatic Injury

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Approval Sheet

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For Mom and 414

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Acknowledgments

I would first like to acknowledge my adviser, Jony. I was once told that great scientists are separated from good ones not by raw intelligence, impact factor, or other metrics, but by *taste*. The precise meaning of "taste" in this context I could never grasp, I only know that Jony has it in spades.

I am grateful for Jony's generosity in giving his students credit, I was corresponding author on my first paper, and opportunities, with his mentorship I've written several reviews/news and views and gone to conferences, but moreso I am grateful for his friendship and council. Jony looks out for me and genuinely cares about my success. I can think of many peers without such an unwavering advocate.

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Apart from learning Art and Biology from her, she's been a guaranteed friend and person to call in times when I was short on them. I've leaned on her many times in the last decade, and without her support and friendship I surely would have given up.

Avani was my first mentor. She has planted many thoughts in my head that have shaped my interests, personality, and work more than I'd like to admit. I remember her commenting on my sloppy handwriting in grade school: "you'll make a good scientist, they all have bad handwriting." At this point I changed life goals from being a firefighter to scientist, and I think subconsciously I've never switched after that.

I respect Avani immensely, she is the coolest person I know of, and I find myself imitating her behavior all the time. I suppose my decision to seek this degree could be just another example of this.

My Mother is an inexhaustible source of joy and love. I am amazed to this day how generous and good to other people she is, its a level of selflessness and empathy I can only aspire to. Her influence was integral in my choosing to focus on medical science, which fundamentally exists to help others and relieve suffering. She has taught me through example to try to be kind, appreciate what I have, and work hard, and it goes without saying was absolutely required for any of my achievement up to this point.

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I finally must acknowledge all other scientists, famous or not, who collectively participate in the search for truth. No one understands reality, the Universe, or life, but I am inspired by anyone who tries.

Chapter 2

Introduction

This Thesis represents the culmination of my graduate work studying neuroinflammation in mouse models of CNS injury. I have focused in particular on the alarmin interleukin(IL)-33, characterizing it as a novel activator of CNS inflammation, and this Thesis will provide a complete summary of my work.

In this Chapter I will begin by introducing CNS injury, outlining the state of treatments and barriers to regeneration (Section 2.1). I will next review our current understanding of the initiation of inflammation, both generally and in the CNS (Section 2.2). Next, I will introduce beneficial and detrimental roles of immune infiltration after CNS injury, reviewing literature by immune cell type (Section 2.3). I will finally provide detailed introductions to IL-33 (Section 2.4) and type 2 innate lymphocytes (ILC2s) (Section 2.5).

2.1 Traumatic Central Nervous System Injury

Central nervous system (CNS) injury is devastating, frequently resulting in severe impairment with little prospect for healing over time. 3-5 million people in the United States, or 1-2% of the population, experience long-term disability from traumatic brain injury (TBI), with most of these cases caused by falls, motor vehicle accidents, or violence (in that order of prevalence) (Hempill, 2014). CNS injury, unlike most other wounds, fails to regenerate significantly over

time and leads to permanent impairment. When the injury affects the spinal cord patients often have irrecoverable paralysis below the injury site, which is particularly difficult to cope with for the most common spinal cord injury (SCI) patient–a young man with median age of 22 (Robert Hansebout, 2014). Despite the devastating impact on patients and high monetary and emotional costs, management options remain extremely limited. Acute SCI cases today are managed with focus on preventing disastrous complications such as respiratory failure, neurogenic shock, or catastrophic thromboembolism (Robert Hansebout, 2014), but there are *no* pharmacologic therapies being used to restore function in the long term. The only pharmacologic treatment approved for use following injury is methylprednisone, a glucocorticoid with immunosuppressive properties. Though it was the standard of care for many years, it has recently fell out of favor in light of concerns over the validity of clinical trials and underestimated detrimental side effects (Robert Hansebout, 2014; Lammertse, 2013; MB, 2004). The absence of treatments is not from a lack of trying; over 30 clinical trials based on promising pre-clinical data have failed to show significant benefit (Maas et al., 2010; Loane and Faden, 2010). These discouraging results are frequently attributed to improperly planned trials or low sample size (Lammertse, 2013), but they highlight the ongoing need to better understand the pathophysiology of CNS injury and to develop more robust therapies.

Why doesn't the CNS recover after injury? This has been a pressing question in neuroscience for decades, and no single reason can explain the deficit. In general, the defined obstacles to healing can be divided into two categories: 1) factors that contribute to the overwhelmingly anti-regeneration post-injury environment, and 2) those that contribute to secondary neuronal death, a phenomenon of neuronal death beyond the original lesion in the days after injury. These phenomena are being targeted by neuroregenerative and neuroprotective agents, respectively, and represent the bulk of efforts for pharmacologic treatment of CNS injury.

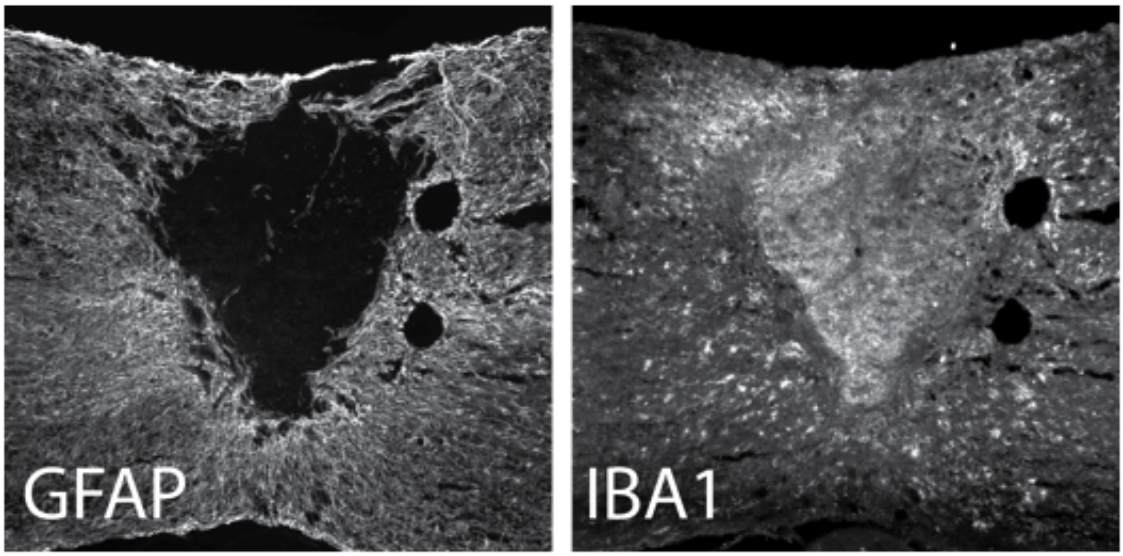

Figure 2.1: **The glial scar is outlined by astrocytes and occupied by microglia.** Spinal cords were collected 14 days after injury and stained for GFAP (astrocytes, right panel) and IBA1 (microglia/macrophages, left panel). The glial scar is delineated by $GFAP^+$ cells, and occupied by $IBA1^+$ cells.

2.1.1 The post-injury environment inhibits regeneration the glial scar, CSPGs, and Nogo

Severe injury to the CNS, for example spinal cord contusion, is characterized by a vigorous initial response from local astrocytes and microglia. Astrocytes undergo a dramatic change to the reactive astrocyte, becoming hypertrophic/hyperplastic, upregulating expression of glial fibrillary acidic protein (GFAP) and laminin, and producing extracellular matrix (ECM) proteins like chondroitin sulfate proteoglycans (CSPGs) as part of their transition (Sofroniew, 2005). Reactive astrocytes sharply delineate the site of injury, outlining the border of the glial scar, a core of unhealing scar tissue characteristic of CNS injury (Figure 2.1). This scar tissue is composed of both cells and ECM protein deposits, including CSPGs, microglia, pericytes and fibroblasts (Goritz et al., 2011; Sharma et al., 2012; Soderblom et al., 2013). Astrocytes were thought to be the major producers of CSPGs, but a relatively recent study actually identified perivascular $GLAST^+$ pericytes as the main producer after injury (Goritz et al., 2011).

The scar itself presents a physical barrier to regeneration–the dense cellular and ECM formation actually prevents axonal outgrowth–but in addition its major protein components, CSPGs, are strong anti-growth signaling molecules (Sharma et al., 2012). The exact mechanism of CSPG inhibition is somewhat unclear. CSPGs bind to numerous neuronal receptors and could be inhibiting growth through any, though among them are the Nogo receptors (NgR) which have known inhibitory properties (Sharma et al., 2012). There are other inhibitory molecules after injury that actively suppress neuronal outgrowth. Key players include the myelin-derived proteins Nogo, myelin-associated glycol protein (MAG), and oligodendrocyte myelin glycoprotein (OMgp), which also bind NgRs on the surface of neurons. NgRs have physiologic roles in the adult brain constitutively inhibiting dendrite and axonal turnover (Akbik et al., 2013), but when engaged by myelin debris after injury they potently inhibit axonal growth (Gonzenbach and Schwab, 2008; Pernet and Schwab, 2012; Wang et al., 2011).

The inhibitory activity of extracellular myelin and protein accumulation is amplified by the profound lack of debris clearance after CNS injury. Myelin debris in the CNS are ineffectively removed by phagocytes, lingering for years and likely contributing to long term inhibition of axonal growth (Brosius and Barres, 2014). This is in contrast to the PNS, a tissue that does regenerate after injury, where debris are efficiently cleared by Schwann cells and infiltrating macrophages (Brosius and Barres, 2014; Vargas and Barres, 2007). The CNS homolog of Schwann cells, oligodendrocytes, do not plagocytose debris after injury and instead apparently die or enter a senescent state (Brosius and Barres, 2014). Microglia and infiltrating macrophages phagocytose some debris (Ma et al., 2002), but are recruited only to the site of injury while myelin and apoptotic material also accumulates distally along the lesioned axon (Brosius and Barres, 2014). It is unclear why local microglia do not clear those distal debris, especially given a recent study showing them to be adept myelin phagocytes acutely after injury (Greenhalgh and David, 2014). Lack of myelin clearance is one of the most prominent differences between PNS and CNS injury, and the regeneration process that normally occurs in the PNS halts at the stage of debris clearance in the CNS.

Some myelin debris are directly inhibitory on axonal growth, and their sustained presence likely contributes to the lack of CNS regeneration (Brosius and Barres, 2014; Vargas and Barres, 2007). Given this, interfering with Nogo and CSPG molecules is a promising method to encourage neuroregeneration, and many studies have used animal models to validate their neutralization in vivo. For example, antibody blocking of Nogo-A improved cognitive function after TBI in rats (Lenzlinger et al., 2005) and enhanced axon sprouting and functional recovery after SCI in monkeys (Freund et al., 2007). Blocking CSPG signaling with CSPG digesting enzymes or receptor antagnoists to the injury site encourages regeneration in vivo (Bartus et al., 2014; Brown et al., 2012; Jefferson et al., 2011; Krekoski et al., 2001; Lang et al., 2015).

The discovery and blocking of these inhibitors generated high hopes for clinical translation. However, the importance of Nogo and other inhibitors was shaken by underwhelming results from knockout mice. One group created triple knockout mice lacking Nogo, MAG, and OMgp, and tested neuronal regeneration after injury. Disappointingly, though these mice had increased axonal sprouting, they showed no enhanced neuron regeneration (Lee et al., 2010). Furthermore, knocking out NgR, the putative receptor for both myelin-derived inhibitors and CSPG, had no affect on inhibition of neurite growth by myelin debris in vitro or regeneration/recovery in vivo (Zheng et al., 2005). Despite the lackluster findings in knockout animals, agents targeting these pathways are in the early stage of clinical translation. BA-210, a drug targeting a downstream molecule of NgRs (Rho), has shown promise in Phase I/IIa clinical safety trials and is currently being tested for efficacy (Fehlings et al., 2011; McKerracher and Anderson, 2013). Direct anti-Nogo antibodies are also being translated, and are being testing for SCI in phase II trials in Europe (Zorner and Schwab, 2010).

Anti-NOGO antibody CNS injury studies are generally assumed to work by blocking the ability of NOGO to bind NgR. A compelling alternative explanation offered by Dr. Ben Barres, however, is that these antibodies are opsonizing (coating) myelin particles to facilitate their clearance (Vargas and Barres, 2007). Myelin antibody opsonization is critical for normal

PNS myelin clearance and healing, and could be a useful therapeutic mechanism in the CNS (Vargas et al., 2010). Intriguigingly, The observation that antibodies against myelin-derived inhibitors like NOGO are more effeciacious at promoting recovery than transgenic removal of myelin inhibitors actually supports this hypothesis (Teng and Tang, 2005).

2.1.2 Secondary neuronal degeneration

Injury to the CNS is characterized by two distinct phases of neuronal death: first, an initial spike representing those cells killed by the trauma itself, and next, over the following hours to days a process of secondary neuronal death leading to increased lesion size and further impairment (Dusart and Schwab, 1994). Secondary degeneration was first hypothesized in 1911 and was the focus of much research extending into the early 90s (Dusart and Schwab, 1994), where its causative agents were hypothesized and tested. It appears to be caused by the combined effects of many noxious stimuli: free radicals generated after injury induce oxidative stress (Algattas and Huang, 2014), glutamate, released from neurons and microglia, causes excitotoxicity (Yawata et al., 2008), swelling of injured tissue crushes it in the limited space (Bareyre et al., 1997), impaired blood flow leads to hypoxia and metabolic dysfunction (a switch from aerobic to anaerobic glycolysis among other things) (Algattas and Huang, 2014), and some aspects of inflammation all work in concert to generate secondary death (Loane and Byrnes, 2010). It is unclear what percent of neurons are killed by secondary versus primary injury, or the degree of functional impairment secondary death instills, but studies manipulating apoptosis after injury have begun to answer these questions. Neurons die apoptotically due to secondary death after injury, followed by a surge of oligodendrocyte death presumably from loss of trophic support (Casha et al., 2005). Cells show apoptotic protein expression changes (ie. downregulation of BCLs and upregulation of BAX) and become TUNEL positive after injury (Casha et al., 2005; Raghupathi et al., 2002), and apoptosis accounted for roughly 10% of total cell death in a rat TBI model (Rink et al., 1995). A 2005 study from Casha et al. addressed the functional implications for this death.

FAS signaling is known to initiate apoptosis, and was also shown to play a role in death after CNS injury. The FAS knockout mouse had more oligodendrocyte, but no difference in neuron, survival after injury, suggesting that neurons die in a FAS-independent manner (Casha et al., 2005). Interestingly, despite the lack of neuronal survival differences the FAS knockout mice displayed increased recovery after SCI (Casha et al., 2005). A plethora of neuroprotective agents have been tested to prevent secondary death. These include glutamate antagonists (Yurkewicz et al., 2005), antioxidants (Kamat et al., 2008), anti-inflammatories like cyclosporine (Yokobori et al., 2013), or macrophage-based cellular therapy (Schwartz and Yoles, 2006). The drug riluzole is one such neuroprotective agent, currently used to treat amyotrophic lateral sclerosis. It has multiple mechanisms that limit secondary death, blocking glutamate release/excitotoxicity (Wang et al., 2004) and reducing cerebral edema (Bareyre et al., 1997), and is currently in phase II trials for acute spinal cord injury (Fehlings et al., 2012; Network, 2016).

2.2 The Initiation of CNS Inflammation: DAMPs, PAMPs, and alarmins

CNS injury results in a vigorous and complex peripheral immune response, recruiting and activating neutrophils, monocytes, and other cells to the injury site. Instead of delving directly into this response and its implications for SCI, however, we will take a brief but useful detour to discuss the general initiation of immune responses. Peripheral recruitment is preceded by an immediate response from local glia, which sense danger and produce chemokines and cytokines that sound the alarm. The process of immune activation is highly controlled, and with good cause; an inappropriate response can result in autoimmune damage while a lack of it when needed could be fatal.

So how do cells discern between friend and foe, sounding the alarm and initiating an immune response only when necessary? A classical explanation is that the immune system

discriminates between self and non-self, using specialized T and B-cell receptors that respond to foreign antigens, and mounts responses to anything deemed non-self. In the early 1990s Polly Matzinger proposed a new model for how the immune system chooses whether or not to respond, the danger theory (Matzinger, 2002a). This theory states that, rather than responding to self vs. non-self, the immune system initially responds to danger or damage signals. Under conditions of infection or trauma the theory posits that host- and pathogen-derived molecular cues, not self versus non-self discrimination, initiates the immune response. Matzinger pointed out that much of the previous evidence for the self/non-self hypothesis, such as experimental graft rejection, inherently involved an injury and therefore could be driven by danger signals (Matzinger, 2002a).

This idea revolutionized immunology, and explained previously unexplainable phenomenon. One example of such a phenomenon comes up in human lactation. At the start of lactation women produce completely novel milk antigens. These proteins should be regarded as non-self and rejected given the earlier model, which they obviously are not. As hypothesized in the danger model, the lack of initiating damage and subsequent danger signals prevents a significant response (Matzinger, 2002a). Her theory also predicted the existence of a new category of molecules–pathogen and damage associated molecular patterns (PAMPs and DAMPs) (Matzinger, 2002a; Seong and Matzinger, 2004). PAMPs and DAMPs are molecules that represent either the presence of pathogen or tissue damage, respectively, and are detected by pattern recognition receptors (PRRs) to initiate and amplify an immune response.

2.2.1 PRRs alert the immune system to pathogens and cell damage

PRRs represent a large group of receptors with an even larger repertoire of potential ligands. There are many types of PRR, including toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG receptors, and C-type lecithin receptors. Each of these categories has multiple representatives and species differences; for example, humans have 10 TLRs and 22 NLRs and

mice have 12 TLRs and 34 NLRs (Bryant and Monie, 2012). Many of these PRRs in turn have multiple ligands, typically a combination of PAMPs and DAMPs. An example of this is the toll-like receptor 4 (TLR4), well known for binding to lipopolysaccharide (LPS) found on the outer core of gram negative bacteria. In addition to LPS, however, TLR4 binds to a wide array of endogenous DAMPs, including heat shock proteins, fibrinogen, heperan sulfate, HMGB1, and many more (Erridge, 2010). Despite the diversity of receptors and ligands, classes of PRRs tend to induce similar intracellular signaling cascades. For example, TLRs excluding TLR3 signal through MyD88 (and also through other adaptors like TRIF), and NLRs signal through the inflammasome protein complex (discussed below).

Myd88 is a key intracellular signaling protein downstream of most TLRs, initiating a chain reaction leading to activation of the NFκB and P38 MAP kinase pathways (Lu et al., 2008). NFκB is a ubiquitous transcription factor, important to many systems including the immune system where it is generally activating and induces transcription of cell-specific inflammatory genes (Lawrence, 2009). NFκB has a huge number of identified target genes and is a large-scale regulator of activation. The Gilmore lab has compiled a list of publications showing genes regulated by NFκB. 479 genes, including cytokines, chemokines, growth factors, surface receptors, and others are represented here (Gilmore Lab, 2014), and computer models predict several hundred additional targets (Naamane et al., 2007; Shelest et al., 2003). The huge number and variety of NFκB targets exemplifies the global impact TLR stimulation can have on a cell, and responding to threats with TLR$\rightarrow$NFκB activation represents a major mechanism of immune induction.

2.2.2 The inflammasome and NLRs

Engagement of PRRs initiates production of inflammatory mediators that go on to orchestrate the immune response. Among these is the cytokine IL-1β, a potent cytokine made initially in a pro-form. IL-1β activation is a tightly regulated process governed by a catalytic protein complex known as the inflammasome (Mariathasan et al., 2006). There are many different

flavors of inflammasome, but typically they consist of three parts: an intracellular PRR, usually a NLR, pro-caspase 1, and an adaptor molecule connecting them. Inflammasomes are named after their NLRs, which play a familiar pattern-sensing role but are modified with an additional pyrin domain (becoming NLRPs). Hence literature will for example refer to the NLRP1 inflammasome when referring to the complex initiated by NLRP1 activation (Walsh et al., 2014a).

The inflammasome cascade begins with NLR engagement and recruitment of adaptor proteins and pro-caspase 1. Pro-caspase 1 is cleaved into its active form, caspase 1, which goes on to cleave and activate pro-IL-1β. In this way two stimuli are required for functional IL-1β: 1) NFκB activation, often by TLR stimulation, to produce pro-IL-1β, and 2) NLR stimulation to activate the inflammasome and cleave pro-caspase 1 (Walsh et al., 2014a).

Inflammasomes differ in their expression profiles and activation stimuli. The NLRP1 inflammasome, was the first identified but has relatively few known ligands, notably the anthrax product lethal toxin (LT). The NLRP3 inflammasome, in contrast, has a huge array of activating stimuli. These range from bacterial, viral, and fungal PAMPs (Walsh et al., 2014a), to DAMPs like reduced intracellular K^+ (Munoz-Planillo et al., 2013), extracellular ATP (Mariathasan et al., 2006), particulate matter (Hornung et al., 2008), ROS/mitochondrial damage (Walsh et al., 2014a), and even ultraviolet light (Feldmeyer et al., 2007). Once danger associated stimuli trigger the inflammasome, it rapidly activates IL-1β. IL-1β goes on to affect numerous cell types, interestingly also through the MyD88/NFκB and P38 MAPK pathways, potentiating the immune response. In this way the inflammasome directly couples pattern sensing with inflammation, and is a key component of immune response initiation.

2.2.3 PRRs and alarmins in CNS injury

Many pattern receptors are abundantly expressed in the CNS. TLRs are mainly expressed by microglia, but astrocytes, oligodendrocytes, neurons, and stem cells also express them (Kigerl and Popovich, 2009; Lathia et al., 2008). Pattern recognition is important in normal wound

healing, and skin healing is impaired in mice lacking MyD88 (Macedo et al., 2007) or an NLR (Campbell et al., 2013). A similar trend seems to be emerging in CNS injury, though fewer studies are available on this topic. Results from our lab have shown impaired recovery after both spinal cord and optic nerve trauma in MyD88$^{-/-}$ mice (Walsh et al., 2015). A 2007 paper by Kigerl et al. showed upregulation of TLR2, TLR4, and MyD88 on macrophages and astrocytes after SCI, and knocking out either of these resulted in impaired motor recovery and increased demyelination (Kigerl and Popovich, 2009). Interestingly, these impairments were associated with reduced IL-1β expression and increased macrophage activation (Kigerl and Popovich, 2009).

These studies are of TLR function in sterile conditions, presumably in response to DAMPs like HMGB1. Regardless, if TLR signaling is indeed beneficial, what would happen if it were strongly potentiated? Early works testing the affect of PAMPs on injury outcome addressed this question. In the 1950s Windle and colleagues treated spinal cord injured cats and monkeys with a systemic pyrogen (of which the active component was LPS) with the intention of studying neural centers for temperature regulation. Doing these studies they made an unexpected observation about the affect of their pyrogen on injury itself the pyrogen treatment increased sensory recovery in these animals (Popovich et al., 2012; Windle and Chambers, 1950). In further studies LPS increased axonal outgrowth and was associated with increased macrophage recruitment to the site of injury, supporting the beneficial role for TLR engagement and macrophages after CNS injury (Clemente and Windle, 1954; Popovich et al., 2012).

Toll and toll-like receptors represent an ancient family of proteins, highly conserved among species ranging from Drosophila to C. elegans (Tenor and Aballay, 2008), and though frequently involved in innate immune activation they have other roles. For example, TLR2 is highly expressed on neural precursor cells (NPCs) and TLR2 knockout animals have impaired adult neurogenesis (Rolls et al., 2007). This suggests that some endogenous TLR2 ligand, of which many are identified (Erridge, 2010), could be actively used in physiologic NPC

signaling and homeostasis (or the receptor acts without a ligand). Mature neurons also express TLRs. TLR3, an intracellular TLR recognizing viral dsRNA among other things, in particular is highly expressed in neurons and localized to neuronal growth cones (Cameron et al., 2007). Administration of Poly I:C, a synthetic ligand for TLR3, negatively affects neurite outgrowth (Cameron et al., 2007). These two vignettes, from NPCs and adult neurons, are a brief glimpse of what is known about healthy TLR function in CNS cells, and are meant to illustrate the roles for TLR signaling in homeostasis and the complexity that must arise in pathology. CNS TLRs (and likely other PRRs) have normal signaling functions, and after injury when extracellular PAMPs/DAMPs are abundant alterations of these functions must be considered alongside the classical one of immune activation.

The NLR/inflammasome complex is highly expressed in the brain and is activated after injury. All of the NLRP1 and NLRP3 inflammasome components are expressed in microglia and neurons, the NLRC4 inflammasome (primarily responsive to infection) is also expressed in microglia, and the NLRC2 inflammasome is expressed in astrocytes (Walsh et al., 2014a). Only a few factors that activate inflammasomes after sterile CNS injury have specifically been identified, but common stimuli such as ROS or extracellular ATP likely play a role. In neurons the NLRP1 inflammasome is activated rapidly after injury (de Rivero Vaccari et al., 2008), possibly in response to high extracellular K^+ (Silverman et al., 2009). Interestingly, though not directly relevant to CNS injury, amyloid β was identified as a potent activator of the NLRP3 inflammasome, and it may play a major role in initiating inflammation during AD (Halle et al., 2008).

One group has tested how the inflammasome affects injury outcome, testing both TBI and SCI models. In both cases neutralizing the NLRP1 and NLRP3 inflammasomes with anti-ASC antibodies significantly decreased pathology after injury (ASC is an intracellular target, and though they show anti-ASC reduces inflammasome activity and gets into neurons it is unclear conceptually how this happens) (de Rivero Vaccari et al., 2008, 2009). Though these papers focused on and attributed their affects to neuronal inflammasomes, their treatments

would also in theory affect microglia. It remains unclear whether microglia, neurons, or astrocytes have the most potent inflammasome activity/IL-1β production after injury, or whether activation of different subsets may be differentially beneficial or detrimental on outcome. From these few works, however, the overall affect of the inflammasome after injury seems to be detrimental.

Pro-IL-1β is rapidly activated after CNS infection, trauma, or stress, and much of what is known about NLR activation comes from studying the potent effects of active IL-1β. The IL-1R is highly expressed on astrocytes and microglia, particularly after injury, and triggers inflammatory cytokine production by those cells through MyD88 and NFκB (Chakraborty et al., 2010). Interestingly, certain neurons also express the IL-1R (Friedman, 2001). IL-1β has a quite different effect on neurons than on glia, affecting LTP and synaptic plasticity (Kelly et al., 2001). This difference is thought to be due to different intracellular signaling, with IL-1β triggering NFkB activation in glia and p38 MAPK activation in neurons (Srinivasan et al., 2004).

NLRs and TLRs, like most other PRRs, have many bacterial and endogenous ligands, some of which probably have not yet been identified. The variety of potential ligands makes it difficult to isolate the most important ones in CNS injury. Certain TLRs are beneficial after injury while the inflammasome seems to be detrimental, but these conclusions are based on a paucity of data.

Two endogenous alarmins have been described to act in the CNS: HMGB1 and ATP. HMGB1 is a nuclear protein alarmin. It is expressed in virtually all cells of the CNS (Daston and Ratner, 1994; Enokido et al., 2008; Gao et al., 2011; Tenenbaum et al., 2006), and once released by injury, HMGB1 signals through TLR4 and RAGE receptors to potentiate the migration, proliferation, and differentiation of immune cells (Degryse et al., 2001). In the CNS, HMGB1 strongly upregulates several chemokines in astrocytes, including neutrophil and T-cell chemoattractants (Pedrazzi et al., 2007). ATP, when released abundantly and in the presence of other DAMPs, is another potent CNS alarmin. It promotes inflammation

by activating the NLRP3 inflammasome (Di Virgilio, 2007), resulting in production of the inflammatory cytokines IL-1β and IL-18, promoting neutrophil recruitment, and activating microglia (Gadani et al., 2015a). IL-33 is, like HMGB1, a nuclear protein alarmin that has been described in the periphery, but its alarmin function in the CNS remains unknown. A major aim of this thesis is characterizing IL-33 in the CNS, and it will be introduced further in Section 2.4.

2.3 Inflammation After CNS Injury: Beneficial or Detrimental?

The immune response to CNS injury is an intensely controversial subject, with groups arguing that it is generally either harmful or beneficial. Perhaps originating from the early success of methylprednisone (which acutely suppresses inflammation) treatment, the immune response was long considered wholly detrimental. In recent decades the beneficial aspects of immune cells have come to light, and current strategies are often focused on harnessing these beneficial aspects rather than non-specifically suppressing inflammation. The initial immune response following CNS injury is characterized by a rapid influx of granular cells, mainly neutrophils, which are first responders after many insults. They are followed by monocytes, recruited to the injury site by chemokines (signaling proteins that are frequently chemoattractive) like CCL2 and differentiate into macrophages (Shi and Pamer, 2011). At weeks post injury lymphocytes, T-cells and B-cells, are prominent at the injury site (Figure 2.2) (Ankeny et al., 2009; Walsh et al., 2014a).

2.3.1 Neutrophils

Neutrophils are the first peripheral cells on the scene after most injuries. They are granular cells continuously produced in the bone marrow, and after a short life in circulation (˜5 days in humans and 12 hours in mice (Pillay et al., 2010), are cleared by bone marrow and liver

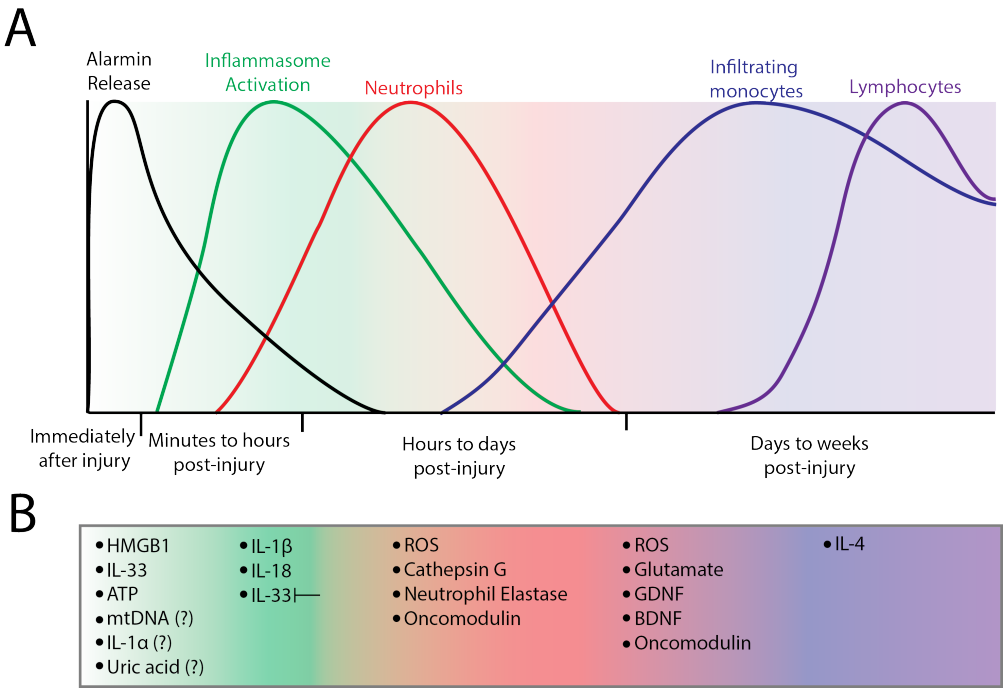

Figure 2.2: **Kinetics of the molecular and cellular immune response to CNS injury.** (A) The phases of molecular and cellular inflammation after CNS injury. DAMPs such as IL-33, HMGB1, and ATP are released immediately following CNS injury. The inflammasome is activated soon after and produces mature IL-1β and IL-18. Neutrophils arrive hours after injury and stay for several days, while monocytes begin infiltrating within the first day and remain present. Lymphocytes begin to arrive days to weeks post-injury. (B) Specific inflammatory molecules active at each time post-injury are listed. GDNF, glial cell line-derived neurotrophic factor; BDNF, brain-derived neurotrophic factor; ROS, reactive oxygen species.

macrophages (Shi et al., 2001; Summers et al., 2010). Their lifespan increases considerably after extravasating to injured or infected tissues, however, and once there they initiate an anti-bacterial response, releasing cytokines, chemokines, lytic enzymes, growth factors, and phagocytosing agents of infection (Summers et al., 2010). As in other injuries, neutrophils arrive in large numbers within hours of CNS injury. There are many factors that could promote neutrophil recruitment, including chemokines like KC or MIP2 (CXCL1 and 2, respectively), but a recent study showed almost complete inhibiton of recruitment in a sterile CNS injury model using direct application of purinergic receptor antagonists (Roth et al., 2014).

Neutrophils are specialized to combat pathogens, but what is their impact in sterile injury? After extravasating, neutrophils secrete toxic factors in hopes of killing pathogens, but these

efforts could be nonspecific and harmful. Indeed, blocking the neutrophil secreted enzyme elastase improved recovery of rats after spinal cord injury (Tonai et al., 2001). Multiple studies have gone on to tie beneficial effects of drugs to decreases in initial neutrophil recruitment (Naruo et al., 2003; Ozevren et al., 2014). A 2009 study by Stirling et al. sought to directly address the role of neutrophils in spinal cord injury by depleting them using a ly6G/GR-1 (a neutrophil surface protein) depleting antibody. Mice depleted of cells in this way actually had worse functional hindlimb recovery and delayed reactivity of astrocytes, suggesting that neutrophils have effects beneficially instruct the local glial response (Stirling et al., 2009). An important caveat to this study, however, is the non-specific nature of cell depletion. Though neutrophils were severely reduced, both circulating monocytes and lymphocytes were lowered in the antibody treated mice (Stirling et al., 2009). Another similar study looking at neutrophil depletion in an optic nerve crush injury demonstrated a similar beneficial role of neutrophils tied to their production of the growth factor oncomodulin (this time using a more neutrophil specific Ly6G clone) (Kurimoto et al., 2013). Literature remains unclear about the overall role of neutrophils in injury, specific products like elastase are detrimental but depleting the cells altogether is also detrimental, and the likely explanation is that they have a combination of effects (Figure 2.3).

2.3.2 Monocyte-derived macrophages

Monocyte-derived inflammatory macrophages (abbreviated from now on as macrophages to distinguish them from microglia) are prominent cells at the injury site. Blood monocytes come in two flavors described by unique functions and surface marker profiles: 1) $Ly6C^{hi}$ monocytes are the most prevalent and express the chemokine receptor CCR2, and 2) $Ly6C^{lo}$ monocytes are less common and express the receptor CX3CR1. $Ly6C^{hi}$ monocytes circulate through blood, and extravasate by rolling/diapedesis given appropriately activated endothelial cells and chemokines signals. $Ly6C^{lo}$ monocytes behave very differently than their $Ly6C^{hi}$

counterparts, instead of circulating they patrolling along the inner surface of blood vessels using CX3CR1 and LFA1 and rapidly extravasate at sites of injury (Auffray et al., 2007).

Both of these cell types differentiate into macrophages after entering the tissue (the term monocyte specifically refers to intravascular cells) (Auffray et al., 2007; Shi and Pamer, 2011), where among other healing activities they promote angiogenesis, scarring and clear debris. Their activities are clearly beneficial in regular healing, as ablation of macrophages impairs normal resolution in a skin wound model of healing (Koh and DiPietro, 2011), and for regeneration of limbs in amphibians (Godwin et al., 2013). Macrophages are robustly recruited along the entire nerve following PNS injury, where they play the beneficial role of clearing myelin and apoptotic debris (Brosius and Barres, 2014). The role of macrophages following CNS injury , however, is more controversial, with works showing them to be either beneficial (Figure 2.3) (Batchelor et al., 1999; Kotter et al., 2001; London et al., 2011; Prewitt et al., 1997; Shechter et al., 2009, 2013; Yin et al., 2006) or harmful (Evans et al., 2014; Horn et al., 2008; McPhail et al., 2004; Popovich et al., 1999).

2.3.3 Beneficial roles of macrophages in CNS injury

Macrophages have several functions at the injury site that promote both neuroregeneration and neuroprotection. They secrete survival and growth-promoting molecules including BDNF, GDNF, and oncomodulin (Dougherty et al., 2000; Hashimoto et al., 2005; Yin et al., 2006), phagocytose inhibitory debris (Ma et al., 2002), and promote pathogen clearance (in non-sterile injury) (Shi et al., 2001). A 2009 study by Shechter et al. addressed the impact of macrophages on SCI in three different ways: increasing the pool of monocytes with I.V. injection, depleting myeloid cells using the CD11C-Dtr mouse (dipthera toxin receptor is only expressed on $CD11C^+$ cells , allowing them to be selectively targeted), and preventing monocyte recruitment using a CCL2 blocking antibody (Shechter et al., 2009). Surprisingly, in all three conditions macrophages proved beneficial, adding macrophages increased functional recovery while removing them or restricting their recruitment decreased it (Shechter et al.,

2009). One of the major functions of macrophages also identified in this paper was production of IL-10, a cytokine that dampens and promotes resolution of inflammation (Shechter et al., 2009).

The beneficial aspects of adding additional macrophages have been well established for some time (Bomstein et al., 2003; Lazarov-Spiegler et al., 1998; Rapalino et al., 1998; Schwartz, 2010; Schwartz et al., 1999; Schwartz and Yoles, 2006). In one line of experiments spanning multiple works/decades, homologous macrophages are activated in vitro with tissue that heals well, typically skin or peripheral nerve, and then delivered directly to the CNS injury site. The intention here is to add phagocytes to encourage debris clearance, with the activation by DAMPs from the skin will instructing macrophages to become better tissue healers. This strategy is beneficial in animal models of SCI (Schwartz, 2010), and was tested in phase I (Knoller et al., 2005) and II clinical trials (Jones et al., 2010a; Lammertse et al., 2012). The clinical trials took patient derived monocytes and activated them with autologous skin for 24 hours. The monocytes were then purified (by CD14 expression), and 4 million cells were delivered by injection to 4 locations evenly spread through the contusion site (Knoller et al., 2005). The phase I study was promising, out of 8 SCI patients treated there was a higher than expected recovery rate (Knoller et al., 2005). These encouraging results led to a phase II study; 43 patients (23 treatment/17 control) were included in the trial, but it was suspended before completion because of a strong trend favoring the control group (P=0.053). This may not mean the treatment is harmful, however, as the control group had uncommonly high recovery, and the failure could be explained by improper delivery methods and low sample size (Lammertse et al., 2012; Lammertse, 2013).

2.3.4 Detrimental roles of macrophages in CNS injury

Other, detrimental products of macrophages on recovery have also been identified. The two most prominent of these are glutamate and nitric oxide (NO). Both microglia and macrophages release glutamate upon stimulation, which contributes to neuronal excitotoxicity and secondary

degeneration (Yawata et al., 2008). Macrophages also contribute to neurotoxicity through free radical production by the induced nitric oxide synthase (iNOS) enzyme. iNOS expression is upregulated within hours of injury on macrophages and perivascular cells and contributes to neuronal apoptosis (Satake et al., 2000).

Work by Jerry Silver and colleagues have discovered direct contacts between macrophages and neurons, both in vivo and in vitro, which appear to precede axon retraction (Evans et al., 2014; Horn et al., 2008). The nature of these interactions, its molecular mediators or purposes, are unclear. However, their evidence suggests a directly harmful effect of macrophages on neurons. Axon retraction in vivo is sharply reduced by clodronate liposome treatment, liposomes that kill engulfing phagocytes (mainly macrophages and microglia) (Horn et al., 2008). Recently they discerned the major culprit to be monocyte derived macrophages rather than microglia using a GFP bone-marrow chimera (Evans et al., 2014).

2.3.5 The importance of macrophage skew in CNS injury

Macrophages seem have both beneficial and harmful affects after injury, but how can the same cell type have such divergent functions? One explanation applied by the CNS injury field is the concept of macrophage skew. This is the idea that macrophages have different subtypes, typically divided into M1 (classically activated) and M2 (alternatively activated, tissue healing), which could both exist at the injury site and have opposite affects. The concept of macrophage M1 and M2 skew has been adopted by the CNS injury field, but originally derives from other systems where in certain conditions macrophages indeed adopt these distinct expression profiles and can behave very differently. For example, M2 macrophages are usually generated in allergic or anti-helminth immune responses by cytokines like IL-4 and IL-13 and characteristically express markers like arginase 1 (Arg1), CD206, and YM-1. M1 macrophages, on the other hand, are induced by stimuli such as IFN$\gamma$and LPS and express iNOS, TNF, and CCL5 (Sica and Mantovani, 2012).

An important 2009 paper by Kigerl et al. brought the M1/M2 concept to CNS injury, using RT-qPCR and immunofluorescence of select markers to describe a time course of M1 and M2 macrophage presence (Kigerl et al., 2009). Notably M2 macrophage markers were transiently upregulated at the injury site early on (1-3 DPI; based mainly on Arg1, other markers are not consistent), but M1 markers were elevated and persisted at later times (7-28 DPI; based mainly on CD16/32) (Kigerl et al., 2009). They next moved in vitro, co-culturing M1 (IFN$\gamma$and LPS treated) or M2 (IL-4 and IL-13 treated) macrophages with neurons. M1 macrophages promoted neuronal death and M2 macrophages encouraged axonal outgrowth, and given the persistent presence of M1 markers after injury it appeared that aberrant macrophage skew could be underlying significant pathology (Kigerl et al., 2009). Many subsequent works have built upon these findings. One recent paper proposed that macrophage phenotype is based on their route of entry. They described M2 macrophages arriving through the choroid plexus, where they presumably received skewing signals, and promoting healing. M1 macrophages, on the other hand, arrived by conventional transendothelial routes and are destructive (Shechter et al., 2013). M1/M2 skew is a convenient paradigm for explaining divergent macrophage effects at the injury site, and numerous studies have looked further at macrophage skew after injury (Girard et al., 2013; Kumar et al., 2013; Stirling et al., 2014; Turtzo et al., 2014; Wang et al., 2013). Though these studies universally use M1/M2 terminology, it is difficult to understand them together because they differ in macrophage skew markers used. Generally it is accepted that both subtypes are present at the injury site.

The situation is more complex, however, as demonstrated in a fascinating study by Hsieh et al. Here they used a mouse expressing YFP under the Arg1 promoter, causing putative M2 macrophages to fluoresce. They, like in other works, see robust Arg1 upregulation early after injury, with distinct Arg1 positive and negative populations (Hsieh et al., 2013). However, after sorting cells based on YFP and analyzing their transcription profiles they found that Arg1 positive cells, which should be M2, do not express many other M2 markers and actually express some M1 markers (Hsieh et al., 2013). These findings are not necessarily surprising,

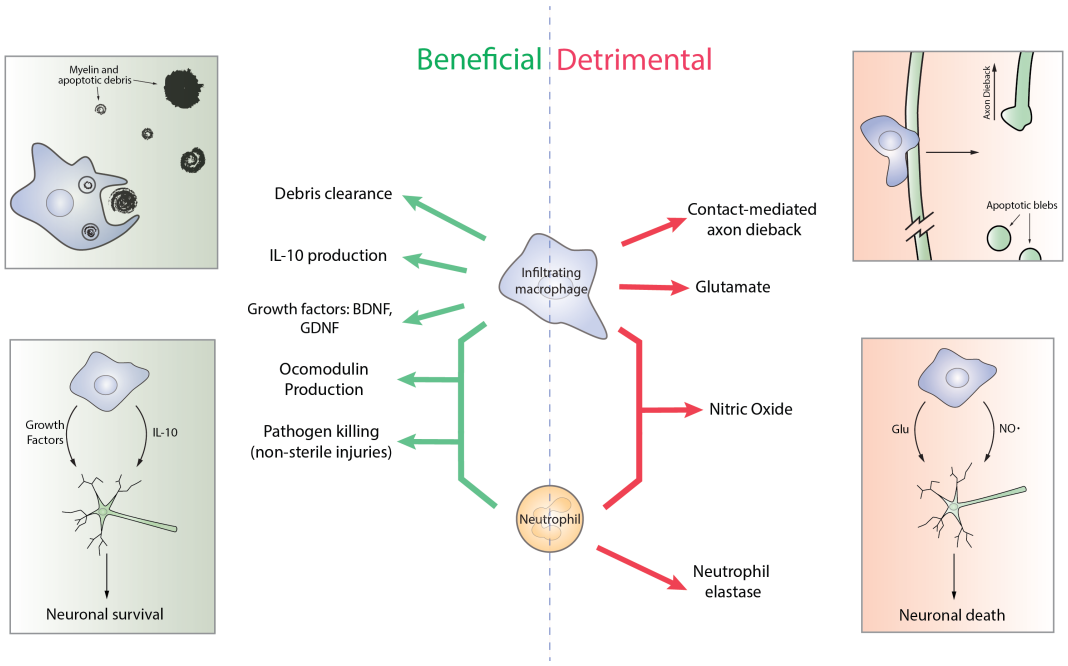

Figure 2.3: **Beneficial and detrimental roles for macrophages and neutrophils in CNS injury.** Macrophages and neutrophils have been described to promote both beneficial and detrimental outcomes following CNS injury. Whether these cells orchestrate CNS repair or exacerbate tissue damage following CNS trauma depends on the specific factors that are generated. Beneficial roles for macrophages (top left) in the CNS include their ability to clear cell debris and produce growth factors and other protective molecules, including BDNF, glial cell line-derived neurotrophic factor (GDNF), and IL-10. Detrimental roles for macrophages (top right) include production of glutamate and contact-mediated axon dieback. Both macrophages and neutrophils beneficially produce the atypical growth factor oncomodulin and clear pathogens in non-sterile injuries (left) and detrimentally produce the free radical nitric oxide (right). Neutrophils additionally secrete the enzyme elastase, which was shown to be detrimental following injury (right).

and it is well known that macrophage polarization is not starkly defined. Macrophages can exhibit simultaneous M1 and M2 marker expression, and may be able to switch phenotypes given appropriate stimuli (Sica and Mantovani, 2012). M1/M2 classifications as they exist in other situations do not apply neatly to macrophages after CNS injury, and the findings from Hsieh et al. highlight the need to move away from adopted nomenclature. Instead of trying to fit CNS infiltrating macrophages into inappropriate categories, efforts would like be better spent understanding the unique subsets existing at the injury site.

The ultimate role of macrophages in CNS injury is still an area of active investigation, and they have aspects that can clearly be good or bad (Figure 2.3). The degree of impairment

caused by depleting them suggests that their overall role is protective (Shechter et al., 2009), but soluble factors such as glutamate and NO are harmful. As phagocytes, macrophages help clear debris (Ma et al., 2002), but not sufficiently (Brosius and Barres, 2014), and promoting their engulfment of debris is a promising avenue of future research.

2.3.6 Lymphocytes

Lymphocytes are the major cell class of the adaptive immune system. Composed of T cells and B cells, lymphocytes share the ability to rearrange regions of their genome, creating unique receptors. The B cell receptor is a surface-bound antibody, which often becomes a secreted effector in inflammation or immunity. The T cell receptor always remains cell surface bound, and allows T cells to detect peptides that have been processed and presented on MHC molecules. When a T cell encounters its antigen, given the proper environmental and secondary cues, it can result in T cell activation. The outcomes of activation can be broadly defined based on two large subsets of T cells, termed $CD4^+$ and $CD8^+$ T cells based on surface marker expression. $CD8^+$ T cells generally detect antigen on non-immune cells through MHCI and induce killing of the MHC expressing cell, which presumably is infected by an intracellular pathogen. $CD4^+$ T cells, on the other hand, typically detect antigen through MHCII on antigen presenting cells (APCs; e.g. dendritic cells, B cells), and proliferate and differentiate to combat the particular threat. $CD4^+$ T cells have multiple differentiation states, including T_h1, T_h2, T_h17, and T regulatory cells (Tregs).

As mentioned earlier, the CNS parenchyma has no lymphocytes, neither T nor B cells, in the naive state. After traumatic injury, however, there is robust recruitment of T-cells, typically peaking at 7-10 days post injury (Raivich et al., 1998; Walsh et al., 2014b). This recruitment appears to be dependent on the adhesion molecule VLA4, frequently targeted to limit T cell infiltration in multiple sclerosis (Schwab et al., 2015), as administration of anti-VLA4 reduces T cell infiltration into the optic nerve after crush (JT Walsh unpublished observations).

Given the clearly pathologic roles for T cells in diseases like multiple sclerosis, it seemed likely that T cells would be pathologic after CNS trauma. Seminal experiments, however, suggested the opposite. In the early 2000s it was observed that athymic nude or $RAG^{-/-}$ mice, which have a greatly reduced number T cells, surprisingly had impaired neuronal survival after injury relative to WT (Serpe et al., 1999, 2000; Kipnis et al., 2002). In an optic nerve crush and nude mouse model, this impairment could be rescued by addition of spleenocytes, suggesting that T cells are required for normal survival after injury (Kipnis et al., 2002).

The story is not so straightforward, however, and it has increasingly been appreciated that not all T cells are beneficial. Though neuronal survival could be rescued in the nude mouse by spleenocytes, there was an increased benefit in adding back spleenocytes depleted of Tregs ($CD4^+$/CD25+ cells) (Kipnis et al., 2002). Tregs dampen other T cell responses through production of modulators like IL-10 and competing for the T cell survival/growth signal IL-2 (Josefowicz et al., 2012). Perhaps then Tregs are inhibiting the full impact of a beneficial T cells response?

This conclusion was challenged by results using a more potent Treg depletion strategy–the DEREG mouse. When the majority of Tregs were depleted in this model, recovery from optic nerve crush was actually impaired (Walsh et al., 2014b). Confusingly, addition of Tregs into WT mice after injury was also detrimental (Walsh et al., 2014b). The authors conclude that Treg numbers have to be strictly controlled, with too few Tregs leading to overactive inflammatory damage but too many Tregs suppressing beneficial T cell inflammation. Indeed, both extreme Treg conditions resulted in pathologic changes of myeloid cells, linking Treg over/under-suppression to dysfunction of the innate immune system (Walsh et al., 2014b; Walsh and Kipnis, 2011).

Based on the above discussed findings, T cells clearly have a net beneficial impact after injury, though their activity must be carefully titrated by an appropriate Treg response (Walsh et al., 2014b). What then are the beneficial activities of T cells? A series of exciting early studies into this question suggested that autoreactive, brain antigen specific, T cells,

were actually mediating benefit (Kipnis et al., 2000; Moalem et al., 1999; Hauben et al., 2000; Kipnis et al., 2001; Yoles et al., 2001). Mice injected with myelin basic protein-reactive T cells (T_{mbp}) immediately after optic nerve crush had less secondary neuronal death, as assessed by RGC enumeration, outgrowth, and optic nerve electrophysiology studies (Moalem et al., 1999). These findings were repeated in the spinal cord injury model (Hauben et al., 2000), and linked to an increase in overall T cell numbers at the injury site (Moalem et al., 1999; Kipnis et al., 2000). Another study reported that T_{mbp} cell administration caused a dramatic increase in neurotrophic factors produced in the optic nerve, giving a potential mechanism to previously observed neuroprotection (Barouch and Schwartz, 2002).

These studies regarding T_{mbp} largely focused on addition of autoreactive T cells to enhance recovery, and do not necessarily address the physiologic beneficial function of T cells uncovered by studies in immunocompromised mice.

This topic was recently investigated by our lab, and unexpectedly we observed an antigen *non*-specific mechanism working to produce neuroprotective T cells. MHCII is required for APC activation of and differentiation of $CD4^+$ T cells, and $MHCII^{-/-}$ animals therefore lack both T cells and the ability to activate their TCR should they be introduced. As expected, $MHCII^{-/-}$ animals had impaired neuronal survival after optic nerve crush (Walsh et al., 2015). The critical experiment that tested the relevance of antigen specificity came in rescue experiments, where T cells are given to $MHCII^{-/-}$ animals. If antigen specificity were important to generate neuroprotective T cells, as was suggested by earlier studies, this reconstitution should have no effect. Instead, giving back T cells in $MHCII^{-/-}$ fully rescued deficits. Two strange things were observed about this beneficial T cell response: that it was dependent on MyD88 and that the T cells produced IL-4. The potential ligands that signal through MyD88 that could give rise to IL-4 producing T cells are few, and we identified IL-1β as the likely signaling molecule (Walsh et al., 2015). Finally, we demonstrated in vitro that T cell derived IL-4 could be directly neuroprotective on neurons through neuronal IL-4R signaling, completing the antigen-independent mechanism of beneficial T cell activity (Walsh

et al., 2015).

2.4 IL-33 is a Nuclear Alarmin and Reporter of Cell Death

IL-33 is a chromatin-associated alarmin expressed at baseline in many tissues. IL-33 is somewhat unique among cytokines, typically acting as a DAMP rather than an actively secreted signaler. Upon release after necrotic cell death, IL-33 escapes the nucleus and can act on multiple cells through its specific receptor (IL-33R). The IL-33R is a heterodimer of ST2 and the IL-1 receptor associated protein (IL-1RAcP). There is also a soluble splice form of ST2 that acts as a non-signaling decoy receptor. Upon binding, IL-33 signals through MyD88, the same adaptor protein used by most TLRs and the IL-1R, and the pathway results similarly in activation of NFκB and MAP kinase. Though IL-33 is constitutively expressed in the nucleus, a clear role for it there has not been found. It appears to participate in gene silencing through association with heterochromatin (Schmitz et al., 2005), but this has been contested by high resolution imaging methodologies showing it actually associating with euchromatin (Kakkar et al., 2012). Mice specifically lacking IL-33 are viable and have no gross abnormalities, demonstrating that its baseline nuclear functions are not necessary to survival, or are redundant with other proteins (personal observations).

IL-33 is a member of the IL-1 cytokine family along with IL-1β , IL-1α , and IL-18. As discussed earlier, IL-1β and IL-18 require inflammasome activation and caspase 1 cleavage to become activated, and this was initially thought to be the case for IL-33. Detailed studies refuted this notion, revealing that in contrast to IL-1β and IL-18, caspase 1 cleavage actually inactivates IL-33 (Cayrol and Girard, 2009). The same group later showed that certain cleavage forms of IL-33 are more potent than the native form, particularly the products of neutrophil elastase and cathepsin G cleavage (Lefrancais et al., 2012). This makes sense given IL-33s alarmin nature. IL-1β is produced by effector cells and requires multiple signals

to be fully active. IL-33, on the other hand, is a rapid post-mortem message from innocent bystander cells and therefore (logically) requires no processing for activity. Furthermore, neutrophils generally degranulate at the injury site, secreting enzymes like elastase that can cleave native IL-33 to a more active form and reinforce the effect of IL-33 release early after injury. These features portray IL-33 as a player immediately after injury, initiating a response but being quickly neutralized by the inflammasome.

2.4.1 Amplification of type 2 immune responses by IL-33

IL-33 was identified in 2005 as a ligand for the then orphaned receptor ST2 in a landmark paper (Schmitz et al., 2005). The group discovered IL-33 using a bioinformatics approach to scan the genome for new IL-1 family members, going on to identify IL-33, its receptor, and characterize its basic tissue localization and function. Screening many tissues, they found the highest IL-33 mRNA expression in skin, lung, brain, and spinal cord (Schmitz et al., 2005). ST2 had previously been connected to type 2 immune responses , and they go on to show amplified cytokine production of Th2 but not Th1 cells when stimulated with IL-33 (Schmitz et al., 2005). They also injected large doses of IL-33 into mice, again finding an amplification of the type 2 immune response, with increased plasma eosinophils and type 2 cytokines (IL-4, 5, and 13) after treatment (Schmitz et al., 2005).

The intracellular cascade induced by IL-33 is common to many other DAMPs and PAMPs, most of which do not specifically amplify type 2 immunity. How then does IL-33 accomplish this? The answer is in tightly regulated expression of ST2, being highly expressed in particular on type 2 immune cells such as mast cells, type 2 innate lymphocytes (ILC2s), and Th2 cells. Th2 cells are highly responsive to IL-33, not because it drives activation of a special transcription factor or unique signaling pathway, but simply because ST2 is highly expressed on Th2 and not Th1 cells (Lohning et al., 1998). Similarly mast cells, tissue resident granular cells and histamine producers, and ILC2s, a newly defined class of cells that produce type 2 cytokines, express high levels of ST2 and are therefore sensitive to IL-33 stimulation (Moulin

et al., 2007; Price et al., 2010). Other immune cells are also affected by IL-33–it activates basophils (Suzukawa et al., 2008), eosinophils (Cherry et al., 2008), potentiates macrophage activation/skew (Joshi et al., 2010), and can encourage dendritic cells to skew T-cells to Th2 (through OX40L costimulatory molecule expression) (Besnard et al., 2011). The route through dendritic cells is one of the few ways IL-33 can actually affect T-cell phenotype, and is the exception to the rule of pure activation (Halim et al., 2014).

2.4.2 IL-33 signaling underlies pathology and protection

Long before the discovery of IL-33, ST2 was already associated with type 2 immune responses and recognized as an important player in the pathology of allergy (a disease characterized by overactive type 2 inflammation) (Kamradt and Burmester, 1998). The field was invigorated by the discovery of ST2's ligand, and subsequent studies have confirmed a vital role for IL-33/ST2 in potentiating allergic inflammation (Ohno et al., 2012). IL-33 signaling is pathologic in several disease models: neutralizing its signaling ameliorates colitis (Oboki et al., 2010; Sedhom et al., 2013), arthritis (Schmitz et al., 2005), asthma (Liew et al., 2010), and allergy (Oboki et al., 2010) in mice.

IL-33 also has key roles in health. Evolutionarily its purpose was likely pathogen, particularly parasite, defense. IL-33 production in the gut is stimulated by intestinal helminth (parasitic worm) infections, and it is critical for promoting T_h2 T cell skewing there (Humphreys et al., 2008). The $ST2^{-/-}$ mouse is also more susceptible to T. gondii-induced encephalitis (Jones et al., 2010b). IL-33 promotes wound healing in the skin (Yin et al., 2013a,b) and gut (Lopetuso et al., 2012), and is protective in models of atherosclerosis and obesity (Miller, 2011). Like many cytokines, unchecked IL-33 signaling is pathologic, but a normal response is critical for the response to parasites, wound healing, and disease resistance.

2.4.3 Cellular expression and effector functions of IL-33 in the CNS

Schmitz et al. originally reported high IL-33 expression in both mouse and human CNS (Schmitz et al., 2005), and this was confirmed by a study using a LacZ element downstream of the IL-33 promoter (Pichery et al., 2012). Despite these studies, the actual expression of IL-33 in the CNS remained uncertain due to many studies that contradicted initial reports. Several papers observed that IL-33 is not expressed highly in the CNS at baseline, only being expressed after an inflammatory stimulus (Jiang et al., 2012; Hudson et al., 2008; Jones et al., 2010b). Another group searched for IL-33 expression in development, finding it in OLIG2^{+} cells, though they too detected no expression in the adult (Wicher et al., 2013). Using a different antibody with high specificity (as controlled by staining the IL-33$^{-/-}$ mouse), we demonstrated that IL-33 is abundantly expressed in the healthy brain, being expressed by myelinating oligodendrocytes and gray matter astrocytes (See results) (Gadani et al., 2015b). This observation has since been repeated by other groups. Zarpelon et al. described an identical distribution of IL-33 expression at baseline, but interestingly also saw upregulation in the spinal cord after a sciatic nerve injury (Zarpelon et al., 2016). Another recent study addressed IL-33 expression in the retina, finding it expressed in both mouse and human vimentin^{+} Müller glia (Xi et al., 2016). In analysis of retinas from human macular degeneration patients, they also demonstrated upregulation in the diseased tissue relative to healthy controls (Xi et al., 2016). Though there is some disagreement in the literature, most recent studies agree that IL-33 is heavily expressed in the healthy CNS, primarily by subsets of neuroglia. Though IL-33 mRNA is abundant in mouse and human (Schmitz et al., 2005), further studies are needed to study IL-33 protein expression the human brain.

IL-33 is a potent stimulator of type 2 inflammation, largely due to enriched ST2 expression by type 2 immune cells. Many other cell types are capable of responding to IL-33, however, including those in the CNS. In the healthy state ST2 protein expression is difficult to detect on glia by conventional means such as flow cytometry or immunoflouresence (personal

observations), but it can be detected indirectly by treating glia and measuring the response, or by measuring mRNA via RT-PCR. IL-33 treatment on mixed glia induces production of cytokines like TNF and IL-1β, and the chemokine CCL2, a monocyte and lymphocyte chemoattractant (Hudson et al., 2008). Further studies have confirmed that IL-33 is a critical inducer of chemokines in various CNS injury models (Gadani et al., 2015b; Xi et al., 2016), and it is likely affecting both astrocytes and microglia (Gadani et al., 2015b). By RT-PCR ST2 mRNA is highest on microglia at baseline, but interestingly is downregulated by microglia and upregulated by astrocytes after injury (Gadani et al., 2015b).

2.4.4 IL-33 in CNS pathology

We are increasingly becoming aware that inflammation has a role in most—if not all—neurologic diseases. IL-33 is uniquely positioned to initiate and drive inflammation in the CNS, and it has justifiably received much recent attention in studies of disease models. IL-33 has garnered interest as a serum biomarker for various CNS insults given its mechanism of release from dying cells (Qian et al., 2016). IL-33 has also been recognized in GWAS studies of neurologic disease (Chapuis et al., 2009).

Mechanistic studies probing the importance of IL-33 in disease models typically use two strategies: addressing the role for endogenous protein by using the IL-33$^{-/-}$ or ST2$^{-/-}$ animals, and asking whether additional IL-33 is beneficial through administration of recombinant protein. IL-33 has critical roles outside the CNS, creating several caveats that should be kept in mind when analyzing data using these strategies. IL-33 plays a central role adaptive thermogenesis and adipose tissue homeostasis (Brestoff and Artis, 2015), causing a number of changes in healthy IL-33$^{-/-}$ animals versus WT, for example having higher weight at baseline (Brestoff et al., 2015). Systemic IL-33 treatment similarly has profound effects independent of the CNS. IL-33 activates the bodies type 2 immune cells, resulting in profound and global type 2 inflammation (Schmitz et al., 2005). Therefore any effects of IL-33 treatment can

not be assumed to be through IL-33 acting on neuronal cells, but any number of changes associated with type 2 inflammation.

Summarized below is the role of IL-33 in Alzheimer's disease, multiple sclerosis, and CNS injury. Studies exist regarding IL-33 in other CNS conditions, including stroke (Korhonen et al., 2015; Luo et al., 2015), neuropathic pain (Zarpelon et al., 2016; Liu et al., 2015), infection (Peng et al., 2013; Hudson et al., 2008; Palomo et al., 2015; Tong and Lu, 2015), and age related macular degeneration (Xi et al., 2016), but given a dearth of literature these will not be specifically discussed here.

IL-33 in Alzheimer's disease

There is evidence that IL-33 is important in Alzheimers disease (AD); a screen of >2500 genes for transcript expression from AD versus healthy patient brain samples showed a significantly lower level of IL-33 mRNA in diseased patients (Chapuis et al., 2009). These findings of decreased IL-33 were confirmed at the protein level (Chapuis et al., 2009), and correlated with increased levels of the decoy IL-33 receptor, sST2 (Fu et al., 2016). Furthermore, three distinct polymorphisms in the IL-33 gene were enriched in AD patients relative to control, and they were succesfully predictive of AD risk in unbiased prospective studies (Chapuis et al., 2009; Yu et al., 2012).

In light of the reduced amount of IL-33 seen in AD patients, a recent study tested the effects of adding back IL-33 into a mouse model of AD (the APPswePS1de9 mouse, overexpressing APP and a mutant presenlin) (Fu et al., 2016; Jankowsky et al., 2004). IL-33 treatment had impressive effects, reversing LTP and behavioral deficits and reducing soluble Aβ levels (Fu et al., 2016). Reduction in Aβ was linked to in vivo increases in microglia phagocytosis of amyloid and an altering of myeloid phenotypic markers to a more m2 state (Fu et al., 2016). Amazingly these changes in Aβ clearance occurred after as little as 2 days of moderate (200ng/day) IL-33 treatment (Fu et al., 2016).

IL-33 in multiple sclerosis and experimental autoimmune encephalomyelitis

Multiple sclerosis (MS) is an autoimmune neuroinflammatory disorder characterized by demyelination, pathology inflammation, and neuronal death (Steinman, 2014). In contrast to AD, patients with MS have elevated IL-33 in the CSF (Jafarzadeh et al., 2016; Sosvorova et al., 2015), serum (Christophi et al., 2012; Jafarzadeh et al., 2016; Sosvorova et al., 2015), and on a per cell basis in the CNS (Zhang et al., 2014; Christophi et al., 2012). 2–3 fold elevations in IL-33 are typically seen regardless of disease progression in the CSF and serum, and IFNβ treatment partially restores normal levels (Jafarzadeh et al., 2016; Christophi et al., 2012).

The functional role of IL-33 in MS has largely been addressed in the mouse model experimental autoimmune encephalomyelitis (EAE), where animals are injected with activated autoimmune T cells or auto-antigen and adjuvant to elicit an MS-like condition. These studies have produced conflicting results, showing overall detrimental (Li et al., 2012), beneficial (Jiang et al., 2012; Milovanovic et al., 2012; Chen et al., 2015), or no effects (Oboki et al., 2010) of IL-33 signaling on EAE outcome. An early study by Li et al. demonstrated a beneficial effect of anti-IL-33 neutralizing antibodies on EAE (Li et al., 2012). They made a similar observation in the converse experiment, administering recombinant IL-33 and impairing EAE outcome. Blocking IL-33 decreased levels of pathologic cytokines like IFNγand IL-17, and this was the proposed mechanism linking it to detrimental inflammation (Li et al., 2012).

Li et al. suggested that IL-33 is harmful in EAE, but this study was limited by the lack of genetically modified mice. Indeed once developed these mouse tools, such as the $ST2^{-/-}$ mouse, seemed to show the opposite for IL-33 in EAE. $ST2^{-/-}$ mice had exacerbated disease course, and in apparent contradiction to Li et al. IL-33 treatment was beneficial to outcome (details in treatment regimen and background strain differ between studies) (Jiang et al., 2012). Another study revisited anti-IL-33 treatment, showing that i.c.v. administration was detrimental in EAE (Chen et al., 2015). Finally, adding to the confusion, another study induced EAE in the $IL\text{-}33^{-/-}$ animal and reported no difference compared to WT (Oboki et al.,

2010). The reasons for these contradictory results remain unknown, studies were performed in homologous mouse models using similar EAE induction strategies. Despite this, the basic finding that IL-33 is upregulated in EAE is agreed upon (Li et al., 2012; Jiang et al., 2012; Chen et al., 2015). The current body of mouse and human studies points to a role for IL-33 in MS, though details are clearly uncertain at this point.

IL-33 in CNS injury

We and others have recently begun to address the role of IL-33 in CNS injury. Our results will be briefly described here, and at length in Sections 3.1 and 3.2. As described previously, IL-33 can stimulate CCL2 production by mixed glia in vitro (Gadani et al., 2015b; Kempuraj et al., 2013), and IL-33$^{-/-}$ mice show significantly reduced production of several chemokines at the injury site after SCI (Gadani et al., 2015b). This defect in chemokine expression was coupled with reduced recruitment of peripheral monocytes, impaired recovery, and increased lesion volume after SCI, and with decreased neuronal survival after optic nerve crush (Gadani et al., 2015b). Furthermore, exogenous administration of CCL2 into the site of SCI in IL-33$^{-/-}$ mice was found to promote recovery, suggesting that delayed monocyte recruitment contributes to increased pathology in these animals (Gadani et al., 2015b). Interestingly, IL-33 signaling can also be potentiated to improve outcomes in wild-type mice, as intraperitoneal injection of exogenous IL-33 was found to improve locomotor recovery and reduce lesion size after SCI (Pomeshchik et al., 2015).

Usually when thinking about CNS injury we are considering traumatic injury that results in immediate death of neurons and glia, as well as delayed secondary neuronal death. In theory any necrotic death of oligodendrocytes or IL-33^{+} astrocytes will result in IL-33 release, but there are several reports of IL-33 release in the absence of cell death (Xi et al., 2016; Fock et al., 2013; Kakkar et al., 2012). One example of this is in retinal chronic light exposure injury. Müller glia express IL-33, and were shown to release an activated 19KDa cleaved form without dying (Xi et al., 2016). Once released, the authors demonstrate that it is, as in spinal

cord injury, critical for monocyte recruitment, though in their model this is detrimental to recovery (Xi et al., 2016).

2.5 Type 2 innate lymphocytes are recently described IL-33 responsive cells

As mentioned above, IL-33 is a potent stimulator of type 2 inflammation due to enriched receptor expression on certain cells types. Among the most sensitive cells to IL-33 are ILC2s, tissue resident cells that produce large amounts of type 2 cytokine in response to certain DAMPs. ILC2s are part of a larger group of innate lymphocytes, subdivided into group 1, natural killer (NK) cells and type 1 ILCs (ILC1s), group 2 , ILC2s, and group 3, lymphoid tissue inducer (LTi) cells and type 3 ILCs (ILC3s). These classifications mirror the T cell subsets T_h1, T_h2, and T_h17, respectively in their lineage-defining transcription factors and cytokine production (Sonnenberg and Artis, 2015).

The existence of LTi cells and NK cells has been known for decades, but other ILCs were not formally described until the late 2000s (Diefenbach et al., 2014). There were early hints towards the existence of ILC2s. In 2001 Fort et al. observed that IL-5 and IL-13 are produced after systemic IL-25 administration in $RAG^{-/-}$ animals, though the cellular source was unknown (Fort et al., 2001). A subsequent 2006 study showed that IL-25 administration induced repulsion of the helminth *N.brasiliensis* (Fallon et al., 2006). IL-25-mediated repulsion was unaffected in $RAG^{-/-}$ mice, but did not work in mice lacking IL-4, IL-13, IL-5, and IL-9 (Fallon et al., 2006). These two results suggested the existence of a completely novel cell type, which is not part of the adaptive immune system (exists in $RAG^{-/-}$ mice) but capable of producing T cell cytokines (IL-4, IL-5, and IL-13). Fallon et al. reported this population as IL-25 dependent, $Ckit^{+}$, and $Fc\varepsilon R\alpha^{-}$ (a mast cell marker) (Fallon et al., 2006).

In 2010 ILC2s were comprehensively characterized by three independent studies. These studies all identified a lineage negative ("lineage" referring to a combination of markers used

to identify common immune cell types such as macrophages, neutrophils, T cells, and B cells) population that produced type 2 cytokines in response to IL-25 or IL-33 (Neill et al., 2010; Moro et al., 2010; Price et al., 2010). Though describing similar cells, these studies disagreed on the name, calling then natural help cells (Moro et al., 2010), nuocytes (Neill et al., 2010), or innate type 2 helper cells (Price et al., 2010). The nomenclature was finally resolved in 2013 by consensus among current leaders in the field (Spits et al., 2013).

ILC2s have since been studied as a unified population of lineage$^-$ cells that constituitively express high levels of the transcription factor Gata3 (also found on T_h2 cells) and respond to mainly IL-33, IL-25, PGD2, and TSLP by producing IL-4, IL-5, IL-13, and amphiregulin (Sonnenberg and Artis, 2015). They have been found to play a role in numerous diseases through amplification of type 2 immunity (Halim, 2016), and also are critically involved in thermogenesis/lipid metabolism (Odegaard and Chawla, 2015) and eosinophil homeostasis (Nussbaum et al., 2013).

2.5.1 ILC2 development and life history

Large strides have been made in the past 1-2 years identifying the key events of ILC development. ILCs derive from hematopoetic stem cells in the bone marrow and, despite their striking similarities to T cells, do not enter the thymus as part of maturation. All ILC subtypes share a common progenitor, the αLP cell (Possot et al., 2011). From this cell NK and ILC lineages split, and some αLPs begin expressing GATA3 and PLZF, become the progenitor of ILC1s, ILC2s, and ILC3s, the common helper innate lymphoid cell progenitor (CHILP) (Constantinides et al., 2014; Yang and Bhandoola, 2016). From the CHILP cell ILC2s differentiate through its own progenitor, the ILC2P, which is characterized by sustained GATA3 expression and a lack of RORγt expression (Hoyler et al., 2012).

The details of how a CHILP 'decides' which line of differentiation to follow remain unknown, but there is intriguing evidence that ILC progenitors may migrate into peripheral tissues and be guided by tissue specific signals there (Yang and Bhandoola, 2016). For

example, an ILC precursor population was isolated from the intestinal lamia propria that could give rise to both ILC1s and ILC3s (Possot et al., 2011). The concept of endpoint differentiation could explain the observed variation in ILC ratios among tissues. For example, ILC2s are the most abundant ILC subset in lung, skin, and meninges, but ILC3s are the most predominant in the intestine (Spencer et al., 2014; De Grove et al., 2016). This could be explained by tissue specific signals, such as vitamin A, which are high in the gut. Vitamin A deficiency reduced gut ILC3 and expanded ILC2s, in what the authors propose to be an adaptation to promote helminth defense during malnutrition (Spencer et al., 2014). This is one example of a tissue specific signal, but it raises the possibility that others could be discovered and provide us with insight into how the tissue microenvironment controls ILC subsets and overall inflammation (Matzinger and Kamala, 2011).

Most proteins involved in ILC2 development are also required for development of T cells and/or other ILC populations. It has therefore remained a significant hurdle in the field to develop genetic methods of targeting ILC2s. One key transcription factor that is largely specific to ILC2 development is RORα, and so called "staggerer" ($rora^{sg/sg}$) mice lacking RORα have specifically impaired ILC2 differentiation (Wong et al., 2012; Halim et al., 2012b). RORα is also required for development of neuron subtypes, notably Purkinje cells, hence the label staggerer describes the abnormal gait of these mice (Sidman et al., 1962). Bone marrow chimeras of $rora^{sg/sg}$ into WT mice yields mice that have ILC2s that do not proliferate in response to IL-25 stimulus (Wong et al., 2012)

Though ILCs are similar in many ways to T cells, one fascinating difference is their localization; ILCs tend to concentrate in peripheral tissues, and are rarely present in lymphoid tissues such as spleen or lymph node (Nussbaum et al., 2013). ILCs were therefore tentatively labeled as 'tissue-resident' cells, but a 2015 study by Gasteiger et al. was the first to rigorously demonstrate their lack of migration (Gasteiger et al., 2015). Using a parabiosis model, where the blood supplies of two mice are connected to allow cell cross-over, the authors asked what percent of ILCs crossed between mice. The results clearly demonstrated minimal turnover. At

40 days post parabiosis there was virtually no turnover of ILCs (excluding NK cells) between mice in any tissue tested (Gasteiger et al., 2015). The authors next immune challenged mice during parabiosis, by depleting Tregs or with a helminth infection, showing in both cases no migration of ILCs. A significant and systemic increase in ILC2 numbers was noted after helminth infection, but this was due wholly to local proliferation (Gasteiger et al., 2015).

2.5.2 The role of ILC2s in pathology

ILC2s are implicated in an ever-growing list of pathologies, playing either beneficial or detrimental roles through amplification of type 2 inflammation. Discussed here are ILC2s in helminth defense, ashtma/allergy pathology, and CNS diseases. ILC2s have been implicated in numerous other disorders including colitis (Sonnenberg, 2014), dermatitis (Salimi et al., 2013), cancer (Li et al., 2014), and rhinosinusitis (Ho et al., 2015) (reviewed in (Halim, 2016)).

ILC2s in helminth infection

The clearest beneficial role for ILC2s is in helminth infection, where type 2 cytokines are critical for defense (Anthony et al., 2007). Local ILC2s are major acute sources of IL-13, and in chronic or reinfection work with $CD4^+$ T cells to maintain alternative macrophage activation (Bouchery et al., 2015). Alternatively activated macrophages, induced by ILC2s and Th2 cells, are effectors against helminths (Bouchery et al., 2015). ILC2s also secrete the atypical growth factor amphiregulin , which promotes healing and survival of injured epithelial cells (Monticelli et al., 2011; Turner et al., 2013). Finally, ILC2s promote their own survival during infection, producing IL-9 which maintains them in an autocrine manner (Turner et al., 2013; Halim, 2016).

ILC2s produce cytokine after injury, but what stimulates them? This question was unintentionally addressed by a group looking for an in vivo source of IL-25. IL-25 had been recognized as a potent activator of ILC2s for years, but puzzlingly a role–or even a major producer–of it had not be described in vivo. Von Moltke et al. explored this by building an

IL-25 reporter mouse (RFP driven under the IL-25 promoter) and looking for RFP^+ cells in the healthy mouse (von Moltke et al., 2016). Surprisingly the only cells they found that express IL-25 at baseline were tuft cells embedded in the gut epithelium. This constitutive IL-25 secretion regulates a basal ILC2 activation state, and during helminth infection tuft cells become more abundant, producing more IL-25 and further activating ILC2s (von Moltke et al., 2016). This discovery does not fully explain how ILC2s detect and respond to helminths, instead it merely pushes our original question onto another cell type. It therefore remains to be shown how tuft cells detect and respond to an invading helminth.

ILC2s in allergy and asthma

Asthma and allergy are pathologies driven by excess type 2 inflammation. It is perhaps not surprising then that ILC2s are major players in disease. Asthmatic patients have elevated ILC2 numbers in their blood (Bartemes et al., 2014), and SNPs in several ILC2 associated genes such as *il13*, *il33*, and *il1rl1* (the gene that codes ST2) correlate significantly with asthma (Moffatt et al., 2010). In the lung, inhaled antigens stimulate the release of cytokines IL-33, IL-25, or TSLP, activating ILC2s (Halim, 2016). ILC2s carry out effector functions through acute cytokine production. Autocrine IL-9 secretion maintains ILC2s, and IL-13 and IL-5 drive pathology in allergy and asthma (Mohapatra et al., 2016).

IL-13 promotes goblet cell hyperplasia, excess mucus production, and airway hyperreactivity (AHR) (Wills-Karp, 2004; Halim et al., 2014). Though T_h2 cells are also a source of this cytokine, ILC2-derived IL-13 is thought to be pathogenic. $RAG^{-/-}$ animals get AHR in response to influenza, but not when treated with ILC-depleting anti-Thy1.2 antibodies (Chang et al., 2011). Similar results were found in other cytokine or allergen based models of lung inflammation, where IL-13-competent ILC2s were found to be required to generate AHR. ILC2s also drive mucus production. Papain inhalation increased mucus production in $RAG^{-/-}$, but not $RAG^{-/-}$/IL-$2R\gamma^{-/-}$ mice (Halim et al., 2012a). This effect could be rescued by WT, but not $ROR\alpha^{-/-}$ ILC2s (Halim et al., 2012b).

IL-5 produced by ILC2s is also pathogenic, in particular by promoting eosinophilia. Several studies have compared mice that lack lymphocytes (ie. $RAG^{-/-}$) versus those lacking both ILC2s and lymphocytes (ie. $RAG^{-/-}$/IL-2R$\gamma^{-/-}$), showing that ILC2s are sufficient to drive lung eosinophilia (Doherty et al., 2013; Halim et al., 2012a). Converse experiments, adding back ILC2s into deficient mice, gave supporting results. Transfer of WT bone marrow into $RAG^{-/-}$/IL-2R$\gamma^{-/-}$ mice was able to restored papain induced eosinophilia, but transfer of ILC2-deficient ROR$\alpha^{sg/sg}$ bone marrow did not (Halim et al., 2012b).

From these data ILC2s seem to be a necessary component of pathology type 2 inflammation, but where do T_h2 cells fit? Though a relatively unstudied topic, it appears that ILC2s may be crucial in driving the differentiation of T_h2 cells. For example, ILC2-derived IL-13 activated lung dendritic cells to migrate to local lymph nodes and promote T_h2 differentiation of naïve T cells (Halim et al., 2014). T_h2 cells are major sources for both IL-13 and IL-5, whose pathologic activities were previously discussed, and in this manner ILC2s promote pathology directly through secreted molecules but also indirectly through promoting T_h2 skew.

ILC2s in CNS disorders

The presence or function of potential CNS ILC2s has been studied only at the most peripheral level, with only 2 studies attempting to study them (Besnard et al., 2015; Russi et al., 2015). The first paper by Besnard et al. focused on cerebral malaria, a condition where type 2 immunity was previously suggested to be beneficial (Knowles, 2011). They hypothesized that systemic IL-33 administration would ameliorate disease by acting on CNS ILC2s and, through them, generating alternatively activated macrophages (Besnard et al., 2015). IL-33 treatment was indeed beneficial and correlated with increased ILC2s and M2 macrophages in the spleen, but the CNS was never actually analyzed for presence or activation of ILC2s (Besnard et al., 2015).

The second study by Russi et al. looked at CNS ILC2s in EAE. Originally intending to study mast cells, the authors demonstrated exacerbated disease in male SJL-W/W^v (a

mouse model with mutant C-kit and as a result no mast cells) versus WT mice. Interestingly, when they attempted to rescue the defect with mast cell reconstitution it had no effect (Russi et al., 2015). Puzzled with this result, the authors looked to other cell types, discovering that SJL-W/W^{v} animals were also deficient in ILC2s (Russi et al., 2015). They were able to detect ILC2s in the CNS (pooled brain and spinal cord) by flow in naïve animals, and the numbers increased 10 days post EAE induction. These ILC2s produced type 2 cytokines differentially in male versus female mice, and the authors proposed this as potentially contributing to the clear female bias in MS incidence (Russi et al., 2015).

Neither of these studies rigorously characterized CNS ILC2s, or definitively demonstrated them in the naïve CNS. Given the general lack of immune cells (other than microglia) in the CNS parenchyma, the presence of ILC2s there warrants close scrutiny. Furthermore, no study to date has tested the activation of ILC2s after CNS injury, or the effect of specifically increasing ILC2 numbers on recovery. These are questions I have addressed as part of this thesis, with results described in Section 3.2 and discussed in Chapter 4.

Chapter 3

Results

3.1 The glia-derived alarmin IL-33 orchestrates inflammation after CNS injury

We first examined the expression levels of IL-33 in different regions of the healthy periphery and CNS. There is high variability of IL-33 expression among CNS areas, with a trend towards tissues with higher myelin content having higher IL-33 expression (Figure 3.1A-B).Given the high expression levels of IL-33 in myelinated areas, we suspected oligodendrocytes to be a primary CNS source for IL-33. To test this we performed immunofluorescent labeling for IL-33 and OLIG2, a transcription factor expressed mainly by cells of the oligodendrocyte lineage. There is substantial colocalization of these markers, revealing enriched IL-33 expression in OLIG2$^+$ cells in several CNS regions (Figure 3.2A-B). To identify mature oligodendrocytes we co-labeled IL-33 with CC-1 (APC), again demonstrating a high degree of co-expression (Figure 3.2C). In most tissues analyzed, the majority of IL-33 positive cells were mature oligodendrocytes (Olig2$^+$CC-1$^+$; Figure 3.2D), but there was a significant portion of non-oligodendrocyte IL-33$^+$ cells in regions such as spinal cord gray matter and neocortex (Figure 3.2E).

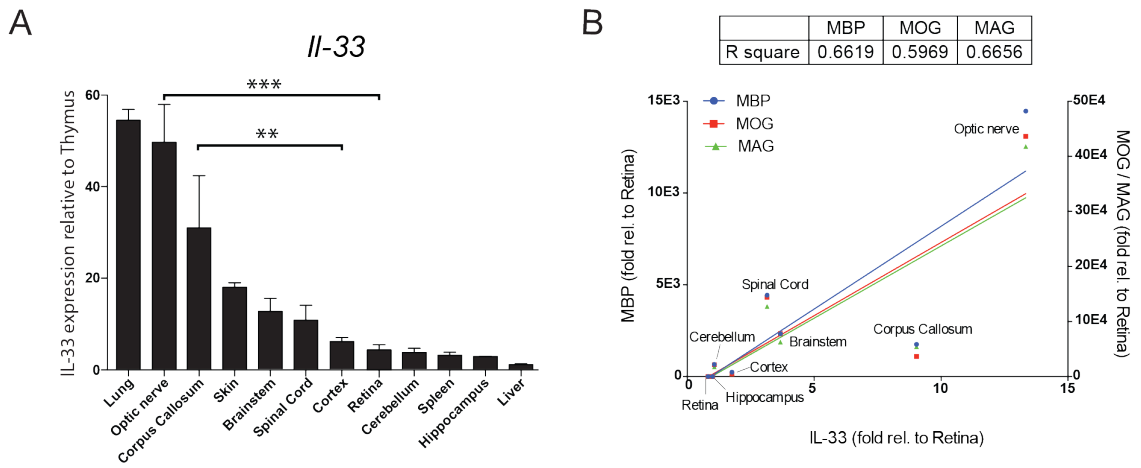

Figure 3.1: **IL-33 expression is concentrated in myelinated CNS regions.** (A) Mouse peripheral tissues and brain regions were isolated and analyzed for IL-33 transcript (N=3; one-way ANOVA with Tukeys multiple comparison test). (B) Transcript levels of IL-33 were correlated with expression of myelin genes in several CNS regions (N=3).

Co-staining of IL-33 with the astrocyte marker ALDH1L1 (Cahoy et al., 2008) demonstrated that these cells were astrocytes, which interestingly express IL-33 in grey matter regions such as the neocortex but not in white matter such as the corpus callosum (Figure 3.2F-G). To further describe the expression of IL-33 in different subsets of the oligodendrocyte lineage, we performed flow cytometry of healthy whole brain (Figure 3.3A). About 33% of isolated brain cells were IL-33$^+$ (Figure 3.3B). IL-33 expression increased in oligodendrocytes with maturity and myelination; myelinating oligodendrocytes (OLIG2$^+$/MOG$^+$) express the most IL-33 both as a percent of cells and by mean fluorescent intensity (MFI), whereas pre-myelinating oligodendrocytes and oligodendrocyte precursor cells (OPCs) express significantly less (Figure 3.3C-D). IL-33 expression was minimal to none in neurons, microglia, or proliferating Ki-67$^+$ cells (Figure 3.4A-C). We conclude that in the healthy CNS IL-33 is expressed by the majority of mature oligodendrocytes and by grey matter astrocytes.

We next sought to test whether IL-33 in the CNS acts as alarmin. Immunofluorescence analysis of the injured spinal cord one day post injury (1DPI) demonstrates a disappearance of IL-33 from the lesion site (Figure 3.5A), suggesting its release from damaged tissue. To further assess the kinetics of release, we measured IL-33 in the cerebrospinal fluid (CSF) after

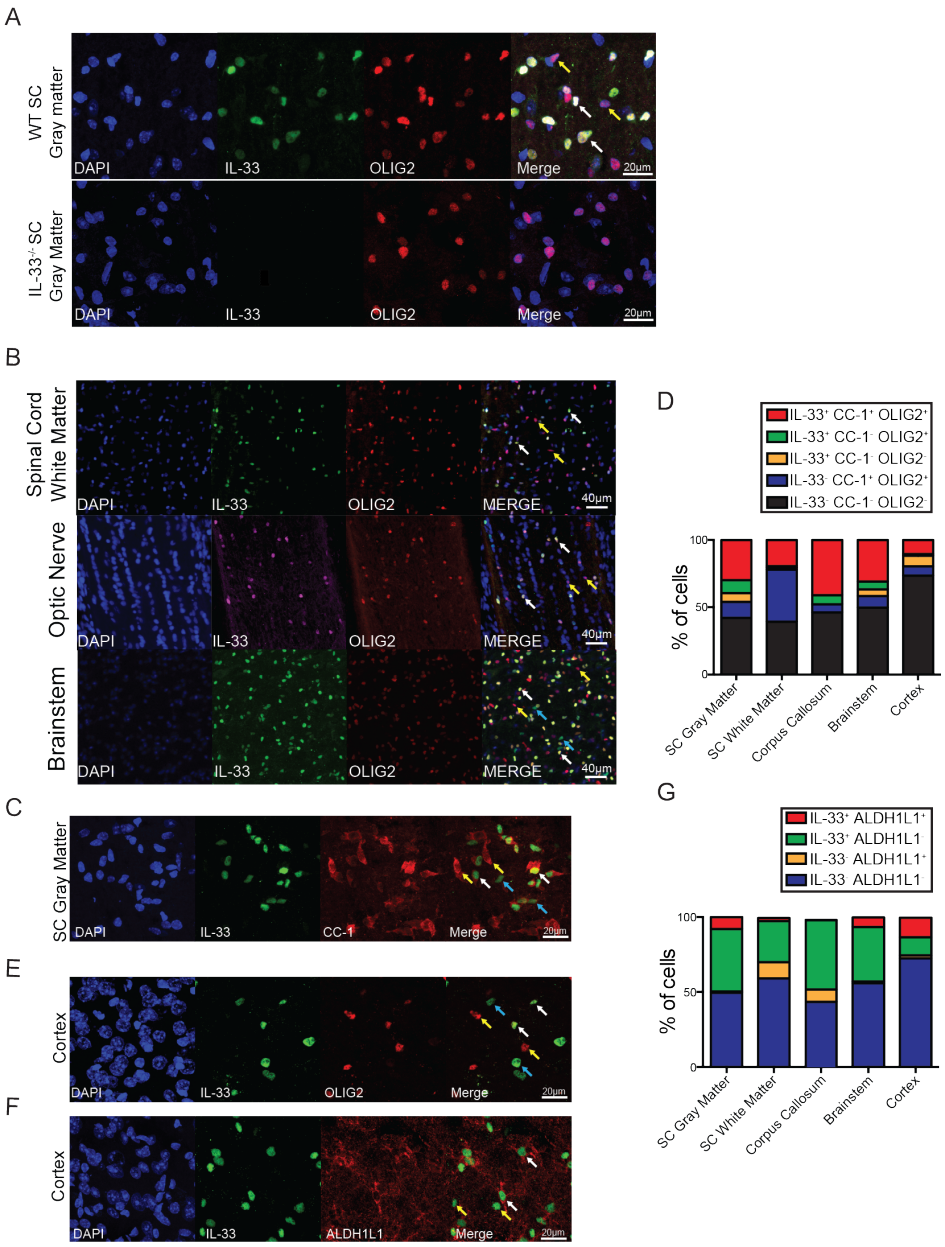

Figure 3.2: **IL-33 is expressed in mature oligodendrocytes and gray matter astrocytes.** (A) Representative labeling of OLIG2 and IL-33 in spinal cord gray matter of (top) WT mice (white arrows=OLIG2$^+$/IL-33$^+$ cells; yellow arrows=OLIG2$^+$/IL33$^-$ cells) and (bottom) IL-33$^{-/-}$ mice as a negative control. (B) Representative images from (top) spinal cord white matter, (middle) optic nerve and (bottom) brainstem demonstrate IL-33 expression and variable OLIG2 colocalization (white arrows=IL-33$^+$/OLIG2$^+$, yellow arrows=IL-33$^-$/OLIG2$^+$, blue arrows=IL-33$^+$/OLIG2$^-$). (C) Representative labeling of CC-1 and IL-33 in WT spinal cord gray matter (white arrows=CC-1$^+$/IL-33$^+$ cells; yellow arrows=CC-1$^+$/IL33$^-$ cells; blue arrows=CC-1$^-$/IL-33$^+$ cells). (D) Quantification of IL-33, OLIG2 and CC-1 positive cells in multiple CNS regions (data is presented as mean counts from 4-5 individual animals). (E) Representative image of OLIG2 and IL-33 labeling in neocortex (white arrows=OLIG2$^+$/IL-33$^+$ cells; yellow arrows=OLIG2$^+$/IL33$^-$ cells; blue arrows=OLIG2$^-$/IL-33$^+$ cells). (F) Representative labeling of IL-33 and ALDH1L1 in the neocortex (white arrows=ALDH1L1$^+$/IL-33$^+$ cells; yellow arrows=ALDH1L1$^-$/IL-33$^+$ cells). (G) Quantification of ALDH1L1 and IL-33 labeling in multiple CNS regions (data is presented as mean counts from 4-5 individual animals).

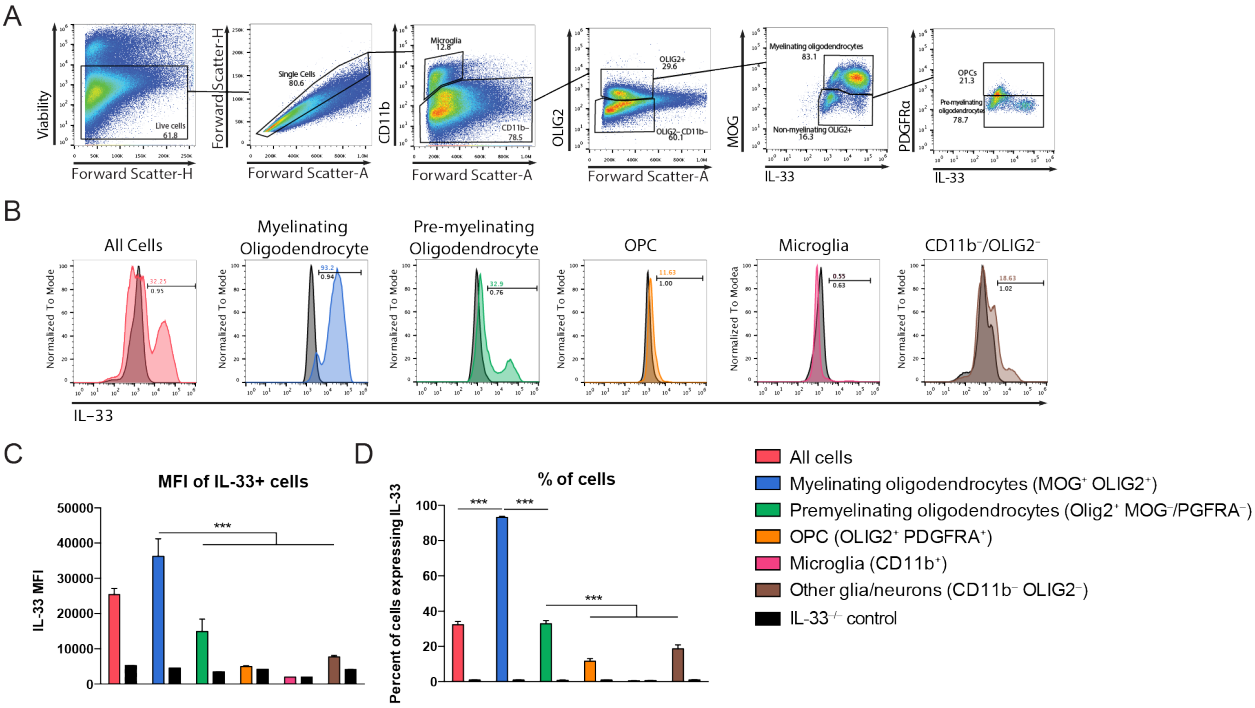

Figure 3.3: **Flow cytometry analysis of CNS IL-33**(A) Representative gating strategy for isolating oligodendrocyte lineage cells from whole brain by flow cytometry. Gates for oligodendrocyte antigens were drawn based on fluorescence minus one (FMO) controls, gates for IL-33 were drawn based on IL-33$^{-/-}$ samples. (B) Histograms showing IL-33 expression in several CNS cell populations isolated from whole brain for flow cytometry. Black lines represent staining of IL-33$^{-/-}$ brain. (C-D) Summary of MFI of IL-33^{+} populations (C) and percent of cells expressing IL-33 (D) (N=3-4; one-way ANOVA with Tukeys multiple comparison test).

spinal cord injury. IL-33 is not detectable in healthy CSF, but it appears 1 hour after spinal cord contusion. IL-33 presence is transient, not being detectable at 3 or 24 hours post-injury (Figure 3.5B) likely due to rapid CSF recirculation.

Spinal cord segments contused in vitro released IL-33 within 10 minutes, with maximum release at 1 hour that persisted after 30 hours in the supernatant (Figure 3.5C). Uncontused spinal cords also released IL-33 due to the injury of excision itself, albeit at a lower concentration (Figure 3.5D). Notably, in vitro release of IL-33 protein occurred without induction of new transcription (Figure 3.5E). This data suggests release of endogenously expressed protein after injury instead of its de-novo synthesis.

To address the role of IL-33 in recovery from CNS trauma, we subjected IL-33$^{-/-}$ and wild-type (WT) mice to an optic nerve crush injury (Yoles and Schwartz, 1998), a model that is dependent on immune assistance for optimal neuronal survival (Kipnis et al., 2004; Walsh et al., 2014b; Yoles et al., 2001). In this assay, retinal ganglion cells (RGCs) are pre-labeled with the retrograde tracer FluoroGold (FG) injected stereotaxically into the superior colliculus. A compression injury is inflicted to the optic nerve three days post labeling, and retinal ganglion cell (RGC) survival is assessed 7 days after the injury by counting labeled RGCs on whole-mounted retinas (Figure 3.6A). IL-33$^{-/-}$ mice showed significantly less RGC survival after optic nerve injury as revealed by FG labeled cell enumeration (Figure 3.6B).

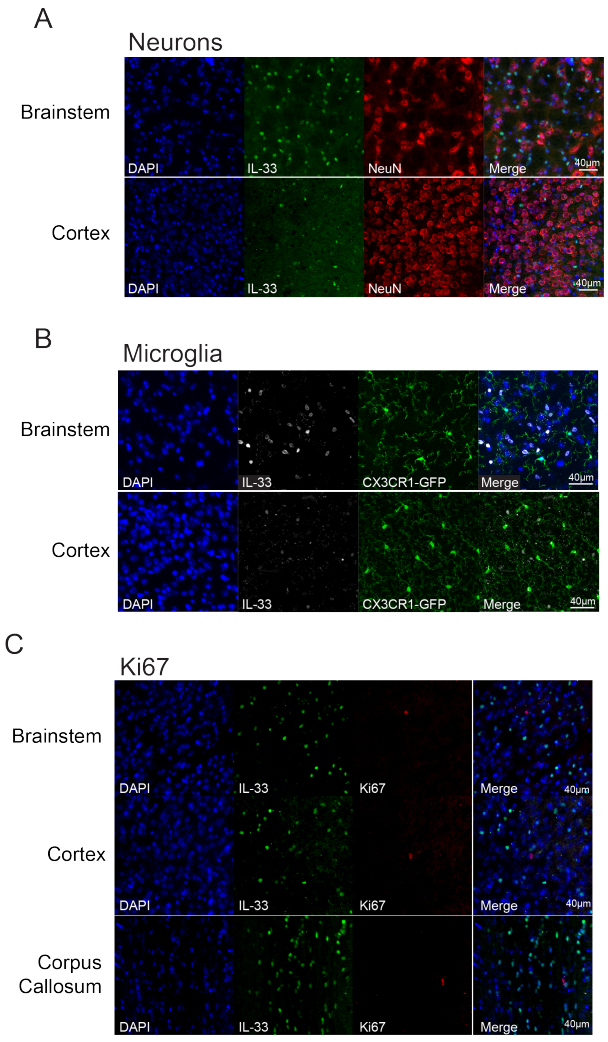

Figure 3.4: **IL-33 expression in neurons, microglia, and proliferating cells.** Representative images of (A) costaining of IL-33 and NeuN, (B) staining of IL-33 in a mouse expressing GFP under the CX3CR1 promoter and (C) costaining of IL-33 and Ki67. Images representative of at least 3-4 individual animals, IL-33 was not detected in NeuN^{+}, CX3CR1^{+}, or Ki67^{+} cells.

To assess whether IL-33 expressing cells must be actually damaged for IL-33 to have an affect, we used a second retinal injury model, intraocular glutamate injection (Schori et al., 2002). IL-33 is abundant in the optic nerve but is not expressed in the retinal cells that would be exposed to glutamate in this model, and therefore we predicted that IL-33$^{-/-}$ mice will not exhibit an impaired response to this injury. Seven days after glutamate injection, we counted RGCs by labeling them using their specific transcription factor Brn3a

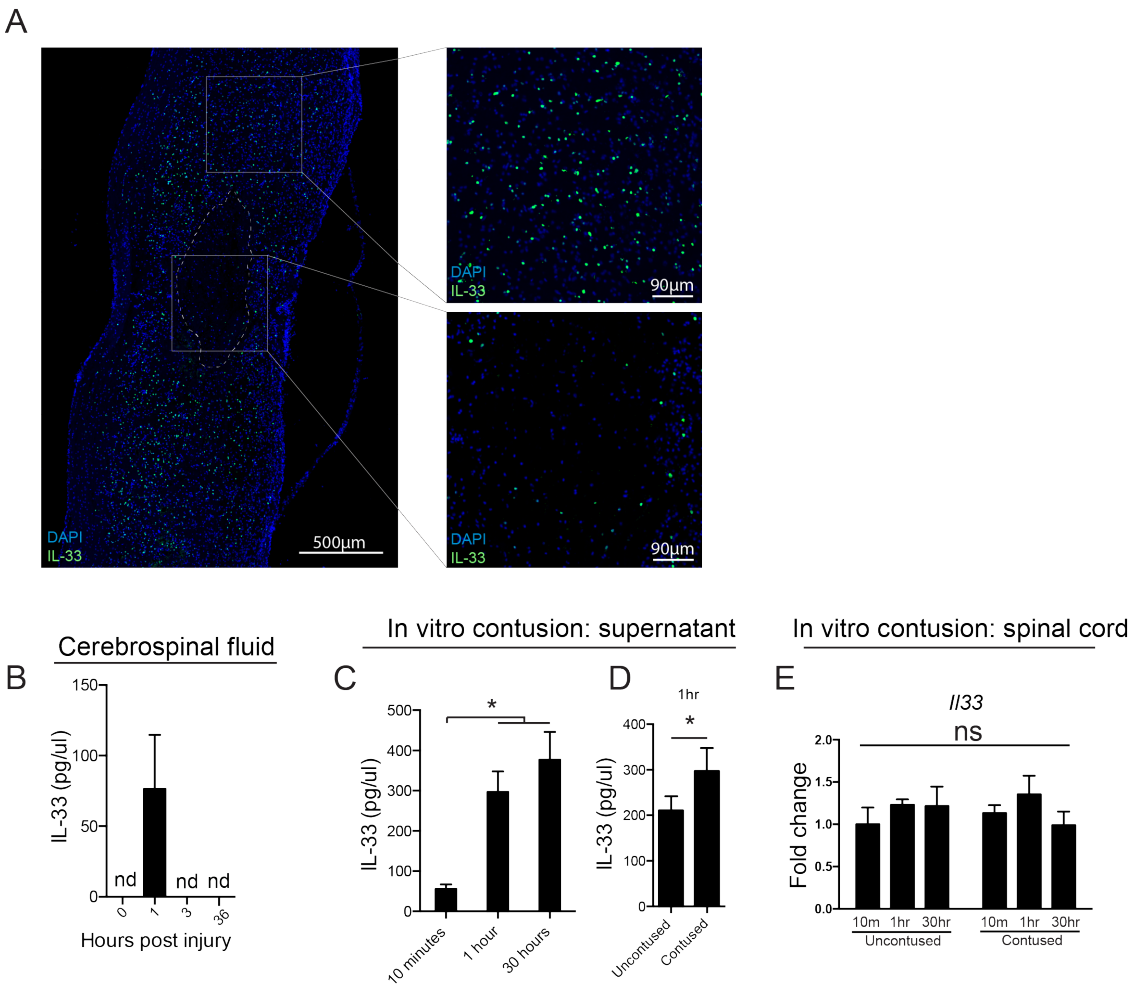

Figure 3.5: **IL-33 is released after CNS injury without induction of new transcript.** (A) Representative image of a spinal cord injury cite 1DPI stained for IL-33. IL-33 positive nuclei are present adjacent to the injury (top right) but absent at the injury site (bottom right). Images are representative of 4 individual animals. (B) CSF was gathered from mice after spinal cord contusion and analyzed for IL-33 by ELISA. IL-33 was detectable only at 1 hour post-injury (N=3 mice per group). (C) IL-33 ELISA of in vitro contused spinal cords. 4mm pieces of spinal cord were contused in individual wells of a 96 well plate and incubated for 10 minutes, 1 hour, or 30 hour before supernatants were collected for ELISA (N=5 individual animals; one-way ANOVA with Tukeys multiple comparison test). (D) Spinal cord segments release more IL-33 with contusion than without (N=5 individual animals; paired two-tailed T-test). (E) In vitro contused spinal cords were harvested and analyzed for IL-33 transcript, showing no induction of IL-33 mRNA (N=5 mice per group; one-way ANOVA with Tukeys multiple comparisons test).

(Figure 3.6C). As expected, direct glutamate toxicity did not lead to exaggerated retinal ganglion cell death in IL-33$^{-/-}$ mice compared to their WT controls (Figure 3.6D).

Optic nerve injury is a model of severe CNS damage. The majority of RGCs die in WT mice, making it challenging to sensitively detect further impairment due to a floor

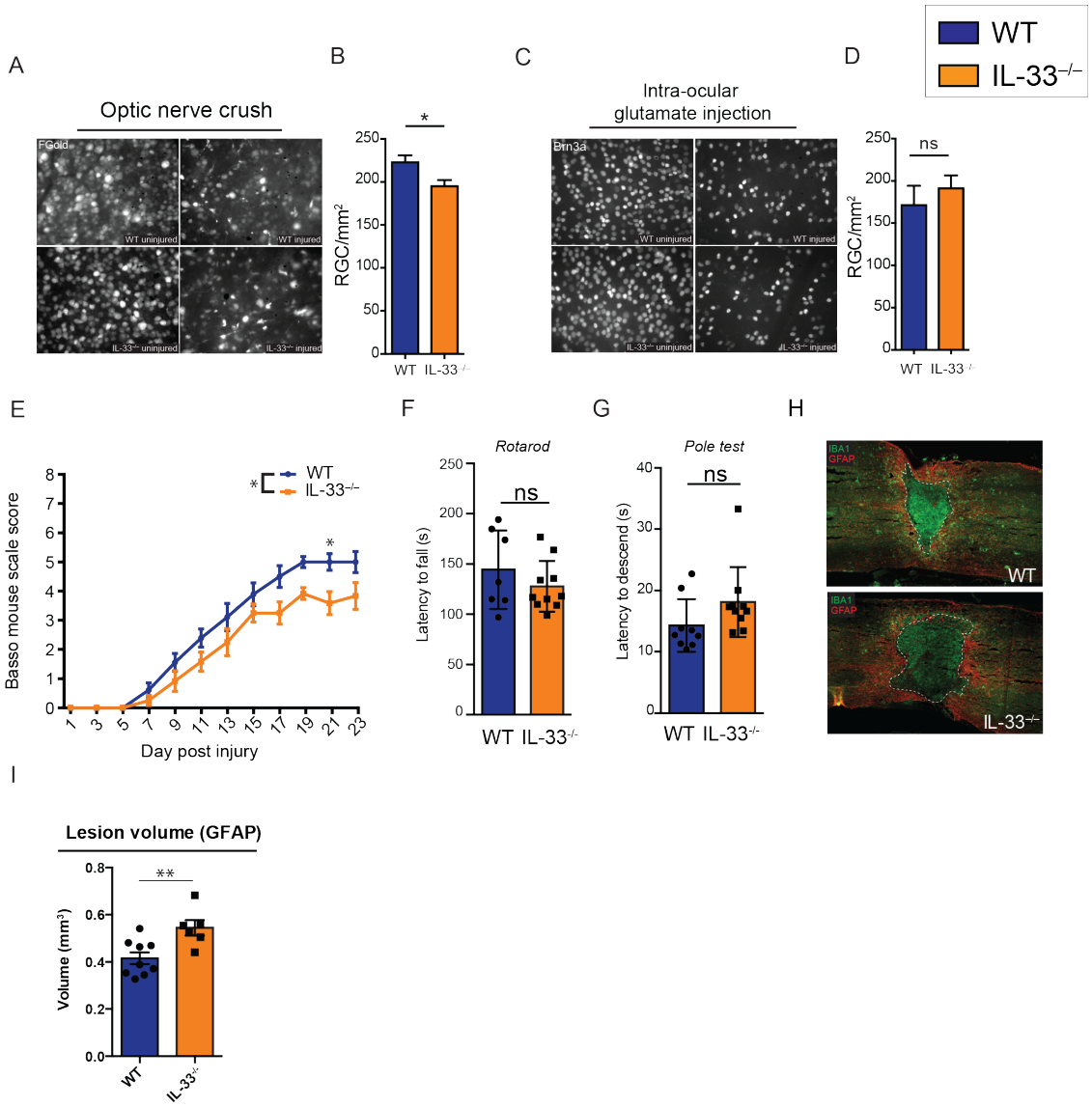

Figure 3.6: **IL-33$^{-/-}$ animals have impaired recovery after optic nerve crush and spinal cord contusion.** (A) Representative images of Fluorogold labeled RGCs with and without optic nerve crush in WT and IL-33$^{-/-}$. (B) Quantification of RGC survival in WT and IL-33$^{-/-}$ animals (N=9-11; two-tailed Students T-test). (C) Representative images of Brn3a labeled RGCs with and without glutamate treatment. (D) Quantification of RGC survival after glutamate toxicity (N=5; two-tailed Students T-test). (E) Recovery after spinal cord contusion (90KDyn) as measured by the Basso Mouse Scale (BMS) (Basso et al., 2006) (N=8 mice per group, representative of 3 experiments with 70-90KDyn severity; repeated measures two-way ANOVA with Sidaks multiple comparisons test). Motor coordination was assessed with the (F) Rotarod and (G) Pole tests. (F) After two training trails, WT and IL-33$^{-/-}$ mice were placed on an accelerating rod and the latency to fall was recorded (N=7-10; two-tailed Students T-test). (G) After one training trial, mice were placed at the top of a narrow pole and latency to descend the pole was recorded (N=9; two-tailed Students T-test). (H) Representative injury sites of WT and IL-33$^{-/-}$ animals 14DPI labeled with GFAP and IBA1. (I) Volume of lesion sites at 23DPI as measured by GFAP delineation.

effect (Levkovitch-Verbin et al., 2000). To repeat our findings in another model with a functional readout, we tested recovery of IL-33$^{-/-}$ mice after spinal cord contusion injury (SCI). Consistent with the high expression of IL-33 in the spinal cord and in line with our findings in optic nerve crush injury, IL-33$^{-/-}$ mice had significantly impaired recovery relative to their WT counterparts after SCI as measured by the Basso mouse scale (Basso et al., 2006) (Figure 3.6E). Notably uninjured IL-33$^{-/-}$ mice showed no deficits in the rotarod (Figure 3.6F) and pole (Figure 3.6G) tests, suggesting that IL-33 deficiency does not affect baseline motor function in these mice.

To analyze the volume of the site of injury, we immunolabeled injured spinal cords with GFAP, a marker of reactive astrocytes, and IBA1, a marker of myeloid cells (Figure 3.6H). Quantification of the lesion volume based on GFAP delineation in coronal sections revealed significantly increased lesion size in IL-33$^{-/-}$ versus WT animals (Figure 3.6I).

Given previous reports showing that IL-33 can polarize towards an M2 (IL-4 activated) macrophage skew (Kurowska-Stolarska et al., 2009) and that M2 macrophages promote neuronal survival (Kigerl et al., 2009), we analyzed the lesion site for expression of several characteristic M2 markers. Indeed there were significant decreases in expression of several M2 genes, but the M1 gene Nos2 was unchanged (Figure 3.7A). To better understand how IL-33 from injured CNS tissue drives macrophage phenotype, we established an in vitro system where bone marrow-derived macrophages (BMDMs) are polarized with IL-4 in the presence of acutely excised healthy CNS tissue (2.5mm of spinal cord or 1 optic nerve per well). WT BMDMs treated with IL-4 display an expected increase of Arg1, which is substantially intensified by co-incubation with injured spinal cord (Figure 3.7C). Of note, spinal cord alone has little affect on Arg1, suggesting that the tissue emits one (or several) alarmins that potentiate rather than direct the macrophage M2 phenotype. Adding an antagonizing antibody to ST2 dampened the amplifying effect of spinal cord tissue (Figure 3.7C). We next tested whether optic nerve will also potentiate M2 skew. Incubating macrophages with IL-4 and excised optic nerves yielded a similar enhancement of Arg1, again attenuated by anti-ST2

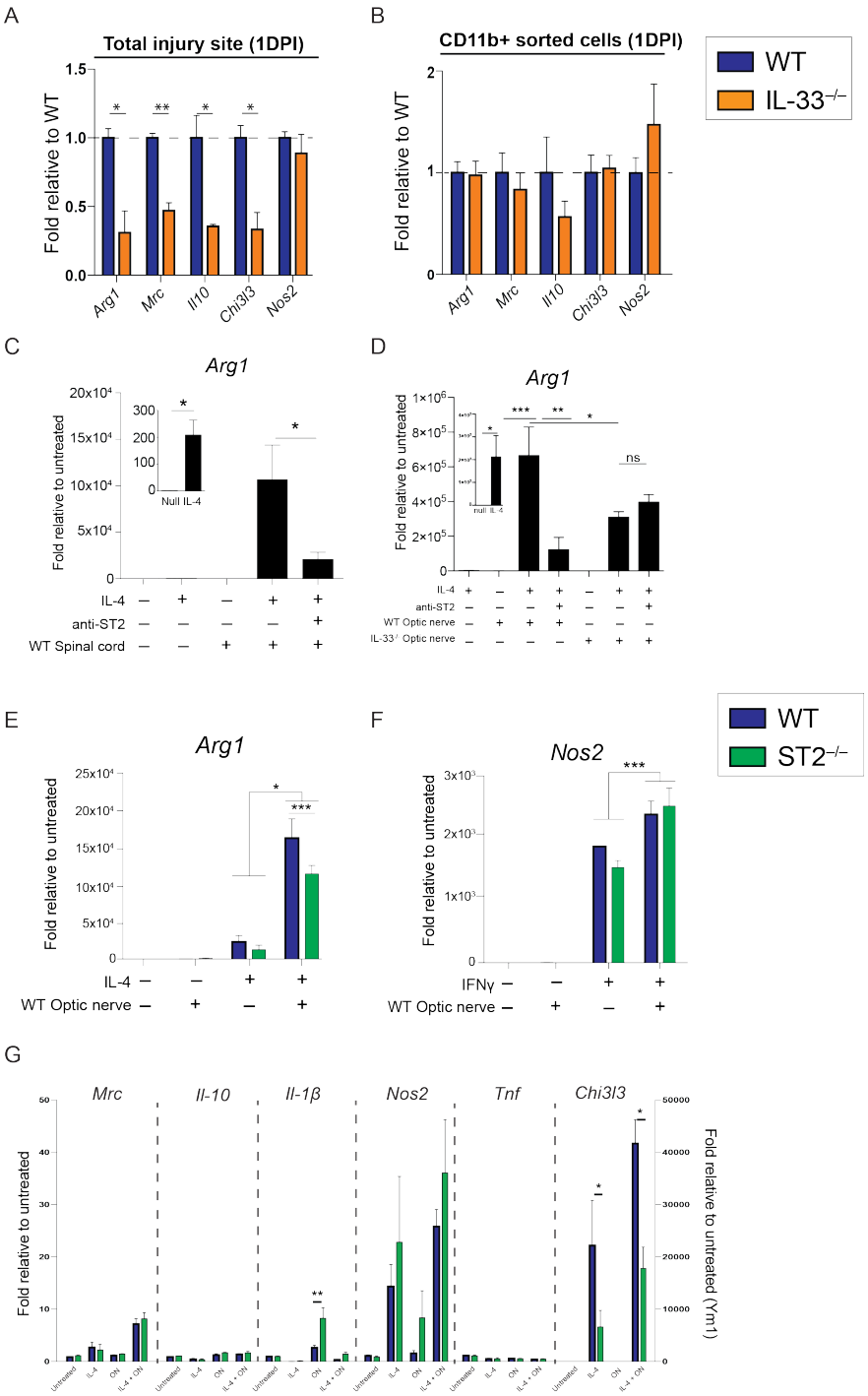

Figure 3.7: **IL-33$^{-/-}$ animals have decreased M2 macrophage activity at the injury site.** (A) 1cm of spinal cord tissue centered on the 1DPI lesion site was analyzed for M2 genes in WT and IL-33$^{-/-}$ animals (N=3, representative of two experiments; Multiple t-tests). (B) CD11b^{+} cells were sorted from the lesion site using magnetic beads and the level of M2 gene transcript was assessed. (N=3; Multiple T-tests). (C) BMDMs were treated with IL-4, anti-ST2, and 2.5mm segments of spinal cord for 24 hours in vitro before lysates were analyzed for *Arg1* (N=3; one-way ANOVA with Tukeys test). (D) BMDMs were treated with IL-4, anti-ST2 antibodies, and optic nerves from WT or IL-33$^{-/-}$ animals (1 optic nerve/well of a 24-well plate, cut into 2 pieces) for 24 hours and analyzed for *Arg1* (N=3, representative of 2 experiments; one-way ANOVA with Tukeys test). (E-F) WT or ST2$^{-/-}$ BMDMs were treated with IL-4, IFNγ, or optic nerves for 24 hours and analyzed for *Arg1* or *Nos2* (N=3, representative of two experiments; two-way ANOVA with Sidaks test).(G) WT and ST2$^{-/-}$ BMDMs were analyzed for several M1 and M2 associated genes after IL-4 and/or optic nerve treatment. IL-4 and anti-ST2, 10ng/mL, IFNγ, 100ng/mL

antibodies (Figure 3.7D). Moreover, optic nerves from an IL-33$^{-/-}$ mouse potentiated M2 skew to a significantly less extent than WT optic nerves (Figure 3.7D). We repeated these experiments using BMDMs from ST2$^{-/-}$ donors and, as expected, potentiation of their M2 skew by injured optic nerves was significantly lower than in WT macrophages (Figure 3.7E). Conversely, injured optic nerve in combination with IFNγdid not potentiate M1 skew of macrophages as measured by *Nos2*(Figure 3.7F), suggesting IL-33 specifically boosts aspects of the M2 response. Analysis of other macrophage phenotype markers revealed changes in other M2-associated genes (Figure 3.7G).Of note, no manipulation to IL-33 signaling alone completely abrogated the potentiating effect of CNS tissue on *Arg1* expression, and IL-33 had little effect on some M2 genes such as *Mrc*, suggesting that IL-33 is likely one of several molecular mediators potentiating a complex M2-like phenotype.

We next assayed M2 associated gene expression on isolated CD11b^{+} cells from the 1DPI injury site, and surprisingly saw no difference in expression of several M2 genes (Figure 3.7B). Given these findings we hypothesized that our prior observation of reduced M2 gene expression in the whole IL-33$^{-/-}$ lesion site represented a reduction in the overall number of M2 cells relative to other brain cells rather than an impaired M2 polarization. To assess the number of peripheral myeloid cells entering the site of injury, we examined the injured spinal cords by flow cytometry. Gating on live, nucleated, CD45^{hi} (excluding CD45^{lo} microglia)/CD11b^{+} cells, we were able to quantify numbers of infiltrating monocytes and granulocytes at the site of injury (Figure 3.8A). At 1DPI there was a striking and specific reduction in the number of Ly6C^{hi} monocytes in the IL-33$^{-/-}$ spinal cords (Figure 3.8B-C). The early lack of monocyte recruitment continued to affect later immune cell composition, with reduced Ly6c^{lo}/F4/80^{+} monocyte-derived macrophages at 7DPI (Figure 3.8D-E). It has previously been reported that the majority of Arg1^{+} cells entering the CNS injury site are infiltrating monocytes (Hsieh et al., 2013), and we confirmed by immunohistochemical analysis of the 1DPI injury site that there are fewer Arg1^{+} cells in IL-33$^{-/-}$ animals relative to WT (Figure 3.8F-G). Previous studies have shown that cultured glia produce the chemokine CCL2 (MCP1) in response to

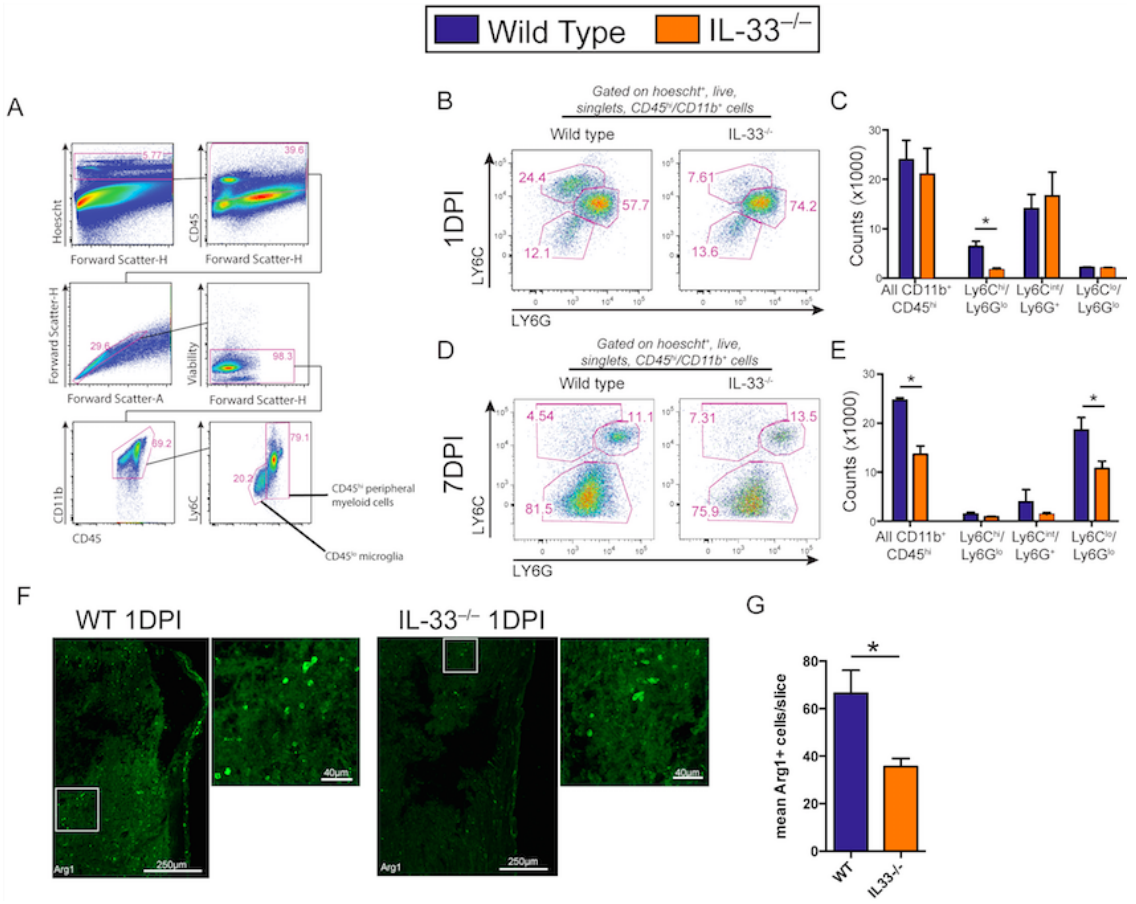

Figure 3.8: **IL-33$^{-/-}$ mice have impaired myeloid cell recruitment after CNS injury.** (A) Example of gating for peripheral myeloid cells at the injury site. Cells were gated as follows: Hoescht^{+}, CD45^{+}, Singlets, live cells, CD11b^{+}, CD45^{hi}. (B-E) Flow cytometry analysis of the injury site 1 and 7 days following spinal cord injury. (B) Representative plots of WT and IL-33$^{-/-}$ sites at 1DPI. (C) Mean cell numbers at the injury site of total peripheral myeloid cells (CD11b^{+}/CD45^{hi}) and subsets of monocytes (Ly6C^{hi}/Ly6G^{lo}), granulocytes (Ly6C^{int}/Ly6G^{+}), and macrophages (Ly6C^{lo}/Ly6G^{lo}) (N=3 mice per group, representative of two experiments; one-way ANOVA with Tukeys multiple comparisons test). (D) Representative flow plots and (E) summarized results comparing WT and IL-33$^{-/-}$ injury sites at 7DPI. (N=3 mice per group; two-way ANOVA with Sidaks multiple comparisons test). (F) Representative images of Arg1^{+} cells at the lesion site 1DPI. (G) 1DPI WT and IL-33$^{-/-}$ injury sites were analyzed by immunofluorescence for Arg1^{+} cell number. Spinal cords were sliced longitudinally and collected at equivalent intervals in all mice, and at least 7 equivalent slices were analyzed per mouse. (N=3; two-tailed Students T-test).

IL-33 stimulation (Kempuraj et al., 2013). We sought to explore other chemokines that could be induced with IL-33 stimulation by performing a luminex chemokine assay on supernatants of IL-33 treated mixed glial cultures. In addition to CCL2, we observed significant secretion of CXCL1 (KC), CXCL2 (MIP2-), CXCL10 (IP-10) and CCL5 (RANTES) (Figure 3.9A).

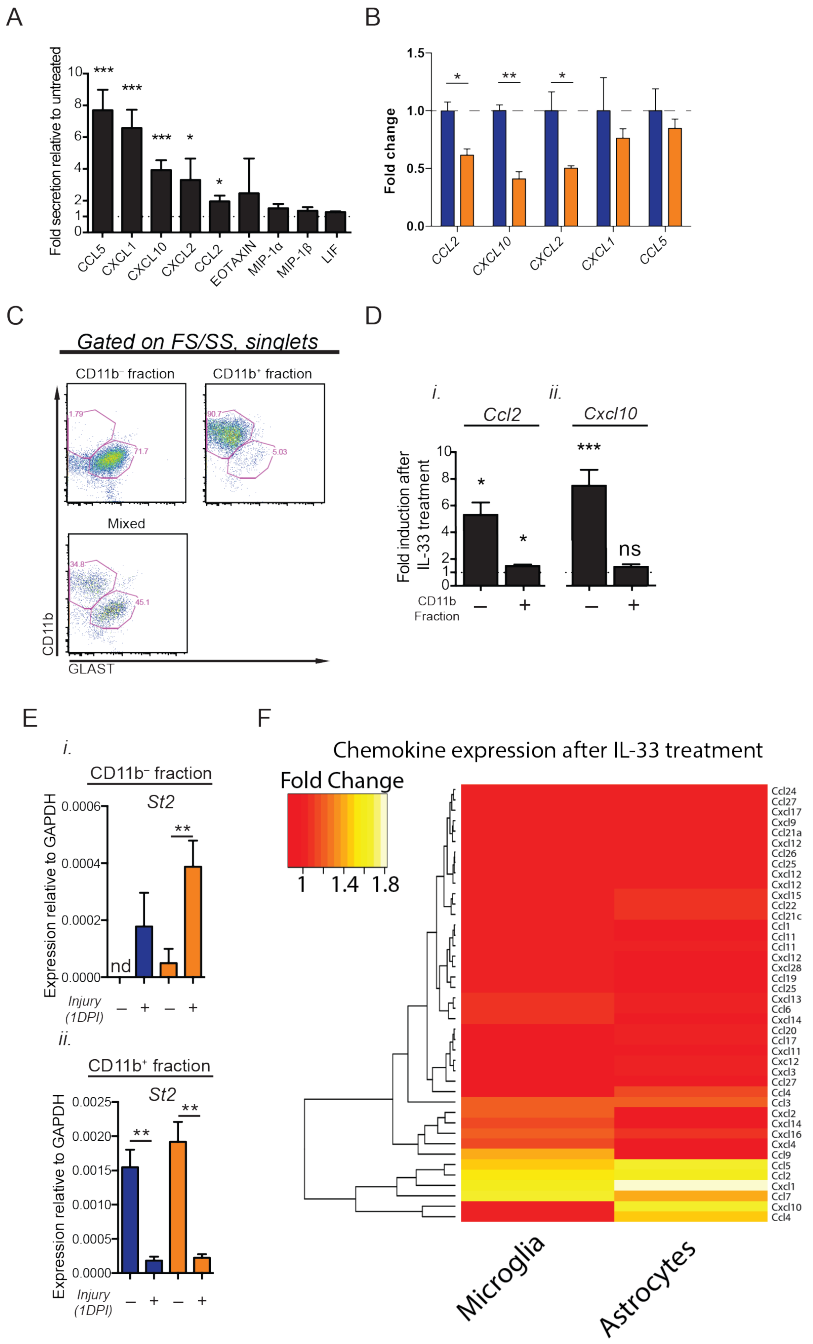

Figure 3.9: **Astrocytes produce chemokines in response IL-33 after CNS injury.** (A) Luminex chemokine analysis of mixed glial cultures after treatment with IL-33 (100ng/mL) for 24 hours. Data shown as fold induction relative to untreated cultures (N=4; Multiple two-tailed Students t-test). (B) Chemokine transcript levels from the injury site at 1DPI in WT vs. IL-33$^{-/-}$ mice (N=3 mice per group, representative of two experiments; Multiple two-tailed Students t-test). (C) Mixed glial cultures were sorted based on CD11b expression using an AutoMACs sorter. (top left) The CD11b fraction has minimal microglial contamination and is primarily GLAST+ astrocytes, (top right) while the CD11b^{+} fraction is 90% pure microglia. (D) qRT-PCR analysis of chemokine induction from sorted CD11b^{+} and CD11b^{-} mixed glial culture populations treated with IL-33 (100ng/mL) overnight. Data are show as fold induction of (i.) *Ccl2* and (ii.) *Cxcl10* relative to untreated (N=3; one-way ANOVA with Tukeys multiple comparisons test). (E) qRT-PCR analysis of ST2 transcript levels from CD11b^{+} and CD11b^{-} sorted from the injury site (N=3; two-way ANOVA with Sidaks multiple comparisons test).(B) Heat plot of chemokine expression in CD11b^{+} (left column) and CD11b^{-} (right column) glia after IL-33 stimulation (100ng/mL). Data is presented as mean fold change relative to untreated of 3 replicate samples.

Looking at chemokine induction at the injury site 1DPI, we observed reductions in several of these chemokines in IL-33$^{-/-}$ mice; *Ccl2*, *Cxcl10*, and *Cxcl2* transcript levels are all reduced at the injury site, while *Ccl5* and *CXCL1* are unchanged (Figure 3.9B).

Mixed glia cultures contain several potential cellular targets for IL-33, and to narrow the specific cell responding to IL-33 with chemokine production we screened different populations of glia after IL-33 stimulation. We sorted CD11b^{+} (myeloid cells) out from a mixed glia culture, resulting in a concentrated population of astrocytes (CD11b^{-} fraction; 70% astrocytes and >95% pure from microglia) (Figure 3.9C). Further RT-PCR analysis reinforced this,

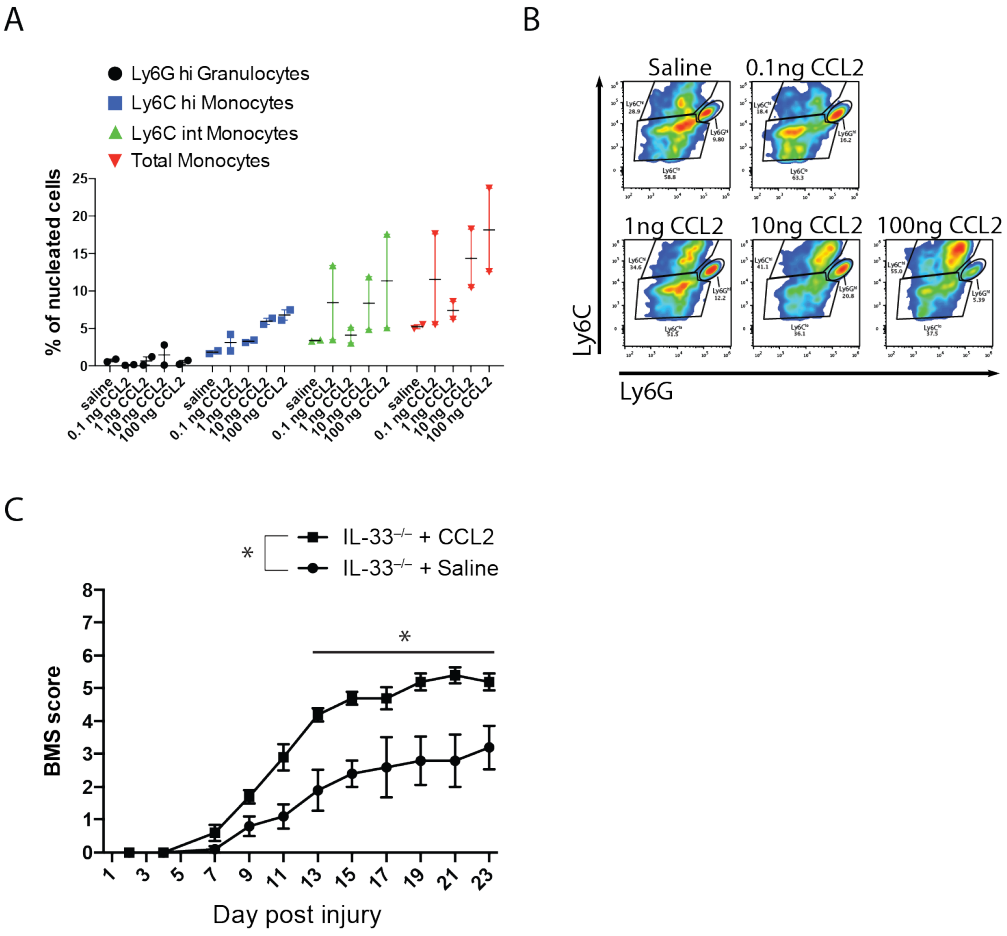

Figure 3.10: **CCL2 treatment rescues impairment in IL-33$^{-/-}$ animals after spinal cord contusion.** (A-B) Titration of CCL2 in vivo injection doses; WT mice underwent spinal cord injury and were immediately treated with CCL2 into the spinal cord at the listed dosages. Mice were sacrificed 1DPI and spinal cords analyzed for Ly6C^{hi} cell recruitment after CCL2 treatment. There was a dose dependent effect of CCL2 on monocyte recruitment.(C) CCL2 treatment (10ng/mouse) rescues recovery deficits in IL-33$^{-/-}$ animals (90KDyn) (N=5-6; repeated measures two-way ANOVA).

demonstrating that IL-33 treatment results in strong induction of monocyte-attracting chemokines such as CCL2 (Figure 3.9Di) and CXCL10 (Figure 3.9Dii) in CD11b^{-} glia. Enriched microglia cultures treated with IL-33 did not exhibit such induction of chemokines (Figure 3.9D). This result compelled us to test the ability of CD11b^{-} glia to respond to IL-33 in vivo. Agreeing with previous literature, at baseline microglia express the highest amounts of *Il1rl1* (the gene coding for ST2) while other glia are lower expressers. Interestingly, however, after injury *Il1rl1* expression levels flip, with the CD11b^{+} fraction downregulating *Il1rl1* and the CD11b^{-} fraction upregulating *Il1rl1*, arguing in favor of CD11b^{-} glia as IL-33 responders after injury (Figure 3.9E). To assess the overall activation of glia after IL-33 treatment, we added IL-33 to sort CD11b^{+} and CD11b^{-} glial cultures cultures and analyzed changes in gene expression by microarray. This screen revealed that, while both fractions respond, CD11b^{-} glia are substantial producers of chemokines compared to microglia (CD11b-enriched cells) following IL-33 treatment (Figure 3.9F).

To reaffirm the critical role of monocyte recruitment in contributing to impairment in IL-33$^{-/-}$ mice, we sought to enhance recruitment by delivering CCL2, a major chemokine for recruitment of peripheral monocytes into the CNS (Ma et al., 2002). We first titrated the dose of CCL2 administration, seeing a dose dependent increase in Ly6Chi monocyte recruitment at 1DPI in WT mice and choosing 10ng as the most efficient dose for further experiments (Figure 3.10A-B). Local administration of CCL2 in IL-33$^{-/-}$ mice indeed had a significant effect improving recovery in IL-33$^{-/-}$ animals after spinal cord injury (Figure 3.10C), strengthening our conclusion that IL-33 is a critical factor important for normal recovery through protective myeloid cell recruitment after injury.

3.2 Characterization of meningeal resident ILC2s in response to CNS injury

Though ILC2s had previously been implicated in CNS diseases (Russi et al., 2015; Besnard et al., 2015), their presence in the healthy meninges has not been established. We therefore attempted to identify ILC2s by flow cytometry in the naïve meninges, successfully identifying them as a population of CD45$^+$, Lineage$^-$, Thy1.2$^+$, ST2$^+$ single live cells (Figure 3.11A). This population had a surface marker profile characteristic of ILC2s, expressing for example C-kit, Sca1, CD25, and IL-7Rα (Figure 3.11B). ILC2s are more abundant in naïve meninges than ILC3s, and interestingly both ILC populations were concentrated in the brain rather than spinal cord meninges (Figure 3.11C). Little to no ILCs were detected in the healthy spinal cord parenchyma (Figure 3.11C).

To explore the physical localization of ILC2s within the meninges we crossed the IL-13^{cre} and tdTomato^{stoplox} (IL-13^{tdT}) mice, generating a model that labels any cell that has even made IL-13. This strategy labels the majority of ILC2s in meninges and lung (Figure 3.12A),

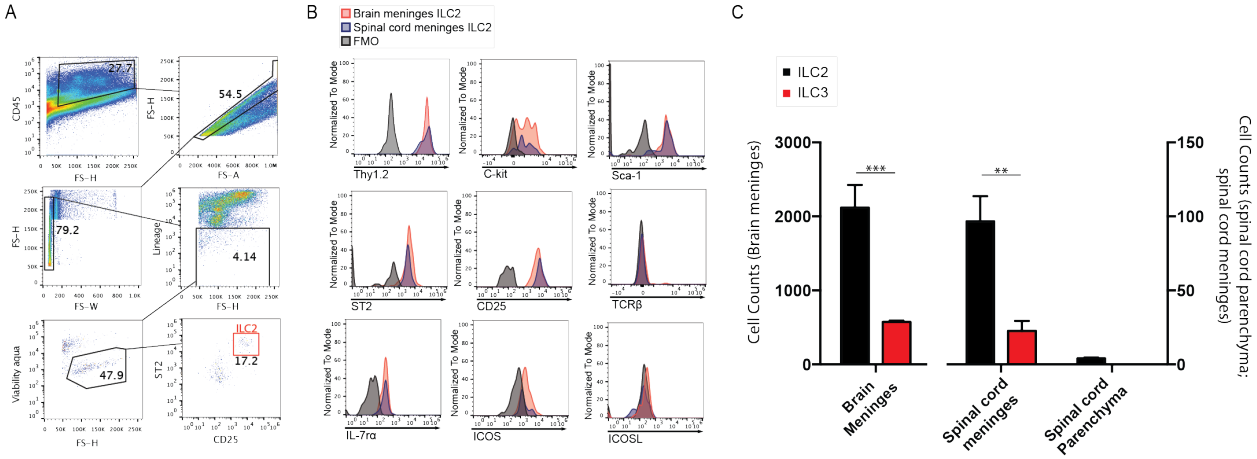

Figure 3.11: **ILC2s are resident in the healthy brain and spinal cord meninges but not in the spinal cord parenchyma.** (A) ILC2s were identified as CD45$^+$/singlets/lineage$^-$/viable/ST2$^+$/CD25$^+$ cells (Lineage cocktail = CD11b, GR1, TCRβ, B220, FCεRα). (B) Representative surface markers of murine ILC2s. (C) Counts of murine ILC2s and ILC3s (CD45$^+$/singlets/lineage$^-$/viable/RORγt$^+$) in healthy mouse brain meninges (left y-axis), spinal meninges, and spinal cord parenchyma (right y-axis) (N=3; Multiple T-tests).

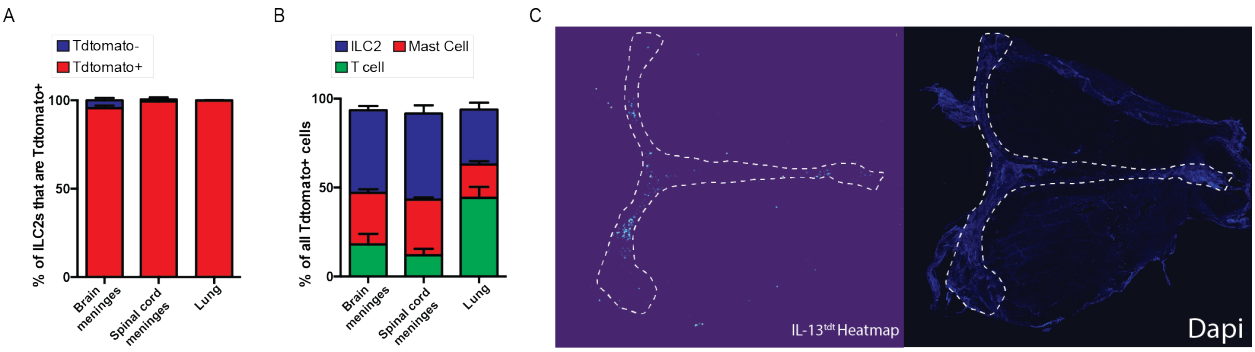

Figure 3.12: **The IL-13^{cre} x tdTomato^{stoplox} mouse labels ILC2s.** (A-B) Characterization of the IL-13^{cre} x tdTomato^{stoplox} mouse by flow cytometry. (A) >95% of ILC2s are tdTomato^{+} in the IL-13^{cre} x tdTomato^{stoplox} mouse (N=3; representative of 3 experiments). (B) ILC2s, T cells, and mast cells make up the majority of total tdTomato^{+} cells in meninges and lung. (C) Heat map demonstrating tdTomato expression in IL-13^{cre} x tdTomato^{stoplox} (IL-13^{tdt}) brain dural whole mounts.

and also labels populations of T cells and mast cells (Figure 3.12B).

Interestingly, a whole mount of IL-13^{tdt} dura mater demonstrated a concentration of tdTomato expression in the transverse and sagittal dural sinuses (Figure 3.12C). In order to specifically identify tdTomato^{+} cell types histologically, we costained IL-13^{tdT} tissues with CD3 and toluidine blue, to identify T cells and mast cells respectively. ILC2s were identified as tdTomato^{+} cells that were not T cells or mast cells in dural sinus, dura mater, and spinal cord meninges whole mounts (Figure 3.13A-C). ILC2s were heavily concentrated in the dural sinus, and found more sparsely throughout the dura mater and spinal cord meninges (Figure 3.13D).

It has previously been noted that ILC2s expand in response to IL-33 stimulation (Molofsky et al., 2013), and we sought to test this phenomenon in meningeal ILC2s (Figure 3.14A-C). Mice treated with IL-33 intraperotineally for 6 days had 3-3.5 fold expansion of ILC2s in the spinal (Figure 3.14B) and brain meninges (Figure 3.14C).

Given our previous observation of IL-33 release into the CSF after spinal cord injury (Figure 3.5B) and the presence of ILC2s in the naïve meninges, we next tested whether ILC2s respond to spinal cord injury in an IL-33 dependent manner. Indeed, 1 day after injury there was an IL-33-dependent increase in ILC2s in the spinal meninges (Figure 3.15A-B), but not

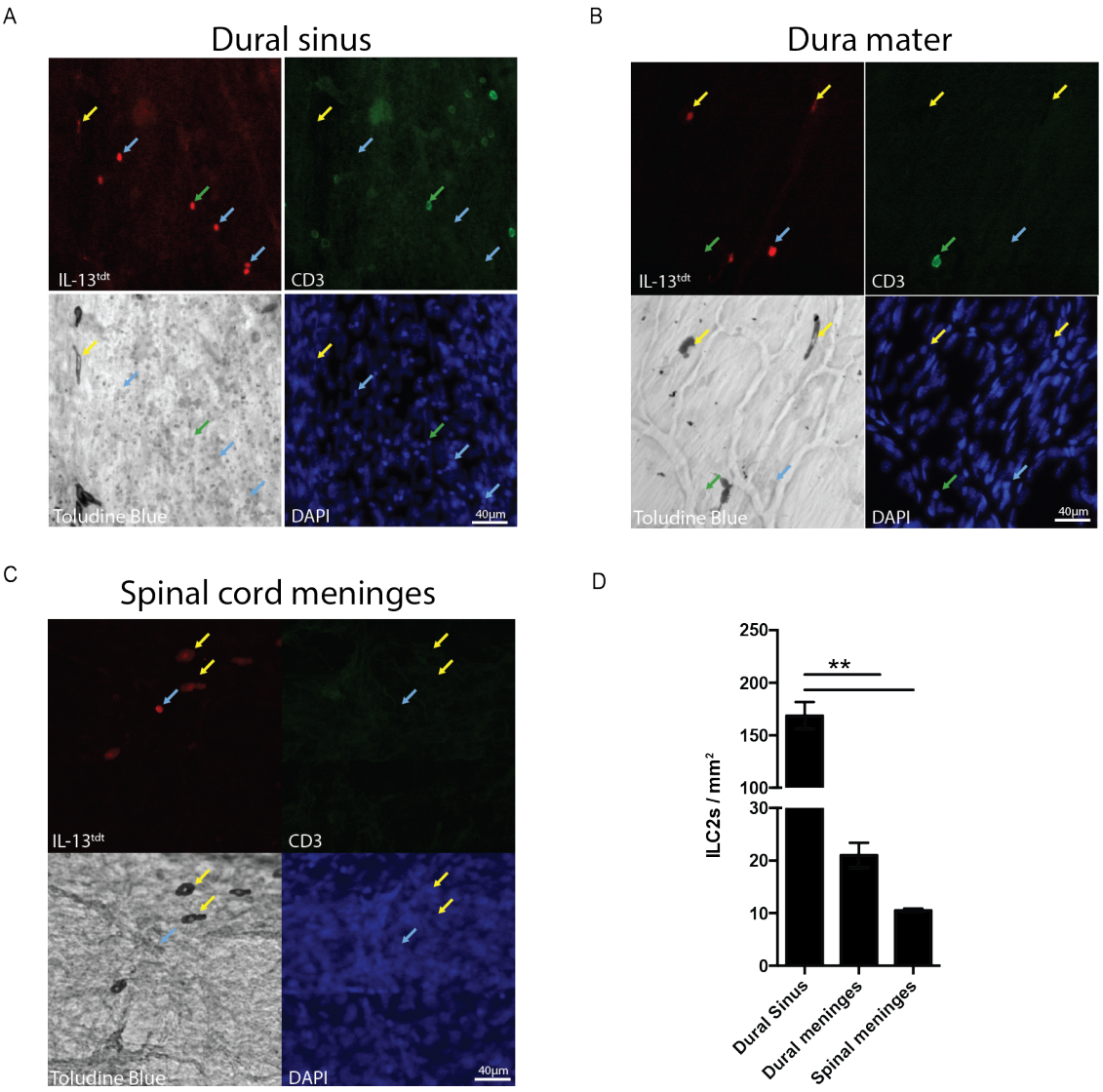

Figure 3.13: **Meningeal ILC2s are concentrated around the dural sinus.** (A-C) Representative images of ILC2s identified as IL-13^{tdt+}/CD3^{-}/toludine blue^{-} cells in (A) Dural sinus, (B) Dura mater, or (C) Spinal cord meninges whole mounts (blue arrows represent IL-13^{tdt+}/CD3^{-}/toludine blue^{-} ILC2s, green arrows represent IL-13$^{tdt+/-}$/CD3^{+}/toludine blue^{-} T cells, yellow arrows represent IL-13^{tdt+}/CD3^{-}/toludine blue^{+} mast cells). (D) Quantification of ILC2 localization demonstrates a concentrating in the dural sinus versus other meninges areas (N=3-4; one-way ANOVA with Tukey's multiple comparisons test).

in the brain meninges (Figure 3.15C). Spinal meningeal ILC2s also demonstrated activation at 1DPI through increased expression of the surface markers CD25 (Figure 3.16A-B) and CD69 (Figure 3.16D-E). This induction was IL-33 dependent, and did not occur in brain meninges ILC2s (Figure 3.16A-F).

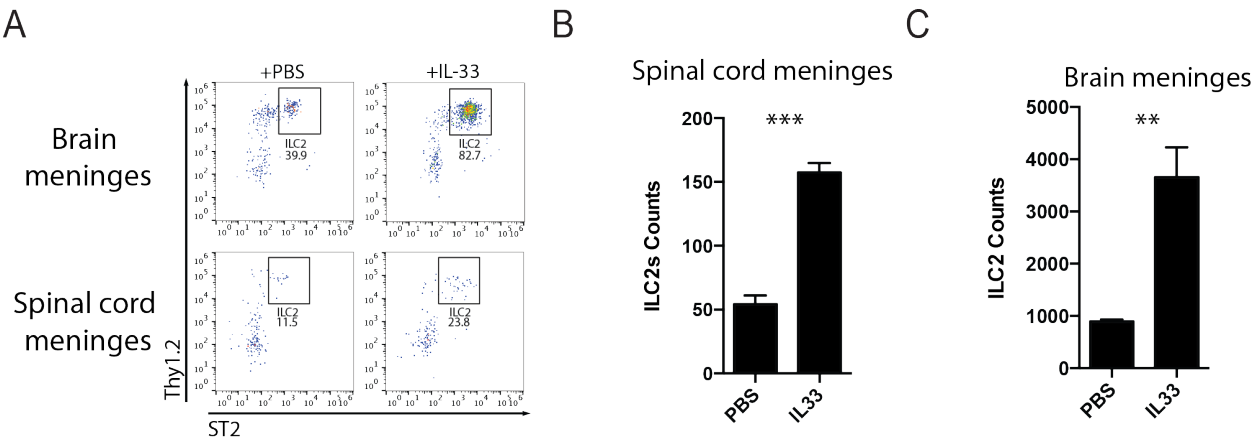

Figure 3.14: **IL-33 treatment expands meningeal ILC2s in vivo.** (A-C) Mice were treated with IL-33 every other day for 6 days intraperotineally, and on the 8th day ILC2 numbers were analyzed by flow cytometry. (A) Representative plots demonstrating expansion of ILC2s in IL-33 treated mice relative to PBS treatment (gated on live/CD45^{+}/singlet/Lineage^{-} cells). (B-C) Counts demonstrating expansion in (B) spinal cord meninges and (C) brain meninges (N=3, representative of 3 experiments; Student's T test).

We next assessed cytokine expression by meningeal ILC2s after SCI. Using the IL-13-YFP transgenic mouse (YET-Cre 13) we compared IL-13 induction in uninjured and 1DPI cells, surprisingly finding a mild induction in brain meningeal, but not spinal meningeal ILC2s (Figure 3.17A). We repeated this observation using IL-13 antibody staining (with 4 hours in vitro PMA/Ionomycin stimulation + Brefeldin A), again finding that brain meningeal, but not spinal cord meningeal ILC2s are induced to produce cytokine 1DPI (Figure 3.17B). Finally, we tested whether brain meningeal ILC2 IL-13 production is IL-33 dependent using the IL-33$^{-/-}$ mice. Indeed, IL-33$^{-/-}$ animals show no significant upregulation relative to uninjured animals (Figure 3.17C).

Given the specific activation seen in brain versus spinal meningeal ILC2s, we assessed cells for differences in basal activity states. Single cell suspensions from brain meninges, spinal cord meninges, and lung were plated at the same density in wells of a 24 well plate. All cells were treated with brefeldin A, and some were also activated with PMA/Ionomycin. Brain meningeal ILC2s shows a strong propensity for cytokine production in response to this stimulation relative to spinal meninges or lung-derived cells (Figure 3.18A). Brain meningeal cells had by far the most IL-13 induction by MFI (Figure 3.18B) but a similar fraction of

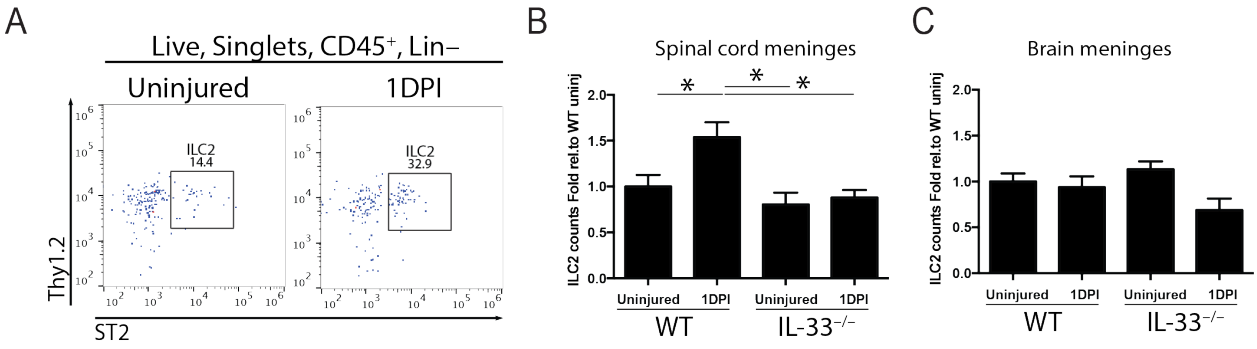

Figure 3.15: **ILC2 numbers increase in spinal meninges in an IL-33 dependent manner after injury.** Meningeal ILC2 counts were analyzed 1 day post spinal cord injury. (A) Representative plots of ILC2 increase 1DPI. (B) ILC2 numbers increased in 1DPI spinal meninges in WT animals, but not IL-33$^{-/-}$ animals or in (C) brain meninges (N=8-12, pooled data from 3 experiments; one-way ANOVA with Tukey's multiple comparisons test).

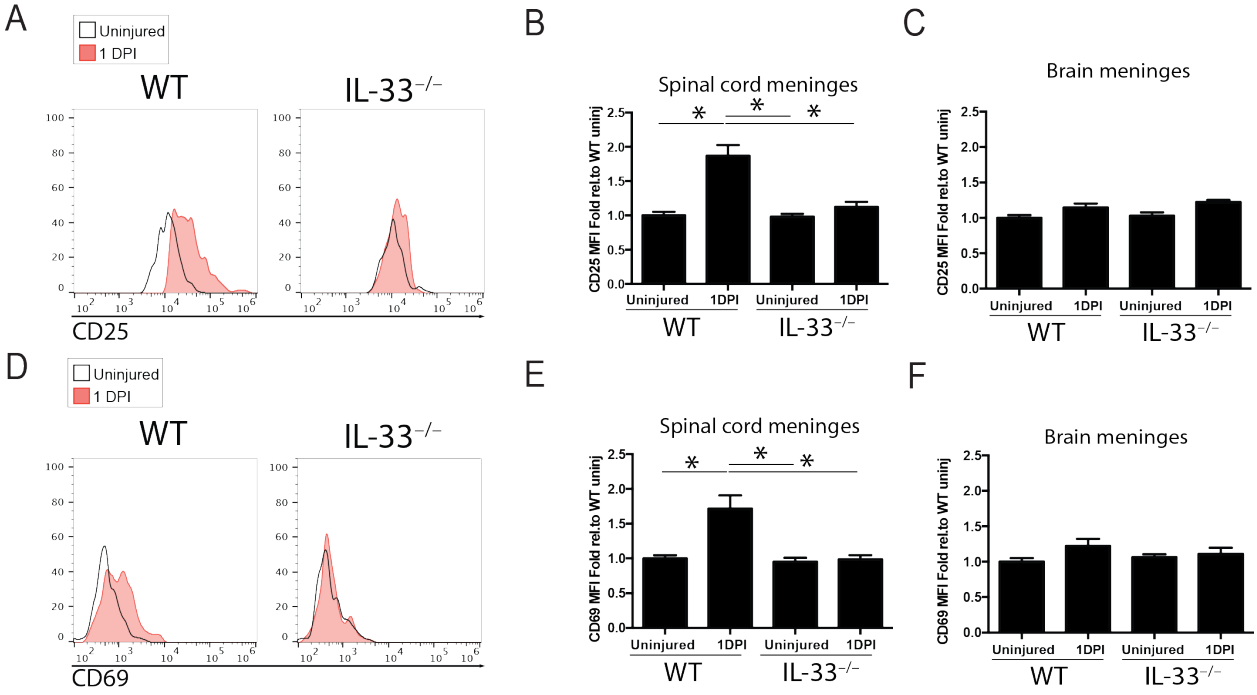

Figure 3.16: **Spinal meninges ILC2s have an activated phenotype acutely after spinal cord injury.** Meningeal ILC2s were analyzed 1 day post spinal cord injury for (A-C) CD25 or (D-F) CD69 expression. (A) Representative histograms of ILC2 CD25 expression 1DPI. CD25 is induced in 1DPI (B) spinal meninges in WT animals, but not IL-33$^{-/-}$ animals or in (C) brain meninges (N=8-12, pooled data from 3 experiments; one-way ANOVA with Tukey's multiple comparisons test). (D) Representative histograms of ILC2 CD69 expression 1DPI. (E) CD69 is induced in 1DPI spinal meninges in WT animals, but not IL-33$^{-/-}$ animals or in (F) brain meninges (N=8-12, pooled data from 3 experiments; one-way ANOVA with Tukey's multiple comparisons test).

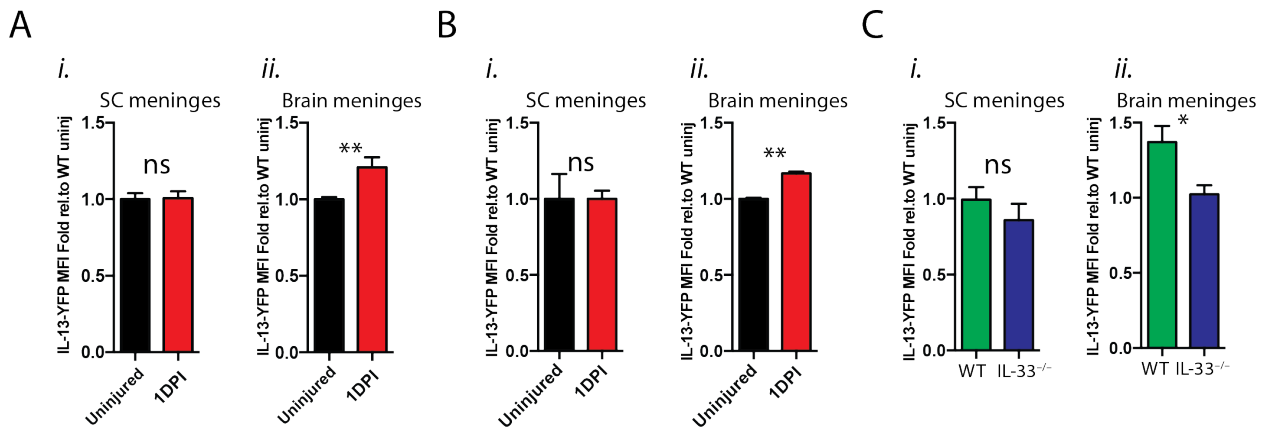

Figure 3.17: **IL-13 production increases in brain meningeal ILC2s 1 day post spinal cord injury.** (A) ILC2 IL-13 expression was analyzed by YFP MFI in YET-cre 13 mice (both YFP and cre are driven under the IL-13 promoter) in (*i.* spinal cord meninges) and (*ii.* brain meninges) (N=9, representative of 3 experiments); Student's T test). (B) Facs analysis of IL-13 expression in ILC2s 1DPI in (*i.* spinal cord meninges) and (*ii.* brain meninges) (N=3, representative of 2 experiments); Student's T test). (C) WT and IL-33$^{-/-}$ animals were injured and meningeal ILC2s analyzed for IL-13 expression 1DPI in (*i.* spinal cord meninges) and (*ii.* brain meninges) (N=4; Student's T test).

ILC2s from all tissues were able to be activated (Figure 3.18C). This suggests that brain meningeal ILC2s produce more cytokine upon stimulation, rather than having an increased propensity to become activated. We confirmed these findings in ILC2s purified by FACS, again noting a heightened ability of ILC2s from brain meninges to produce cytokine in response to PMA/ionomycin (Figure 3.18D-E).

To study the effect of lung microenvironment on activation of brain meningeal ILC2s, we combined lung and meninges samples from Thy1.1 and Thy1.2 tissues during activation. ILC2s from either tissue could then be isolated based on Thy1 expression (Figure 3.18F). As expected, meningeal ILC2s had increased IL-13 and IL-5 expression relative to lung ILC2s after 4 hours of stimulation, but this was significantly diminished when meningeal cells were costimulated with lung. There was no observable effect of adding meningeal cells to lung ILC2s (Figure 3.18F-G).

The healthy CNS parenchyma is void of immune cells besides microglia [ref], and this is also the case for ILC2s. There is, however, evidence to suggest that they accumulate in injury sites and impact recovery there [ref]. To test this in SCI, we used the IL-13^{cre} x

tdTomato^{stoplox} animal to identify ILC2s that have infiltrated the lesion site. Surprisingly, we identified tdTomato^{+}/CD3^{-} cells in the injury site at 10DPI (Figure 3.19A). Using FACs analysis with additional markers, we performed a time course of ILC2 numbers in the spinal cord injury site (Figure 3.19B). ILC2s began to enter early on, but numbers maximized at 10DPI at roughly 40 cells per injury site and persisted through 30DPI (Figure 3.19B). Notably, these cells produced IL-13 relative to FMO without stimulation, and make IL-13 abundantly after in vitro stimluation (Figure 3.19C). We next assessed whether migration was IL-33 dependent, finding that it was not–IL-33$^{-/-}$ mice had similar ILC2 infiltrates at 10DPI (Figure 3.19D).

Finally, we sought to test the functional impact ILC2s have on recovery from CNS injury. Given the relative recent discovery of ILCs, no robust mouse models to specifically removing ILC2s currently exist. We therefore attempted a global ILC depletion strategy: Rag1$^{-/-}$ animals, lacking T and B cells but having ILCs, were reconstituted with Thy1.1 lymphocytes. In this mouse reconstituted lymphocytes will bear a different isoform of the cell surface protein Thy1 than endogenous ILCs (ILCs with have Thy1.2, lymphocytes will have Thy1.1). We then injected mice with a Thy1.2 depleting antibody, planning to target ILCs without affecting lymphocytes, as had been previously reported in the literature (Gorski et al., 2013). This treatment had no effect on functional recovery after SCI (Figure 3.20A). However the strategy of depletion did not produce the expected outcome. Antibody injection did not deplete ILCs, but instead only blocked the Thy1 epitope (Figure 3.20B-C).

As an alternative to depleting meningeal ILC2s, we choose to do the converse experiment. 5000 FACS-purified ILC2s were injected into the CSF of mice 1 day prior to SCI. We found that this moderate increase in CSF ILC2 numbers (increasing them ˜5-fold) produced a significant beneficial effect on functional recovery (Figure 3.20D) and reduced lesion volume (Figure 3.20E).

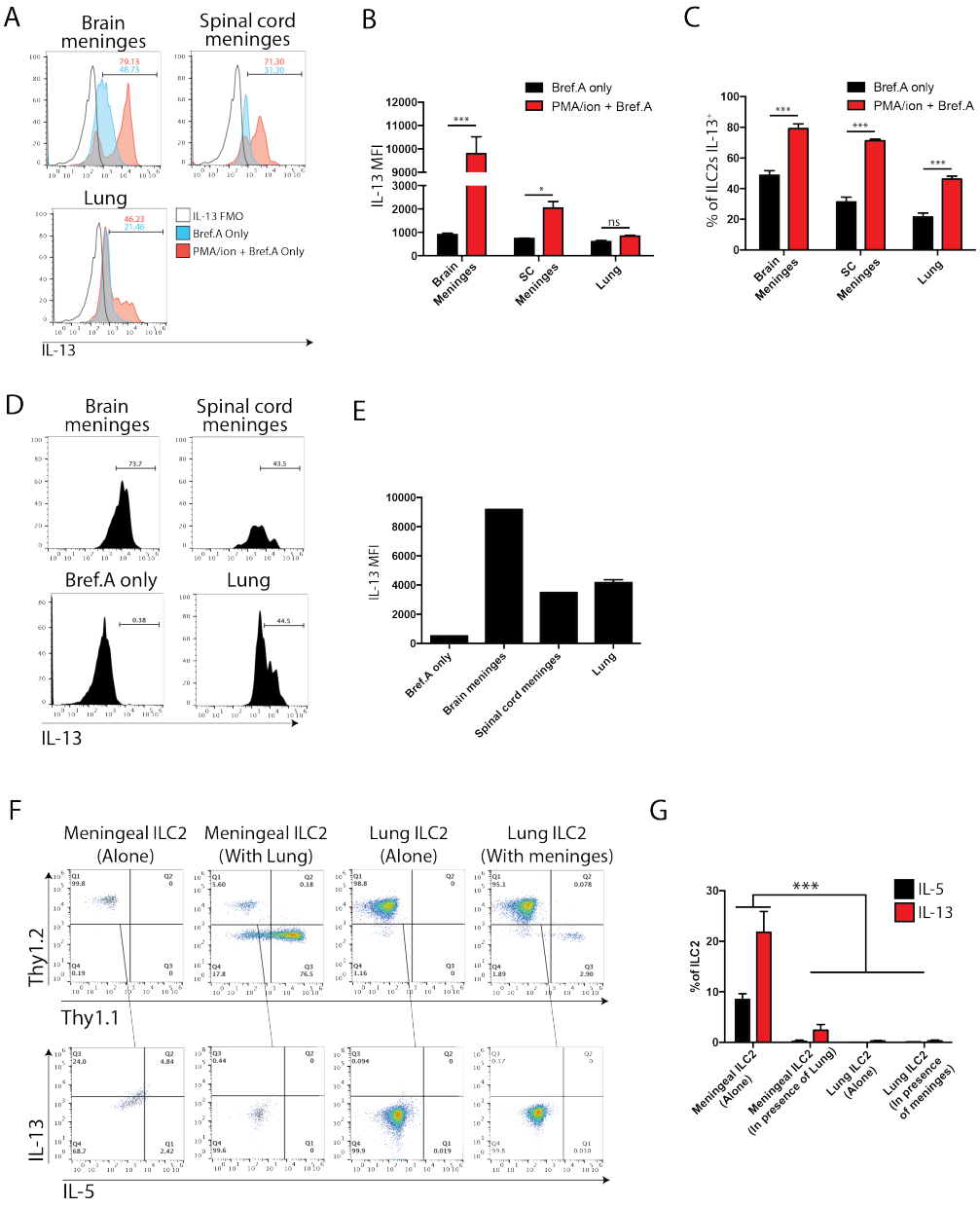

Figure 3.18: **ILC2s derived from brain meninges produce more IL-13 than those from spinal meninges or lung after PMA/ionomycin stimulation.** (A-C) Naïve brain meningeal, spinal cord meningeal, and lung single cell suspensions were incubated for 4 hours with PMA/Ionomycin and brefeldin A or brefeldin A only. (A) Representative histograms of IL-13 expression. Gates are drawn on FMO controls, and numbers represent mean percent of cells positive. (B) IL-13 MFI and (C) % of ILC2s expressing IL-13 (N=3, representative of 3 experiments; Two way ANOVA with Sidak's multiple comparison's test). (D-E) 1500 ILC2s from brain meninges, spinal cord meninges, or lung were purified by FACS and stimulated for 4 hours with PMA/ionomycin and brefeldin A or brefeldin A only. (D) Representative histograms of IL-13 expression. (E) A summary of results in (D), IL-13 MFI in brefeldin A treated and stimulated ILC2s from meninges and lung (N=1, cells sorted from 5 pooled mice). (F-G) Coincubation of lung and meninges during in vitro stimulation. (F) Representative plots of (top row) ILC2s gated on $CD45^+/Lineage^-/Gata3^+$ cells and (bottom row) their cytokine production. (G) Summary of results demonstrating effect of coincubation of meningeal ILC2s with lung (N=3; Two way ANOVA with Sidak's multiple comparison's test).

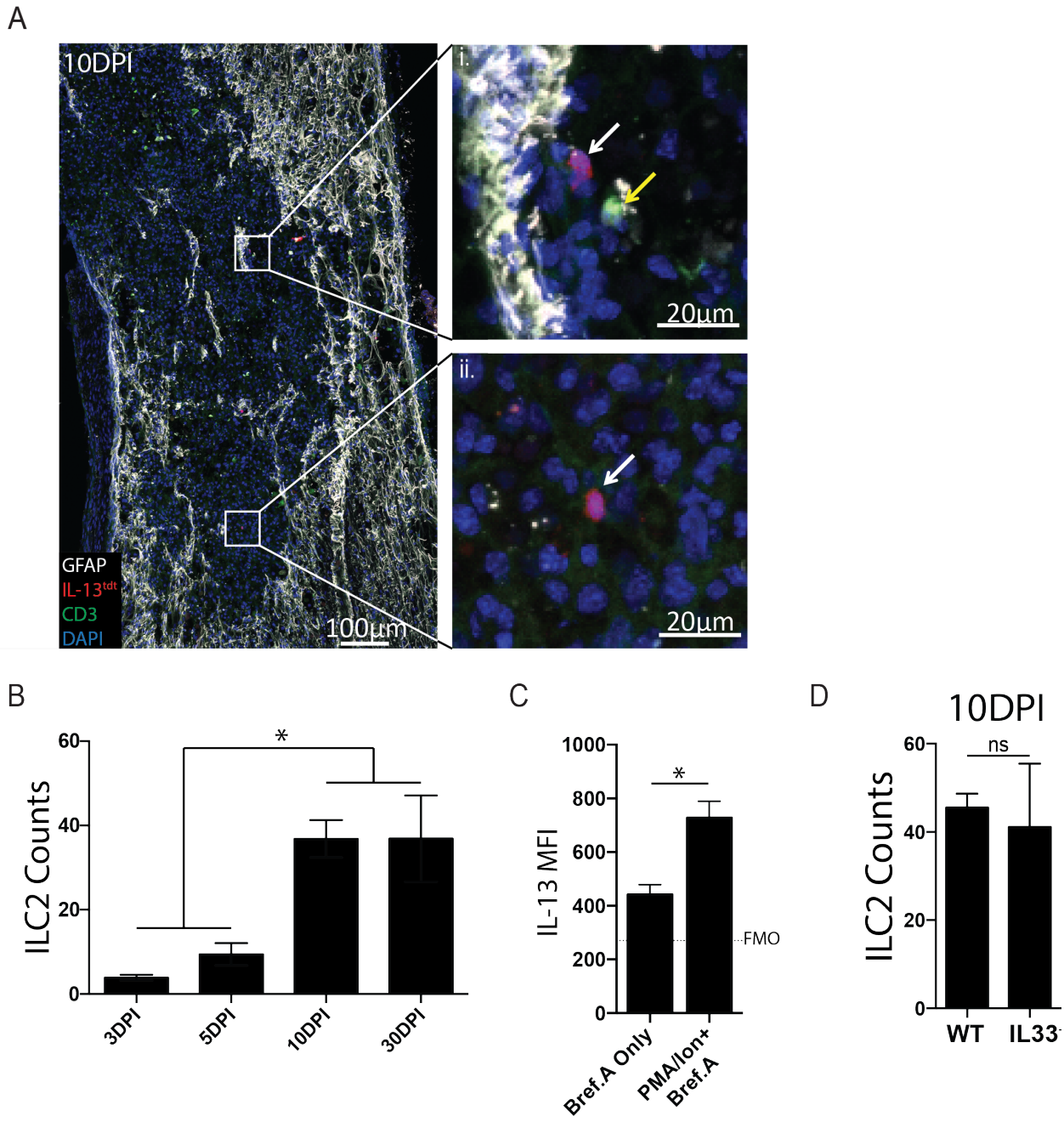

Figure 3.19: **ILC2s infiltrate the injury site in an IL-33 independent manner.** (A) Immunoflourescence staining of the IL-13^{cre} x tdTomato^{stoplox} spinal cord 10 days post spinal cordi injury. Insets show zoomed in images representative ILC2s (white arrows, IL-13^{tdt+}/CD3^{-}) or T cells (yellow arrows, IL-13^{tdt-}/CD3^{+}) in the injury site at 10DPI. (B) Quantification of ILC2 infiltration into the spinal cord injury site by flow cytometry (N=3-4, representative of 2 experiments; one-way ANOVA with Tukey's multiple comparisons test). (C) Single cell suspensions of spinal cord injury sites 10DPI were stimulated with PMA/Ionomycin and brefeldin A or brefeldin A along and tested for IL-13 expression. Infiltrating ILC2s are competent to make cytokine (N=4; Student's T test). (D) WT and IL-33$^{-/-}$ mice were injured, and at 10DPI the injury site was analyzed for ILC2 infiltration (N=4 WT and 3 IL-33$^{-/-}$ mice; Student's T test).

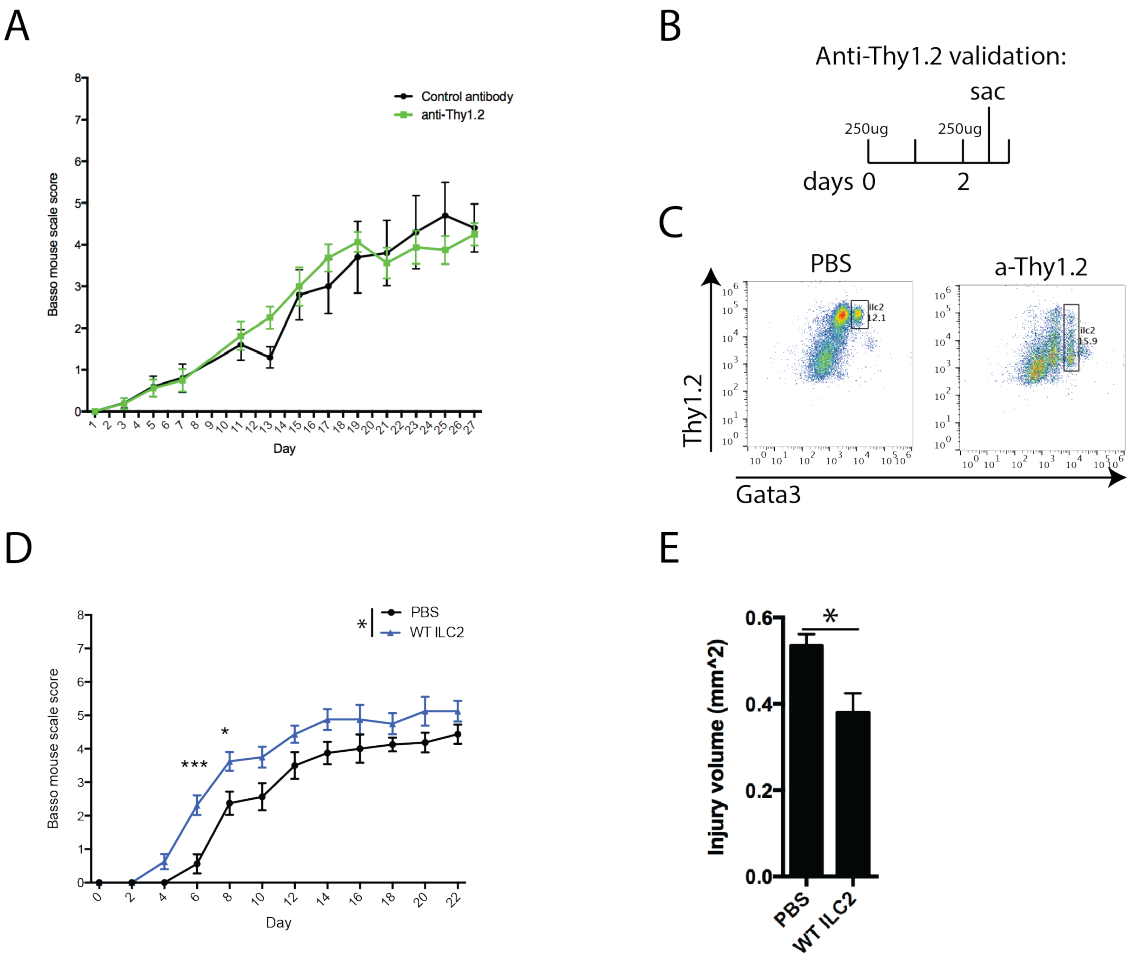

Figure 3.20: **WT ILC2s improve recovery of ST2$^{-/-}$ mice after spinal cord injury.** (A) Mice were injured after 3 injections every other day for 6 days with anti-Thy1.2 to deplete ILC2s in Rag$^{-/-}$ mice reconstituted with Thy1.1 T cells (N=8; two way ANOVA with Sidak's multiple comparisons test). (B) Timeline of depletion for FACs presented in (C), demonstrating blockage of the Thy1.2 epitope but not depletion of GATA3^{+} cells (gated on CD45^{+}/Lineage^{-} live cells; representative of 3 experiments). (D) 5000 WT lung ILC2s were purified by FACS and delivered i.c.v. into ST2$^{-/-}$ animals in 1 µL of PBS. The control group received 1 µL of PBS i.c.v. (N=8; two way ANOVA with Sidak's multiple comparisons test). (E) Lesion volume of ST2$^{-/-}$ mice treated with PBS or ILC2s (N=4; Student's T test).

Chapter 4

Discussion and Future Directions

Whenever we experience injury, whether it is something as simple as a papercut or severe as spinal cord contusion, a rapid immune response is engaged. The response to these injuries will be unique, and the severity, tissue, and type of injury actively influence recruitment of an appropriate immune response. How is this level of control achieved, and what, on a molecular scale, initiates inflammation after injury?

These questions have become a prominent theme in my graduate work, including the two projects presented in this thesis, and were originally inspired by the work of Polly Matzinger. As discussed in the Introduction, Matzinger challenged the "self" vs "non-self" model of immune activation in favor of the danger theory, and in doing so she provided a framework to understand previously mysterious phenomenon (Matzinger, 2002a,b). A perfect example of this is sterile inflammation, where despite the lack of non-self antigens there is a robust immune response. The immune system responds to danger, often in the form of molecular patters that report cellular damage (DAMPs and alarmins) or pathogen presence (PAMPs).

In this thesis I have sought to understand how inflammation is initiated and regulated by alarmins after CNS injury, focusing on IL-33. I described the expression of IL-33 in the healthy CNS, as well as its release and functions. IL-33 acts on glia to initiate chemokine production and monocyte recruitment. I also studied ILC2s, known to be a highly IL-33

responsive cell type, describing their localization and IL-33 dependent or independent actions after CNS injury. The work in this thesis adds IL-33 to the list of currently known CNS alarmins, HMGB1 and ATP, and contributes to a more complete picture of CNS inflammation (Figure 4.1). In this chapter I will discuss my results in more detail, placing them in a broader context and describing their implications. I will also speculate on hypotheses derived from my work, and briefly describe future experiments to address them.

4.1 IL-33 is a novel CNS alarmin

There is longstanding confusion in the CNS injury field about the role of inflammation, whether it is beneficial or detrimental, and this was reviewed at length in the Introduction. The ideal experiment to answer this question would compare injury outcome with and without inflammation. Furthermore, if we completely understand the alarmins that initiate inflammation, we could block them, blocking inflammation along with them, and perform the perfect experiment. Admittedly, complete blocking of alarmins after injury is, at best, an interesting thought experiment, but nevertheless I sought to describe one alarmin in the CNS, IL-33. Our studies on this alarmin alone have provided insights into the role of inflammation after injury, but we remain far from a complete understanding.

IL-33 is an alarmin predominantly studied in the lung and skin. These tissues have high IL-33 expression, and IL-33 has established roles in diseases like asthma and allergy. I focused on IL-33 in CNS injury, where neither its expression nor disease relevance had been well characterized, in an effort to understand the initiation of CNS inflammation. Discussed in this section is the expression and alarmin nature of IL-33.

4.1.1 IL-33 expression in the healthy CNS

To determine the extent and location of CNS IL-33 expression I performed an RT-PCR screen of different brain regions. Interestingly, there is a large disparity of IL-33 mRNA between CNS regions, with white matter generally expressing more IL-33 than gray matter. IL-33 expression correlates with genes associated with myelin, which led us to hypothesize and demonstrate enriched expression of IL-33 in oligodendrocytes. IL-33 expression is initiated with oligodendrocyte development: OPCs express very little IL-33, a subset of premyelinating oligodendrocytes turn it on, and virtually all mature oligodendrocytes express it. Finally, though the majority of IL-33$^+$ cells are oligodendrocytes, IL-33 is also expressed in a subset of gray matter astrocytes.

The fact that IL-33 is expressed in the CNS was expected. The original paper describing IL-33 actually reported the highest IL-33 expression in the mouse and human CNS (Schmitz et al., 2005). Its predominance in oligodendrocytes, however, was surprising and actually differed from previous studies. Astrocytes were identified as the primary CNS source of IL-33 both in vivo using IHC (Pomeshchik et al., 2015) and in vitro by western blot (Yasuoka et al., 2011). The likely reason for our different results is simple: prior studies had never looked for IL-33 expression in oligodendrocytes, instead focusing on astrocytes and microglia. Given our RT-PCR results we had reason to believe these were not the only players, and actively sought to demonstrate expression in oligodendrocytes. Our conclusions regarding IL-33 expression have since been validated by another group (Zarpelon et al., 2016), and agree with an RNAseq resource published by the Allen Brain Institute (interactive dataset: http://casestudies.brain-map.org/celltax# section_ explorea) (Tasic et al., 2016).

Why is IL-33 expressed by glia, and not neurons? Neurons convey information, and their lack of regeneration is the root cause of loss of function after SCI, so it would make sense to alert the immune response whenever they are damaged. On closer consideration glial, especially oligodendrocyte, IL-33 expression is actually better at accomplishing this than neuronal expression. IL-33 is a nuclear alarmin, while neurons can be damaged far from the nucleus. Damage to an axon tract would not damage any neuronal nuclei, but would inevitably damage oligodendrocytes, which intimately contact axons. In gray matter injury, for example to the cortex, IL-33 is expressed and presumably released by astrocytes. The pattern of IL-33 expression in health therefore covers both gray and white matter, but still doesn't explain the lack of neuronal expression. If neuronal cell bodies were damaged their IL-33 would still be released and effective, so why not express IL-33? An alternative rationale for the IL-33 expression pattern is that IL-33 has an undiscovered function outside of injury, and this is discussed further in the next section.

4.1.2 IL-33: A glial death note and nothing more?

After establishing its cellular expression I tested whether IL-33 in the CNS is an alarmin. I approached this question by showing that IL-33, expressed in the healthy CNS, is released after CNS injury into the CSF. We detected IL-33 in the CSF 1 hour have SCI, but not in normal CSF or at later times after injury. IL-33 is also released within minutes from spinal cord contused in vitro. Notably, in vitro injured tissue released large amounts of IL-33 without inducing mRNA transcript, suggesting release of the endogenously expressed protein and not transcription/translation of new IL-33.

These results are consistent with IL-33 being a CNS alarmin, and though this is the function I focused on in my work, IL-33 could have additional, non-alarmin, roles as well. Several such roles have already been described in the periphery; for example, IL-33 is a nuclear chromatin-associated protein, and effects transcription and chromatin structure there (Choi et al., 2012; Roussel et al., 2008). Its role in the nucleus is obviously not necessary for life, IL-33$^{-/-}$ animals are viable and appear grossly normal, but it does modulate specific pathways such as NFκB (Choi et al., 2012). In cell lines nuclear IL-33 sequesters NFκB components, dampening the response to IL-1β. Perhaps IL-33 plays a nuclear role in healthy glial, regulating transcriptional programs associated with mature oligodendrocytes and gray matter astrocytes. There are no gross defects in oligodendrocyte maturation of IL-33$^{-/-}$ mice (personal observations), but its function could be redundant or subtle.

A second potential non-alarmin function of CNS IL-33 is secretion without cell death, which has already established in other systems (Fock et al., 2013; Lee et al., 2015). Xi et al. even noted this in the retina, with IL-33 secretion by Müller cells after chronic light exposure (Xi et al., 2016). If glia-derived IL-33 could be actively secreted, it enormously increase the potential impact IL-33 could have in the healthy CNS. But, what stimuli would induce it? Perhaps mild stressors similar to chronic light, local neuronal firing or even psychological stress, could cause small amounts of IL-33 release in the brain. IL-33 could be regularly active in the healthy brain, reflexively being secreted after a given stimulus to maintain homeostasis.

Studies into non-alarmin functions of IL-33 are currently a minority, and in most scenarios IL-33 is thought to have its most potent effects when released from dying or damaged cells. Given the extensive expression of IL-33 in healthy CNS, however, looking for nuclear or other homeostatic functions remains an enticing avenue of future research.

4.2 Cellular targets of IL-33: infiltrating monocytes and local glia

IL-33 is expressed by oligodendrocytes and gray matter astrocytes and released after CNS injury. Which cell types respond to this IL-33, and how are they activated? In the coming sections I will describe my efforts to answer this question, focusing on infiltrating monocytes, local glia, and meningeal ILC2s.

4.2.1 Infiltrating monocytes

As described in the introduction, IL-4 induces an M2 skew in cultured bone marrow-derived macrophages (BMDMs; more similar to infiltrating monocytes than tissue resident macrophages). Interestingly, IL-33 has no effect on skew alone, but strongly potentiates IL-4 activity (Kurowska-Stolarska et al., 2009). Based on these findings and reports of IL-4 dependent "M2-like" macrophages at the injury site (Kigerl et al., 2009; Fenn et al., 2014), I hypothesized that IL-33 acts on infiltrating monocytes and potentiates an M2 phenotype.

To address this idea I developed an in vitro assay that modeled exposure of macrophages to CNS alarmins, asking whether the cocktail of CNS alarmins, including IL-33, potentiated the effects of IL-4. BMDMs were treated with IL-4 with and without addition of acutely isolated CNS tissue. Our results supported a role for IL-33 affecting macrophage skew–the presence of CNS tissue potentiated the skewing effects of IL-4 in an IL-33 dependent manner. IL-33 is probably not the only CNS derived signal potentiating IL-4 in vitro, however, as $ST2^{-/-}$ BMDMs showed a decreased but not ameliorated response to CNS tissue incubation.

Our in vitro studies were promising, and I sought to validate IL-33's effect on macrophage phenotype in vivo. I started by collecting mRNA from WT and IL-33$^{-/-}$ lesion sites and measuring macrophage M2 markers, finding a striking reduction in all markers in IL-33$^{-/-}$ mice. I next attempted to confirm these phenotypic changes on a per cell basis by analyzing the same genes on CD11b^{+} cells sorted from the WT or IL-33$^{-/-}$ injury site. Surprisingly, this experiment consistently showed no difference between genotypes, forcing me to reassess my hypothesis. M2 Marker expression was reduced in the total lesion site, but on a per cell basis was unaffected. Instead of affecting the phenotype of infiltrating macrophages, these results suggested that IL-33 was more important for their entry into the site of injury–fewer total infiltrating monocytes caused reduced marker expression in bulk IL-33$^{-/-}$ tissue, but when purified and analyzed they were found to have the same phenotype in WT and IL-33$^{-/-}$ mice.

I confirmed this with flow cytometry of the injury site, finding a defect in monocyte recruitment in IL-33$^{-/-}$ animals at 1DPI. At 7DPI the defect in early recruitment resulted in fewer total Ly6C^{low}/CD45^{hi}/F4/80^{hi} macrophages at the injury site. Interestingly, there was no difference between WT and IL-33$^{-/-}$ in neutrophil numbers, suggesting a specific defect in monocyte recruitment.

4.2.2 Glia respond to IL-33 with chemokine production

The realization that IL-33 governs monocyte recruitment, not phenotype, in vivo led me to consider that it participates in chemokine induction. Chemokines like CCL2 are rapidly induced after CNS injury and are critical for normal immune cell recruitment (Ma et al., 2002; Pineau et al., 2010). Microglia and astrocytes are major chemokine producers after injury (Jaerve and Muller, 2012), and I therefore asked the natural question: does IL-33 induce chemokine expression by glia?

I began in vitro by treating mixed glial cultures, largely composed of microglia and astrocytes, with IL-33 and measuring chemokine secretion. Glia secreted numerous chemokines, including CCL2, CXCL10, and CCL5, in response to IL-33. To more specifically assess whether

microglia or other glia are the major responders we performed microarray analysis on sorted $CD11b^+$ microglia and $CD11b^-$ (mostly astrocytes) glia with and without IL-33. Both groups of cells responded to IL-33, though $CD11b^-$ cells are more potent producers of CXCL10 and CCL2. In vivo results supported IL-33 as a chemokine inducer–$IL\text{-}33^{-/-}$ injury sites had decreased chemokine levels relative to WT. Importantly, though both $CD11b^+$ and $CD11b^-$ cells are capable of responding to IL-33, after injury $CD11b^+$ cells downregulate ST2 while $CD11b^-$ cells upregulate it. This suggests that microglia are most sensitive to IL-33 in homeostasis, but after injury lose sensitivity while astrocytes gain it.

A feature of these results that is somewhat puzzling is the specificity of impairment in $IL\text{-}33^{-/-}$ mice to recruitment of monocytes, despite broad reductions in chemokine expression. $IL\text{-}33^{-/-}$ mice had reduced *Ccl2*, *Cxcl10*, and *Cxcl2* transcript at the injury site. CXCL2 is best recognized as a neutrophil chemokine, yet there was no change in neutrophil recruitment in $IL\text{-}33^{-/-}$ mice. This discrepancy could have two potential explanations: we measured a ~50% decrease in CXCL2 mRNA, and either this is not reflective of actual protein changes, or the chemokine program driving neutrophil recruitment is sufficiently redundant not to be affected. It also is important to note that my findings may only pertain to spinal cord injury, and IL-33 could have broader impact on immune cell recruitment after other insults.

The results discussed so far shed light on the process of monocyte recruitment following spinal cord injury, but could broadly apply to neuroinflammation. I demonstrated that glia-derived IL-33 activates local astrocytes and microglia to initiate monocyte recruitment after spinal cord injury, and in theory any necrotic injury to oligodendrocytes will initiate a similar mechanism. IL-33 likely initiates inflammation after numerous disorders, including stroke, traumatic brain injury, CNS infection, or even peripheral nerve injury, and future studies on IL-33 in these conditions are warranted.

Discussed in the next section is a revised understanding of "immune privledge" in the CNS, I argue that meningeal immune cells fill the void of local CNS parenchymal leukocytes

by, for example, responding to CNS derived IL-33.

4.3 Meningeal leukocytes respond to CNS alarmins: focus on ILC2s

The CNS is an immunologically unique site. With few exceptions, the rest of the body is permeated with tissue resident leukocytes: T cells, ILCs, mast cells, dendritic cells, and macrophages. In non-CNS tissues, resident leukocytes mediate antigen drainage/presentation, participate in tissue homeostasis, and are sentinels to detect alarmins. For unclear evolutionary reasons, the mammalian CNS has limited leukocytes from its parenchyma (besides microglia), and it is tempting to assume that the CNS traded these functions of tissue resident leukocytes for some beneficial trait. I disagree with this, and believe the CNS has not lost the functions of tissue resident leukocytes, but merely transferred them to the meninges.

The Kipnis lab has maintained a longstanding focus on the sub-arachnoid space, and in the last several years I have gradually become convinced that meningeal leukocytes can perform all the functions of tissue resident cells elsewhere in the body. For example, through the glymphatic system, antigens drain from the brain parenchyma to the CSF (Iliff et al., 2012). In the CSF, antigens could be picked up by meningeal dendritic cells and presented in draining deep cervical lymph nodes via meningeal lymphatics. Meningeal lymphocytes maintain brain homeostasis, and lack of meningeal T cells, IL-4, or IFNγ impairs higher order function (Brynskikh et al., 2008; Derecki et al., 2010; Filiano et al., 2016). One question therefore remains: do meningeal immune cells also serve as sentinels, detecting alarmins after CNS injury?

I believe the answer to this questions is yes, and in the coming section will discuss my results regarding meningeal ILC2s as evidence. ILC2s are recently described cells, with little known about them in general and almost nothing in the meninges. In the following sections I will discuss meningeal ILC2s, describing my work on their presence in the meninges, their localization, and their response to spinal cord injury, finally revisiting the notion that meningeal immunity takes the place of tissue resident leukocytes, detecting alarmins and

initiating CNS inflammation. I will also propose hypotheses about the uniqueness and functions of ILC2s in particular.

4.3.1 A unique residence for meningeal ILC2s

Are ILC2s in the meninges, and if so, where? Using flow cytometry I detected abundant ILC2s in the healthy meninges. ILC2 are strongly concentrated in the naïve brain versus spinal cord meninges, and are not detectable in spinal cord parenchyma. The concentration in brain meninges was interesting, and I pursued it by developing a method to visualize ILC2s histologically. Using a IL-13^{cre} reporter system I demonstrated a dramatic concentration of ILC2s around the dural sinus that largely accounted for overall differences between brain and spinal cord meninges.

These results are the first to show that ILC2s are in the meninges. We were also unable to detect ILC2s in the spinal cord parenchyma by, apparently contradicting published work that shows them in the healthy CNS (Russi et al., 2015). Notably, we studied different mouse lines than Russi et al. (C57/Bl6 versus SJL), and it is possible that SJL mice have ILC2s in the CNS parenchyma while C57/Bl6 do not. An alternative explanation is that meninges were included in the CNS flow in Russi et al., contaminating CNS preps with meningeal ILC2s. This is a common oversight in neuroimmunology literature, and I believe is the likely reason for differences in our results. Authors frequently analyze the CNS by flow cytometry without removing meninges, and therefore contaminate the preparation with abundant leukocytes from the brain's borders.

The observation that ILC2s concentrate around the dural sinus is intriguing, though still largely mysterious. The dural sinuses are unique structures in the body, and it is therefore difficult to relate the localization of meningeal ILC2s to those found elsewhere or even hypothesize on their function. The abundance of ILC2s in the dural sinus suggests that they have some role there, or at least are attracted to unidentified signals originating from it. Adding even more allure to the dural sinus, recent studies from our lab have identified other

leukocytes also congregate around it; both T cells and MHCII$^+$ cells are highly enriched in the dural sinus relative to dura/arachnoid, pia, or choroid plexus (Louveau et al., 2015). ILC2s are significant presence both in the dural sinus and elsewhere, being about half as prevalent in the sinus as T cells and roughly equivalent to them in the other meninges (Louveau et al., 2015).

All of these cells, ILC2s, T cells, and MHCII$^+$ cells, are not only proximal to the sinus, but also to lymphatic vessels that run along it (Louveau et al., 2015). The riddle of leukocyte concentration around the dural sinus is an enticing problem to work on. The phenomenon is clearly real but entirely not understood, and further characterization of other cells present, the signals that attract them, and the consequences of removing them from the dural sinus all represent promising future avenues of research.

4.3.2 What do ILC2s do after CNS injury?

ILC2s are abundant in the meninges and IL-33 is released into the CSF following injury. Meningeal ILC2s, detecting IL-33 in the CSF, ought to be activated, and I tested this activation by flow cytometry after injury. We observed IL-33 dependent activation of both spinal and brain meningeal ILC2s acutely after injury, though interestingly the details of their activation differed: spinal meningeal ILC2s upregulated activation markers like CD25 and CD69, but showed no change in IL-13 expression, while brain meningeal ILC2s produced more IL-13 acutely after injury but had no change in CD25 or CD69.

ILC2s are tissue resident cells, turning over slowly if at all in vivo and not being detectable in healthy mouse blood (Gasteiger et al., 2015), but I nonetheless checked whether they infiltrate the lesion site. Surprisingly, ILC2s did enter the cord, peaking at ˜10DPI and lingering until at least 30DPI. They are sparse in the injury site, with no more than 50 being detected per injury site. Still, ILC2s are potent cytokine producers and could have important roles in resolving inflammation at the injury site. An important outstanding quesiton is what signal caused ILC2s to infiltrate? Two molecules that promote ILC2s migration have been

described, IL-33 and PGD2, though IL-33 has only been characterized in human ILC2s (Xue et al., 2014; Salimi et al., 2013). ILC2 numbers at the injury site were unchanged in IL-33$^{-/-}$ mice, suggesting that it is not driving entry. Future studies along this line could test the *Gpr44*$^{-/-}$ (*Gpr44* is the gene coding for CRTH2, the PGD2 receptor) mouse, which lacks PGD2 signaling and has known defects in ILC2 accumulation to the infected lung (Wojno et al., 2015).

Overall, these findings regarding meningeal ILC2s support the idea that meningeal leukocytes "fill the void" of tissue resident leukocytes in the CNS. Where in other tissues like lung or skin released IL-33 activates local ILC2s, in the CNS meningeal ILC2s are the target. Through actions on ILC2 and other meningeal cells CNS alarmins could alert a full complement of immune cells despite them not being present in the meninges. Though it remains speculative, meningeal immune cells could perform functions for the CNS normally attributed to tissue resident leukocytes: draining and presenting CNS antigens, maintaining tissue homeostasis, and responding to danger signals.

4.3.3 Tissue-based control of ILC2 activity and phenotype

As described, brain and spinal meningeal ILC2s have different activation programs after CNS injury. The reason this remains unclear, particularly since both effects were IL-33 dependent. I have, however, accumulated evidence that supports one explanation–that ILC2s acquire tissue-dependent and plastic phenotypes which govern their activity. Given the unique localization of brain meningeal ILC2s around the dural sinus, it is conceivable that they would receive a unique set of environmental signals. My results could therefore reflect a difference between brain and spinal meninges-resident ILC2s governed by unique microenvironments. In this scenario the local environment differentially primes ILC2s for a certain activation program, with brain meningeal ILC2s primed for rapid IL-13 production.

In support of this hypothesis, we have observed baseline enhancement in cytokine production of ILC2s derived from brain meninges versus spinal meninges or lung. These differences persist in ILC2s that have been purified by FACs from tissues. Interestingly, co-incubation of brain meningeal and lung single-cell suspensions during in vitro stimulation inhibited cytokine production by brain meningeal cells, suggesting the presence of a dominant inhibitor of ILC2 activity in lung homogenates. An important caveat of these experiments is that tissues are digested and homogenized prior to activation, staining, and analysis. Thus, the increased sensitivity of brain meningeal ILC2s relative to spinal cord meningeal or lung could be an artifact of our tissue preparation and in vitro stimulation. For example, a signal present in the lung homogenate during stimulation could inhibit ILC2 cytokine production, irrelevant to the in vivo situation. Ideally we would measure cytokine responses of ILC2s in vivo, analyzing them by antibody or reporter in fixed tissue slices, but this experiment remains challenging with current technology. Cytokine staining does not work on fixed slices, and the current IL-13 reporter (Yet-Cre 13) is too dim to be detectable by histology. A more sensitive reporter would allow this experiment, and given that tool I would hypothesize that brain meningeal ILC2s would be more responsive to stimulation relative to spinal meningeal or lung ILC2s.

The concept of ILC2 phenotypes is not without precedent. In 2015 Paul and colleagues identified so called "inflammatory" versus "natural" ILC2s (iILC2 and nILC2 respectively) (Huang et al., 2015). iILC2s are generated transiently during *N. brasiliensis* infection or systemic IL-25 administration, and return to an nILC2 phenotype when cultured in vitro (Huang et al., 2015). Brain meningeal ILC2s are not iILC2s, as iILC2s lack ST2 expression, but the description of ILC2 phenotypes in inflammation opens the possibility for tissue-based control of ILC2 activity.

4.3.4 A working hypothesis of meningeal ILC2 function in health and after CNS injury

Meningeal ILC2s respond to injury, becoming activated, producing cytokine, and infiltrating the injury site, and addition of ILC2s i.c.v. improves outcome from SCI. My findings describe ILC2s after CNS injury, but the question remains: *how, mechanistically, do ILC2s improve recovery?* This has been a challenging question to address, particularly given the lack of mouse models specifically deficient in ILC2s. Here I will describe my current working hypothesis of ILC2 function in injury and how it could also apply to the healthy state. This hypothesis is derived from a synthesis of my existing work with several other studies, which are summarized below.

ILC2s promote type 2 inflammation not only in the direct effect of their cytokines on tissues, but also by initiating and recalling the T_h2 response. In lung and skin models of allergy, ILC2-derived IL-13 induces DC migration to local lymph nodes where they activate and skew T cells to a Th2 phenotype (Halim et al., 2014). ILC2-derived IL-13 also works through DCs to recruit and potentiate memory Th2 responses (Halim, 2016). These findings frame ILC2s as critical players in Th2 differentiation, and connect them to recent work from our lab demonstrating that T cell derived IL-4 is neuroprotective after CNS injury (Walsh et al., 2015). Th2 cells enter the injury site and produce IL-4, which acts directly on neurons to promote neurotrophin production and survival (Walsh et al., 2015). The final piece are meningeal lymphatics, mentioned above, which are vessels running parallel to the dural sinus (Louveau et al., 2015). These vessels connect the sub-arachnoid space to the deep cervical lymph nodes (DCLNs), and are a route for molecule and cell drainage (Louveau et al., 2015).

Connecting these pieces, I hypothesize that ILC2s in the dural sinus initiate a beneficial Th2 response via local DCs and the meningeal lymphatics. After injury, IL-33 would induce IL-13 production from brain meningeal ILC2s, which activates DC migration through lymphatics and skewing of naïve T cells in the DCLN. In this hypothetical model, Th2 cells would then enter the injury site and produce IL-4, which is known to be neuroprotective (Figure

4.2). This mechanism would help explain the high concentration of ILC2s around the dural sinus. From that position they are ideally situated to influence cells such as DCs as the migrate through lymphatics to the lymph node, and in that way ILC2s could regulate overall meningeal immunity through this function.

A major role of meningeal ILC2s could be to promote a local Th2 cell phenotype, which would benefit recovery in injury. It is interesting, if speculative, to also consider ILC2s as promoting type 2 immunity in the healthy meninges. Meningeal T cell-derived IL-4 promotes healthy brain function (Derecki et al., 2010), but it remains unknown what initiates and maintains IL-4 producing T cells in the meninges. ILC2s are abundant in the sub-arachnoid space, and could produce IL-13 in response to CSF signals such as PGD2 (a potent ILC2 activator known to be present in the CSF) (Xue et al., 2014; Ram et al., 1997) or even secreted IL-33. Through IL-13 production, ILC2s could be instrumental in supporting a type 2 inflammatory environment in the healthy meninges.

4.4 Why initiate CNS inflammation?

Injury to the brain results in robust inflammation, driven by endogenously expressed CNS alarmins. The expression of brain alarmins is interesting from an evolutionary standpoint, particularly in light of the persistent confusion regarding the role of inflammation in CNS injury. Perhaps the mere fact that alarmins evolved CNS expression is evidence supporting an overall beneficial role for neuroinflammation. Why would IL-33 or HMGB1 expression be selected for in the CNS, if not to confer some sort of immune-mediated protection?

In the case of IL-33 in CNS injury this conjecture seems to hold. IL-33$^{-/-}$ animals have impaired recovery following either spinal cord injury or optic nerve crush. The impairment is only apparent in models where IL-33 expressing glia are traumatically injured, as direct glutamate toxicity on retinal ganglion cells is unchanged between WT and IL-33$^{-/-}$ mice.

What could be the beneficial function of IL-33? So far I have described multiple of its alarmin effects–IL-33 initiates monocyte recruitment and activates meningeal ILC2s–but the relative importance of these mechanisms is unknown. There is data to suggest both are beneficial: CCL2 injections improves SCI outcome in IL-33$^{-/-}$ and enhanced monocyte recruitment. Similarly, WT ILC2 delivery i.c.v. enhanced recovery in ST2$^{-/-}$ animals, where no other cell was capable of responding to IL-33. Neither of these experiments help determine the most critical in vivo function of IL-33, however, and only demonstrate that when artificially enhanced both ILC2s and infiltrating monocytes can be beneficial. Furthermore, as yet unstudied IL-33 targets, for example meningeal mast cells or Tregs, could be influencing injury outcome. A key tool that would answer this question is the ST2^{flox} mouse. By systematically removing ST2 from astrocytes, microglia, and ILC2s (and inevitably other cells, as no ILC2 specific cre exists), the functional outcomes of IL-33's separate actions could be elucidated.

IL-33 expression in the CNS is among the highest of any tissue in the body (Schmitz et al., 2005). Why did the brain evolve such a particularly high expression? I have already mentioned potential non-alarmin functions of IL-33, but my experiments showing no effect of IL-33 in glutamate toxicity suggest a beneficial alarmin function. In one sense it is logical for the CNS, normally void of leukocytes, to have the highest alarmin expression. After injury, the CNS must produce an especially loud "alarm" to activate distal meningeal cells and initiate inflammation. The CNS does not have the benefit of local immune cell sentinels, and compensates for this by expressing high levels of IL-33 that can have far reaching effects.

Though the results presented in this thesis are generally consistent with a beneficial role for inflammation, uncertainty remains in the field and inflammation likely has distinct beneficial and detrimental functions. Despite uncertainty in the field, preclinical studies continue to cite inhibition of inflammation as an explanation for beneficial effects seen in their treatment paradigms (Myers et al., 2014; Benedict et al., 2012; Bao et al., 2011). There is a dire need for consensus among basic scientists about which aspects of inflammation are good or bad to inform accurate and relevant readouts in future preclinical studies.

4.5 Concluding remarks

The field of neuroimmunology is undergoing a renaissance, with fundamental discoveries regarding the homeostatic function of microglia (Stevens et al., 2007; Parkhurst et al., 2013), drainage of CNS antigens (Louveau et al., 2015; Plog et al., 2015), meningeal immunity (Radjavi et al., 2014; Derecki et al., 2010), and neurologic control of inflammation (Tracey, 2016; Talbot et al., 2015) occurring rapidly. It has been fantastic to witness and participate in the creation of a new narrative for neuroimmunology and neuroinflammation. The CNS and immune systems are not enemies, but are intimately engaged in bidirectional regulation, communication, and support.

In this thesis I have described two projects, regarding IL-33's role in recruiting macrophages and activating ILC2s after CNS injury, that have occupied much of my attention for the past 4 years. In this Thesis I have attempted to place these findings in the larger scheme of CNS injury, providing an introduction to inflammation after CNS injury, discussing the unique aspects of IL-33 as an alarmin, its proinflammatory effects, and proposing mechanisms to explain defects seen in IL-33$^{-/-}$ animals after SCI. In summary, I have described a novel paradigm of immune activation after CNS injury, revolving around the nuclear alarmin IL-33.

What hasn't been significantly discussed here, however, are the countless failed hypotheses that led to our current understanding and the final data I described in the Methods. Though anyone who has attempted to do science must be familiar with this, successful ideas are only built upon failed ones. When presented here as a final product the path of experiments are deceptively linear. The actual path has been a maze of false positives, good but wrong ideas, and faulty reagents. It is through understanding how things are not that we gain a sense of how things actually are. "Failed" experiments have taught me at least as much as successful ones, though they receive too little acknowledgement. For this reason I conclude that failures, at least conclusive ones, are incredibly desirable in science.

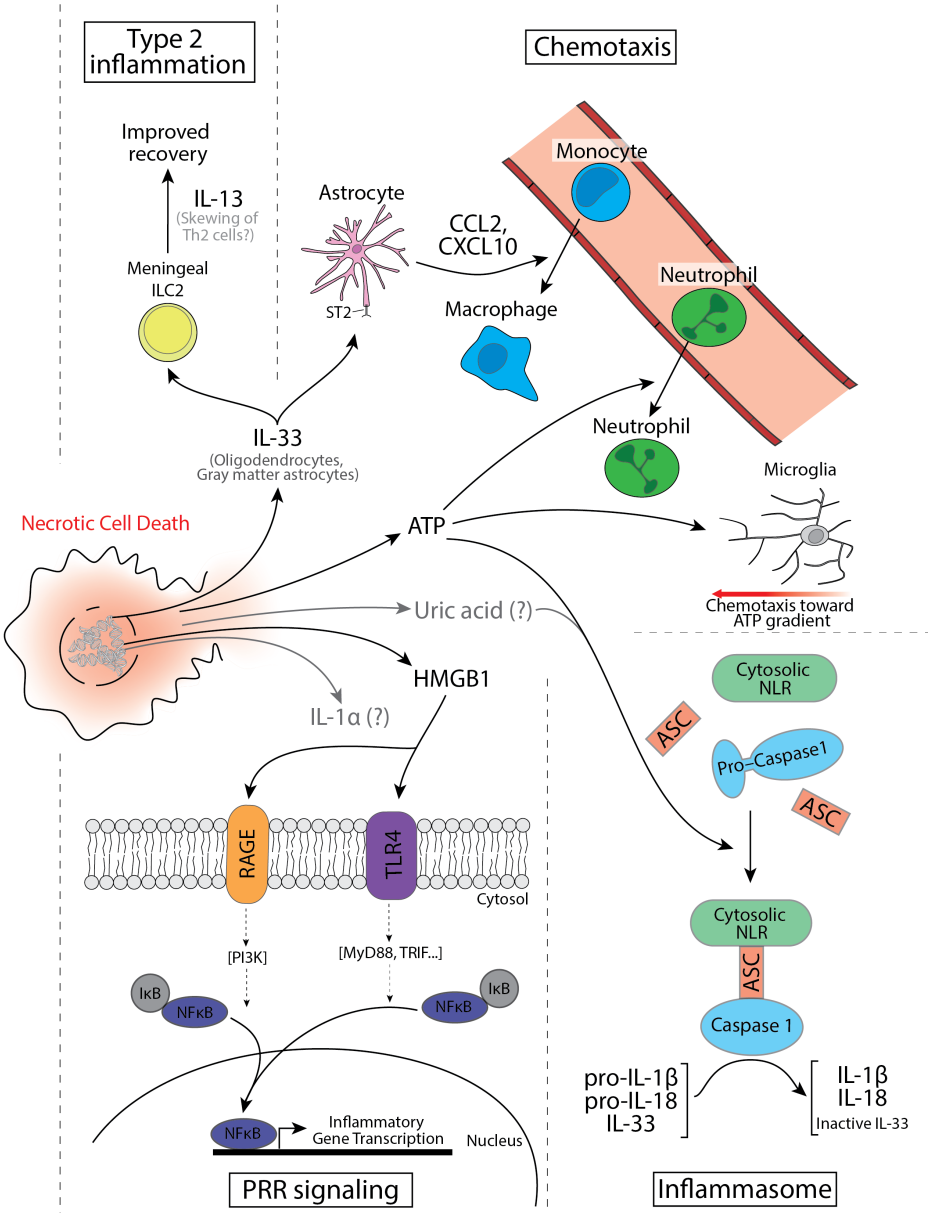

Figure 4.1: **A summary of CNS alarmins and their functions.** Necrotic cell death releases peptide and nucleic acid derivative alarmins that initiate inflammation. IL-33 acts on meningeal ILC2s, inducing them to produce IL-13. ILC2s are beneficial to injury recovery. IL-33 also plays an important role in bringing monocyte-derived macrophages into the CNS through upregulation of astrocytic chemokine expression. ATP promotes chemotaxis of neutrophils (through its activation of the inflammasome), and is directly chemotactic to microglial processes. ATP also activates the inflammasome, stimulating the assembly of the cytosolic NLR, ASC, and pro-caspase 1. Pro-caspase 1 is auto-cleaved to mature caspase 1, which cleaves pro-IL-1β and pro-IL-18 to active forms and IL-33 to an inactive form. HMGB1 signals through TLR4 and RAGE receptors, directly promoting inflammatory cytokine and chemokine production. An important transcription factor downstream of both receptors is NF-κB, important in enhancing inflammation and cellular infiltration, but the RAGE receptor has several other downstream signaling pathways (not shown here). IL-1α and uric acid (gray arrows), are alarmins in the periphery, but their roles in response to CNS injury remained poorly defined. Adapted with permission from (Gadani et al., 2015a).

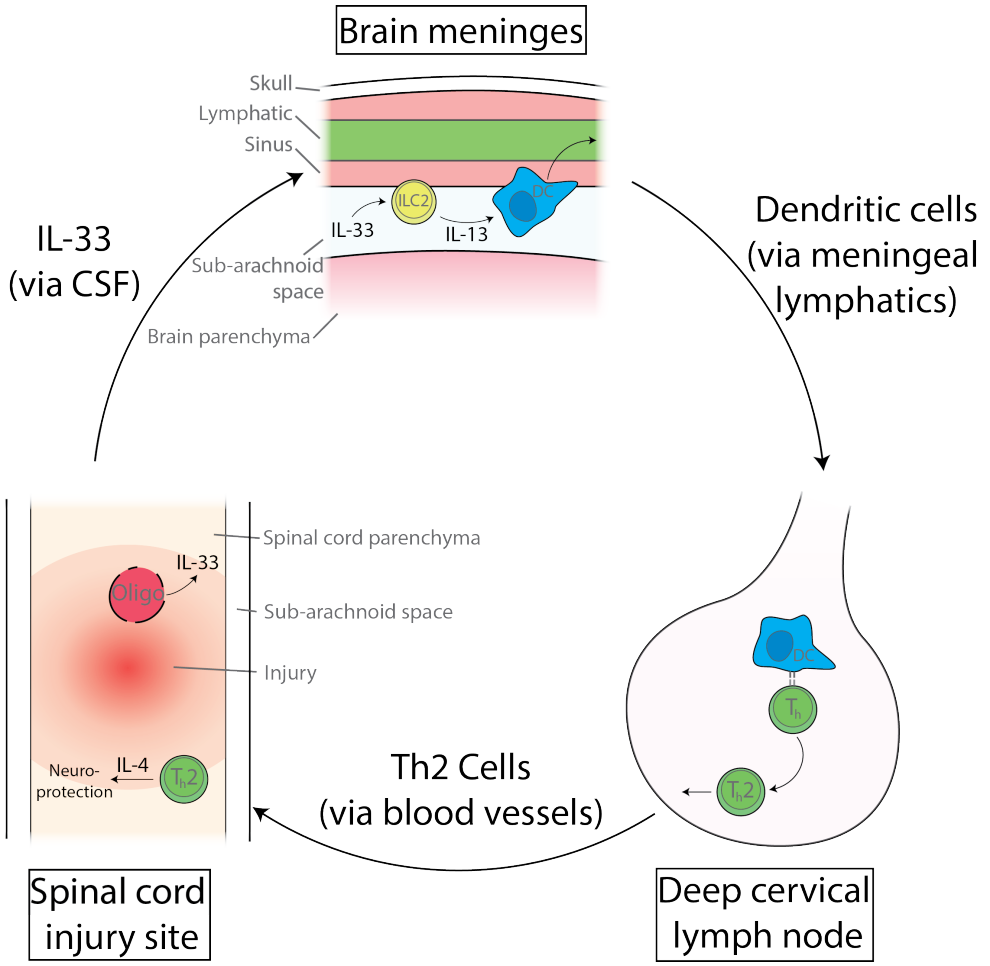

Figure 4.2: **A working model of ILC2 function after SCI.** In this working model, IL-33 released from oligodendrocytes activates the brain meningeal ILC2s in proximity to lymphatics and the dural sinus. ILC2s produce IL-13, which initiates DC migration to local lymph nodes (the deep cervical lymph nodes (DCLNs)). In the DCLN, these DCs activate and skew naïve T cells to T_h2. T_h2 cells then return to the spinal core via blood vessels, where they produce IL-4 that is neuroprotective.

Chapter 5

Materials and Methods

Mice

IL-33$^{-/-}$ mice were generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP repository (www.komp.org). ST2$^{-/-}$ mice were generated in the lab of Dr. A. McKenzie (University of Cambridge) and were gift from Dr. P. Bryce (Northwestern University). YetCre-13 mice were generated by and a gift from Dr. R. Locksley (UCSF). C57/Bl6, Tdtomato^{stoplox}, and CX3CR1-eGFP mice were obtained from Jackson Laboratory (Bar Harbor, Me), Stock # 004999, # 007905, and # 005582 respectively. All animals were housed in temperature and humidity controlled rooms, maintained on a 12 hour light/dark cycle (lights on 7:00 A.M.), and age-matched in each experiment. All strains were kept in identical housing conditions. For survival surgeries, mice were anesthetized with either 200μL of Ketamine/Xylazine (1mL KetamineHCl (1mg/mL), 1mL of 2% Xylazine, 8mL saline) or inhaled isoflurane. All procedures complied with regulations of the Institutional Animal Care and Use Committee (ACUC) at the University of Virginia.

Statistics

Statistical tests performed in Prism (Graphpad) software as described in the text and figure legends. In all figures error bars represent mean SEM; * = P 0.05; ** = P 0.01; *** = P

0.001.

Quantitative real-time PCR

RNA was extracted from cells or tissues using the ISOLATE II RNA Mini kit (Bioline) according to manufacturers instructions, and complementary DNA was then synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). A C1000 Touch Thermal Cycler (BIO-RAD) was used to carry out the reverse transcription reaction. qPCR reactions were carried out using TaqMan Gene Expression Probes (Applied Biosystems) and Luminaris Taqman Mastermix (Fischer Scientific). Taqman probes used in this study were: Arg1 (Mm00475988m1), Nos2 (Mm00440502m1), Il33 (Mm00505403m1), Il10 (Mm00439614m1), Ccl2 (Mm00441242m1), Cxcl10 (Mm00445235m1), Cxcl2 (Mm00436450m1), Ccl5 (Mm01302427m1), Cxcl1 (Mm04207460m1), St2 (Mm00516117m1), Mog (Mm00447824m1), Mbp (Mm01266402m1), Mag (Mm00487538m1), Mrc (Mm00485148m1), Chi3l3 (Mm00657889mH). Reactions were run in triplicate on either an IQ5 or CFX384 real-time thermal cycler (BIO-RAD). Data was normalized to GapDH expression and analyzed by the $2\Delta\Delta CT$ method.

Gene array analysis

RNA was extracted from cells as described above and analyzed using an Illumina genearray by UVA core facilities. Data was analyzed and graphed using R statistical software.

Tissue preparation and Immunofluorescence

For quantification of IL-33$^+$ cells in CNS, brain and spinal cord was harvested from animals after perfusion with heperanized (5U/mL) PBS and 4% PFA. The tissue was then post-fixed for 48 hours in 4% PFA, followed by cryoprotection in 30% sucrose for 48 hours. 40μm sections were sliced into a 24-well plate containing PBS + 0.05% Na Azide. For staining of injured spinal cords, tissue was harvested, post-fixed, and cryoprotected as described. Spinal cords

were cut into 20μm sections, mounted on gelatin-coated slides, and stored at -20°C until use. Slices were blocked for 1hr at RT in blocking buffer (2% serum (of the 2ndary's species); 1% BSA; 0.1% triton; .05% tween; .05% Na Azide) followed by overnight incubation in primary antibody at 4°C. The following antibodies were used for immunofluorescence staining: Goat anti-IL33 (R& D Systems AF3626; 1:100), Rabbit anti-OLIG2 (Millipore AB9610; 1:1000), Mouse anti-APC (EMD Millipore OP80; 1:300), Rabbit anti-ALDH1L1 (Abcam ab87117; 1:300), Rabbit anti-Iba1 (Wako 019-19741; 1:300), Chicken anti-GFAP (Millipore AB5541; 1:1000), Goat anti-BRN3a (Satna Cruz sc-31984; 1:300), Goat anti-arginase1 (Santa Cruz sc-18354; 1:100), Rabbit anti-Ki67 (Abcam ab15580; 1:300), Rat anti-CD3 660 (eBioscience 50-0032; 1:300), Rat anti-CD3 biotin (eBioscience 13-0032; 1:300). Slices were washed 3x 10 minutes, incubated for 2 hours at RT with the appropriate secondary antibodies (all from Life Technologies; 1:1000), washed again 3x10 minutes and mounted with aquamount (Thermo Scientific) and DAPI. For ALDH1L1 staining antigen retrieval was performed. Slices were incubated in sodium citrate buffer (10mM sodium citrate, 0.05%, Tween-20, pH 6.0) at 80°C for 30 minutes, washed 3x in PBS, and followed by the standard block/staining protocol.

Toluidine blue staining

For staining meningeal mast cells toluidine blue staining was performed on samples after being stained and imaged for florescence markers. Meninges were stained in 0.5% Toluidine blue O (Ph 2.5; Sigma-Aldrich) for 15 mins at room temperature. Toluidine blue was washed overnight at 4°C, and images acquired using brightfield microscopy.

IL-33 treatment

For IL-33 treatments to expand ILC2s, mice were injected i.p. with 500ng carrier-free recombinant IL-33 (eBioscience) every other day. After 3 injections, mice were sacrificed and ILC2 numbers analyzed by flow cytometry.

CSF collection

Mice were anesthetized with ketamine/xylazine and placed in a prone position with the head elevated relative to the body. The skin, fascia, and muscle over the dorsal neck were removed to expose the dura mater of the cisterna magna. CSF was collected with a pulled glass capillary inserted into the cisterna magna and stored at -80°C until analysis.

Retrograde labeling of retinal ganglion cells

Mice were anesthetized with isoflurane, the skull exposed, and the head immobilized in a stereotactic device. Holes were drilled bilaterally in the skull above the superior colliculus (2.9 mm caudal to bregma and 0.5mm lateral to midline). 1μL of 4% Fluorogold (Fluorochrome) was injected 2mm below the meningeal surface at a rate of 0.5μL/min using a Hamilton syringe and a motorized injector. The dye was allowed to diffuse into the tissue for 1 minute before the syringe was removed. The scalp was then sutured closed and the mice allowed to recover on warming pads at 37°C.

Optic nerve injury

Three days following Fluorogold injection the optic nerve was injured. Mice were anesthetized with ketamine/xylazine and the tissue around the nerve was carefully dissected and the optic nerve exposed. N5 self-closing forceps were closed around the optic nerve 5mm behind the globe and held for three seconds. The mice were then allowed to recover at 37°C on a warming pad before returning to their cages.

Intra-ocular glutamate injection

Glutamate injections were performed as previously described (Schori et al., 2002). Briefly, mice were anesthetized with ketamine/xylazine and the upper sclera punctured with a 25 gauge needle. A Hamilton syringe was inserted into the eye and 1μL of 400mM L-glutamate

(Sigma G1626) was delivered into the globe. Mice were sacrificed 7 days after injection and retinas harvested for counting.

Retina excision

Mice were enucleated, and the cornea removed at the corneal limbus. The lens was removed and the retina was separated from the sclera and pigment epithelium. Three cuts were made toward the optic disc to allow the retina to lay flat. Retinas were mounted on nitrocellulose paper and fixed in PFA prior to counting or staining.

Image quantification

For assessing RGC survival, pictures (of Fluorogold or BRN3a) from four quadrants of the retina were taken at equal distances from the optic disc using an Olympus IX-71 microscope. The pictures were counted in a blinded fashion to determine the number of RGCs/field. The contralateral uninjured eye was counted as a control for staining and to exclude baseline differences between genotypes. Images of IL-33 staining were acquired using a Leica SP8 confocal microscope. Counting was done in ImageJ with the Cell Counter plugin (Kurt De Vos, University of Sheffield).

To quantify spinal cord lesion size 20μm coronal sections were stained with GFAP and imaged. Lesion area per slice was quantified using ImageJ and total volume calculated using Microsoft Excel.

Spinal cord contusion

Female mice were first anesthetized with ketamine/xylazine. The back fur was shaved and underlying skin sterilized with an iodide/betadine solution. An incision was made over the T9-T10 vertebrae and the skin held back with retractors. The fascia overlying the spinal cord was removed to expose the vertebrae. The T10 vertebra was removed with fine rongeurs to expose the spinal cord. The IH-0400 Impactor (Precision Systems and Instrumentation,

Lexington Ky) was used to contuse the spinal cord centrally, after which the muscles and skin overlying the spinal cord were sutured closed and the mouse was allowed to recover on warming pads. The force of impact was computer controlled and set to 70 or 90 KDyn as noted in the text/legends. Mice were maintained on sulfa water and twice daily manual bladder expulsion. Two blinded observers assessed recovery of hind-limb locomotor activity with the Basso mouse scale (BMS)(Basso et al., 2006) following injury.

CCL2 treatment

Mice were contused as described above and injected with either saline or 10ng of carrier-free CCL2 (BioLegend) in a 1μL volume. Injections were made into the center of the injury site directly after contusion using a Hamilton syringe.

In vitro spinal cord contusion

Spinal cord segments were sterilely dissected after PBS perfusion and the meninges were carefully removed. The central portion of the spinal cord was cut into 6 sections 4mm in length and individually placed in wells of a 96-well plate. The IH-0400 Impactor (Precision Systems and Instrumentation) was oriented above cords and the computer set to contuse at a force of 50KDyn. After contusion, 200μL of sterile media (DMEM + Antibiotic-Antimycotic) was added to each well and the plate was incubated at 37°C. Supernatants were collected, centrifuged to separate any contaminating tissue, and analyzed for IL-33 protein by (IL-33 ELISA ready-set-go, Ebioscience 88-7333) following the manufacturers protocol. Absorbance was measured with a Multiskan FC microplate reader (Fischer Scientific).

MACs cell sorting

Samples were sorted based on CD11b expression using CD11b-FITC antibody (Ebioscience) and anti-FITC microbeads (Miltenyi). Briefly, samples were labeled with 1μL CD11b-FITC and incubated at 4°C for 30 minutes in MACs running buffer. Cells were then washed and

resuspended in 10μL anti-FITC microbeads and 40L MACs running buffer per 10^7 cells. After 10 minutes of staining, cells were washed and sorted using an autoMACs (Miltenyi).

Flow cytometry and cell sorting

To prepare single cell suspensions, spinal cords were dissected from PBS-perfused mice, and 1cm of cord centered on the lesion was isolated, minced, and digested in 4U/mL papain and 1mg/mL DNAse for 30 minutes with titration with a large-bore fire-polished pipette after 15 minutes. The samples were then triturated with a small-bore fire-polished pipette and passed through a 70μM filter. Meninges (brain and spinal cord) were dissected with fine forceps and digested in RPMI + 1.4U/mL Collagenase VIII (Worthington) + 1mg/mL DNase1 (Sigma) for 15mins at 37°C. Lungs were dissected, minced, and digested for 30mins in RPMI + 1.4U/mL Collagenase VIII + 1mg/mL DNase1 at 37°C. Digested meninges and lung were dissociated by pipetting and passed through 70μm filters. Whole brain samples were resuspended in a 40% percoll gradient, spun @650g for 25mins and the pellet was collected.

Samples were washed in FACs buffer (PBS, 0.05% Na Azide, 1mM EDTA, 2% FBS) and stained with antibody cocktail and viability dye for 30 minutes at 4°C in FACs buffer. For lineage staining, cells were labeled with biotinylated lineage antibody cocktail, washed, and the incubated with strepdavidin-PeCy7 or -Fitc for 30mins at 4°C. Zombie aqua fixable viability dye (Biolegend) was used to discriminate live cell populations. To obtain absolute cell counts, counting beads (eBioscience) were added to the samples. After staining, samples were washed in FACs buffer and resuspended in 4% PFA. Cultured glial cells were isolated and incubated in antibody after one wash in FACs buffer.

The following antibodies were used (all from Ebioscience unless otherwise noted): CD45-af700, Lineage-biotin (CD11b, B220, CD3, GR1, TER-119, FcεRα), Thy1.2-Fitc, ST2-Pe, C-kit-ef780, Sca1-af700, CD25-APC, TCR-Fitc or APC-Cy7, IL-7Rα-PeCy7, ICOS-Pe, ICOSL-APC, Gata3-660, Rort-PeCP594, IL-13-PeCy7, CD69-PeCy7, CD11b-PeCy5, CD11b-

FITC, CD45-APC, Ly6C-PerCP5.5, Ly6G-PeCy7, Glast-APC (Miltenyi; 1ug/test), rat anti-PDGFRα (APA5; 3μg/test), mouse anti-MOG (MAB5680 EMD Millipore; 1μg/test).

To stain intracellular antigens cells were fixed after extracellular staining with BD cytofix/cytoperm, washed in perm buffer, and stained for 30 minutes at RT in rabbit anti-OLIG2 (1:300) and goat anti-IL-33 (1:100). Samples were read on an LSR Fortessa (BD) or Gallios (Beckman Coulter) cytometer and analyzed using Flowjo software (Tree Star). ILC2s (selected as $CD45^+$/Lineage/$Thy1.2^+$/$ST2^+$ cells) were sorted on an Influx cell sorter (BD Biosciences) in the UVA flow cytometry core facility.

In vitro ILC2 stimulation

For in vitro stimulation and cytokine staining of ILC2s, normalized numbers of cells were maintained in RPMI + 10% FBS + Anti-anti at 37°C. Cells were treated with golgi block (Brefeldin A) and stimulated with PMA/Ionomycin for 4 hours before being washed and stained as described.

Bone Marrow-Derived Macrophages (BMDMs)

Bone marrow was collected from mouse tibias and femurs and passed through a 70μm screen. Cells were cultured in DMEM F12 + 10% FBS and 10ng/mL MCSF on non-TC treated petri dishes. Macrophages were fed additional media with 20ng/mL MCSF on days 3 and 6 after plating. On day 8-9, cells were harvested and replated in 24 or 6 well plates, and allowed to adhere for 24 hours before beginning the experiment.

Mixed Glial Cultures

P0-P3 pup cortices were harvested, minced, and digested in a solution containing Papain (15U/mL) and DNAse (1mg/mL) for 1 hour at 37°C with agitation. Tissue was triturated and passed through a 70μm screen. $5x10^6$ cells were plated per T75 flask in Hi glucose DMEM/F12 + 10% FBS. Media was changed weekly and cells were passaged at least once prior to use. For

Luminex analysis, MGCs were treated overnight with IL-33 and the supernatant collected 24 hours later and stored at -80°C until use.

Accelerating Rotarod

The rotarod assay was performed as previously described (Derecki et al., 2012) on an Economex accelerating rotarod (Columbus Instruments). Mice were given two training phases, the first consisting of a stationary rod and the second a rod with constant acceleration (2.0 rpm), followed by a testing phase where the rod started at 2.0 rpm and accelerates at a rate of 0.1rpm/s. During the training phases mice were placed on the rod facing away from the experimenter and required to remain on the rod for 60s consecutively, and in case of a fall it was placed back on the rod. During the testing phase, mice were placed facing away from the experimenter on the moving rod with acceleration started after placement. Latency to fall after the start of acceleration was recorded. Mice that turned around during the testing phase were omitted from analysis.

Pole Test

Pole test was performed as previously described with minor modifications (Matsuura et al., 1997). Mice were tested on a vertical pole (55cm in height, 0.8cm in diameter) with a roughened surface. Animals were placed head upwards at the top of the pole, and required to turn around and climb down the pole. Mice were given one training trial for a maximum of 120s, and in the case of a fall were immediately placed back on the pole for another 120s or until descent. During the testing phase, latency to descend the pole was recorded, and in the case of a fall the maximum time (120s) was recorded.

All behavior experiments were performed on 8-10 week old age- and sex-matched mice by an experimenter blinded to the identity of experimental groups.

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