Effect of Chronic Thoracic Spinal Cord Injury (SCI) on the Peripheral Immune System in Mice

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EFFECT OF CHRONIC THORACIC SPINAL CORD INJURY (SCI) ON THE PERIPHERAL IMMUNE SYSTEM IN MICE

By
Ji Zha

A DISSERTATION

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EFFECT OF CHRONIC THORACIC SPINAL CORD INJURY (SCI) ON THE
PERIPHERAL IMMUNE SYSTEM IN MICE

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Patients with chronic spinal cord injury (SCI) are at higher risk than the general population of developing infections and their prognosis is often much poorer. The disruption of the normal balance between central nervous system and immune system following SCI induces immunodepression, leading to a compromised capacity to combat infections such as influenza virus in patients with SCI. Using a contusion model of SCI at thoracic level 9, we analyzed the chronic effect of SCI on the peripheral immune system in mice, with a focus on T-cell immunity. We found that although the splenic T-cell number was not changed during chronic SCI, their function as measured in cytokine production, upon ex vivo stimulation, was diminished. We further explored the mechanisms of SCI-induced T-cell dysfunction and demonstrated that chronic SCI increased sympathetic nervous system (SNS) activity and elevated norepinephrine level in the spleen. Higher splenic norepinephrine level correlates with a T-cell exhaustion phenotype as shown by higher expression of exhaustion marker PD-1 on T-cells. Up-regulated PD-1 expression contributes to the CD8⁺ T-cell functional deficiency in chronic SCI mice, as blocking PD-1 signaling in vitro restored the CD8⁺ T-cell function.

We next investigated how chronic SCI affects the antiviral immunity in mice using a well-established influenza virus mouse model. Virus specific immunity was analyzed in chronically injured mice at different times post-infection in comparison to
uninjured controls. The data indicates that chronic SCI injury impairs the ability of the animals to mount an antiviral immunity. While all the control mice cleared the virus from the lungs 10 days post-infection, significant number of SCI mice did not clear the virus. This was attributed to severe deficit in both virus-specific antibody and CD8+ T-cell response in injured mice.

Taken together, our study indicates that the alteration of sympathetic activity following chronic SCI induces T-cell exhaustion, which in turn impairs T-cell function and contributes to immune depression. Moreover, we demonstrate that the antiviral immunity against influenza virus infection is compromised in mice with chronic SCI. Our results highlight the important role of central nervous system and neurotransmitters in regulation of immune cell function. Our study also suggests that blockade of PD-1 pathway is a potential therapeutic strategy to restore immunity in patients with chronic SCI.
To my parents, Shuwei Zha and Lili Chen, for always supporting me, encouraging me and loving me.
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AISA, American Spinal Injury Association
APC, antigen presenting cell
β2AR, β2 adrenergic receptor
BAL, broncholaveolar lavage
BBB, blood brain-barrier
BCR, B-cell receptor
CIDS, CNS injury-induced immunodepression
CTL, cytotoxic T lymphocyte
CTLA-4, cytotoxic T-lymphocyte antigen 4
CNS, central nervous system
DC, dendritic cell
FACS, flow cytometry
FO, follicular
HA, hemaglutinin
HBSS, Hank's Balanced Salt Solution
HIV, human immunodeficiency virus
IFN-γ, interferon gamma
Ig, immunoglobulin
IML, intermediolateral
ISNCSCI, International Standards for Neurological Classification of Spinal Cord
LAG-3, lymphocyte activation gene-3
LCMV, lymphocytic choriomeningitis virus
LPS, lipopolysaccharide
MDCK, Madine-Darby canine kidney
MHC, major histocompatibility complex
MHV, mouse hepatitis virus
M1, Matrix 1
MZ, marginal zone
NA, neuraminidase
NE, norepinephrine
NK, natural killer
NFAT, nuclear factor of activated T-cells
NLR, NOD-like receptor
NP, nucleoprotein
p38 MAPK, phosphor 38 mitogen-activated protein kinase
PA, acidic polymerase protein
PB2, basic polymerase protein 2
PD-1, Programmed cell death-1
PCA, perchloric acid
PAMP, pathogen-associated molecular pattern
PMA, phorbol myristate acetate
PSNS, parasympathetic nervous system
PRR, pattern recognition receptors
RLR, RIG-I-like receptor
SEM, standard error of the mean
SCI, spinal cord injury
SNS, sympathetic nervous system
T9, thoracic level 9
TBI, traumatic brain injury
TCR, T-cell receptor
TGF, transforming growth factor
Tfh, follicular helper T-cell
TLR, toll-like receptor
TBST, Tris-buffered saline with Tween-20
TH, tyrosine hydroxylase
Th1, T helper 1
Th2, T helper 2
TIM-3, T cell immunoglobulin mucin-3
TNF, tumor necrosis factor
Treg, regulatory T-cell
Chapter 1: Introduction

1.1 Spinal cord injury overview

Spinal cord injury (SCI) is an unexpected and devastating condition that causes lifelong challenges including loss of sensory, motor and autonomic functions in patients. Description of the symptoms of cervical spinal cord injury can be found in ancient Egyptian medical documents dating back to about 3500 years ago. The Greek physician Hippocrates (460-370 B.C.) started trying to treat spinal cord injuries (Lin, Cardenas et al. 2003). However, spinal cord injury was still considered fatal until the 1940s. In the last several decades, the life expectancy of spinal cord injured individuals has improved dramatically due to medical developments including new surgical procedure, better healthcare, antibiotics, rehabilitation medicine, and respirators (Liverman, Altevogt et al. 2005).

Spinal cord injuries can be classified based on the injury location along the spinal cord, the pathology and outcomes (i.e. complete and incomplete). The spinal cord is divided into four anatomic levels: cervical, thoracic, lumbar and sacral. Injury at one level often disrupts the function below that level. Contusion, laceration and solid cord injuries are the three most common types of spinal cord injury, with contusion being the most frequent injury (Liverman, Altevogt et al. 2005). The International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) developed by the American Spinal Injury Association (ASIA) is widely used in assessing the level and the outcomes of spinal cord injury in patients. ISNSCI classifies spinal cord injuries based on the sensory and motor level, the ASIA Impairment Scale and the zone of partial preservation.
ASIA Impairment Scale, which assesses the motor and sensory function at different neurological levels in patients, is the standardized measure for the completeness of the injury and neurological outcome after SCI (Committee, Burns et al. 2012).

### 1.1.1 Spinal cord injury demographics

According to the annual statistical report by the National Spinal Cord Injury Statistical Center, an estimated 273,000 people who have spinal cord injury are living in United States in 2013, with about 12,000 new SCI cases each year. The average age when injury occurs is 42.6 years old. The most frequent cause of SCI cases is car accidents (36.5%). Incomplete tetraplegia (40.6%) is the most common type of SCI, followed by incomplete paraplegia (18.7%), complete paraplegia (18.0%) and complete tetraplegia (11.6%). The life time cost and life expectancy for patients with SCI depends on the neurological category. Compared within uninjured person, the life expectancy for SCI patients are still significantly lower and have not increased since 1980’s. Follow-up study in patients with chronic SCI indicates that the leading causes of death are infection and cardiometabolic complications (National Spinal Cord Injury Statistical Center. 2012).

### 1.1.2 Spinal cord injury progression and chronic complications

The human biological response to SCI is divided into three phases: acute phase (seconds to minutes after SCI), secondary phase (minutes to weeks after SCI), and chronic phase (months to years after SCI) (Liverman, Altevogt et al. 2005). Experimental spinal cord injury using animal models has characterized the events during the three phases of SCI. The events in the acute phase includes systemic hypotension, spinal shock induced by systemic hypoxia, neuronal necrosis, action potential disruption accompanied
by excessive release of neurotransmitters, hemorrhage, local edema and vasospasm (Osterholm and Mathews 1972, Young and Koreh 1986, Faden and Simon 1988, Tator 1991, Hulsebosch 2002). These events continue in the secondary phase. In addition, lipid peroxidation, free-radical formation, and cell apoptosis also occurs in the secondary phase (Demopoulos, Flamm et al. 1980, Hulsebosch 2002). The local immune response is initiated in the cord marked by leukocyte infiltration and microglia activation, which will be discussed later.

The lesion site within the spinal cord begins to form a cavity surrounded by the glial scar after injury in animal model (Inman, Guth et al. 2002, Byrnes, Fricke et al. 2010). The gap physically blocks axon growth, while oligodendrocytes and astrocytes produce molecules that are inhibitory to axon growth (Fawcett and Asher 1999). Some of the SCI patients (1~4%) develop syringomyelia, which refers to the formation of a cyst that expands and elongates in the cord at months to years after injury (Terre, Valles et al. 2000). The neurons exhibit chronic changes after traumatic injury: the expression and function of ion channels and receptors is altered (Boulenguez, Liabeuf et al. 2010, Bouhy, Ghasemlou et al. 2011); demyelination caused by the loss of oligodendrocytes decreases the conductivity of the neuron (Kigerl, McGaughy et al. 2006); some degree of plasticity may occur in the surviving neurons, as shown by increased expression of the neuron plasticity marker GAP-43 in the cord of SCI animals (Helgren and Goldberger 1993, Weaver, Cassam et al. 1997, Liverman, Altevogt et al. 2005).

Chronic SCI affects multiple body systems of the patients. In the cardiovascular system, SCI patients show abnormal lipid profile associated with higher risk of atherogenesis (Zlotolow, Levy et al. 1992, Bauman, Adkins et al. 1999, Demirel, Demirel
et al. 2001, Orakzai, Orakzai et al. 2007). Sleep disordered breathing is more prevalent in the respiratory system of SCI individuals (Cahan, Gothe et al. 1993). For the endocrine system, chronic SCI impairs the levels of testosterone, human growth hormone and insulin-like growth factor-1 (Wang, Huang et al. 1992, Bauman, Spungen et al. 1994, Cheville and Kirshblum 1995, Tsitouras, Zhong et al. 1995). Persons with chronic SCI also have higher levels of fat mass, reduced lean tissue and rapid bone loss (Bauman, Spungen et al. 1999, Dauty, Perrouin Verbe et al. 2000, Spungen, Wang et al. 2000, Garland, Adkins et al. 2001, Jones, Legge et al. 2003). Chronic effects of SCI on renal function depends on the age at the time of injury (Grigorean, Sandu et al. 2009, Hitzig, Eng et al. 2011). Chronic SCI also impairs the immune system, which is related to its higher death rate from infection. Chronic SCI-induced immunodepression will be the major focus of this thesis.

1.1.3 Immune response in the spinal cord following injury

Animal studies have characterized the infiltration of immune cells into the lesion site of spinal cord. The temporal profile of leukocyte infiltration and activation at the injury site is well described. Neutrophils enter the cord within 6h after injury, reaching a peak at 24 hours post-injury (Dusart and Schwab 1994), decreasing over the next few weeks but still persists for 6 weeks post injury (Kigerl, McGaughey et al. 2006). Increased numbers of macrophages/microglia (CD11b+) are observed in the injured cord within the first week, and reaches the maximal at around 7 days post-SCI and decline thereafter but still remains elevated compared with the uninjured controls at 6 weeks after injury (Kigerl, McGaughey et al. 2006, Kostyk, Popovich et al. 2008). Adoptive cell transfer experiments and bone marrow chimeras have been performed to distinguish
infiltrating macrophage from resident activated microglia in the SCI mice. When the total number of CD11b+ macrophage/microglia in the cord declines after one week post-SCI, the number of infiltrating macrophages continues to increase weeks after SCI (Shechter, London et al. 2009). However, it should be taken into consideration that bone marrow irradiation may increase the permeability of blood brain barrier and lead to increasing macrophage infiltration into the cord (Fauquette, Amourette et al. 2012). T-cell infiltrates into the spinal cord between 7 and 14 days after injury and reaches a peak of infiltration at 14 days post-injury. T-cell number declines between 2 and 4 weeks and show a secondary increase after four weeks (Popovich, Wei et al. 1997, Kigerl, McGaughy et al. 2006). T-cell infiltration persists until at least 8 weeks after injury (Sroga, Jones et al. 2003). B-lymphocytes are present in the lesion site 3 hours after injury and are predominant for the first 3 days following SCI, then their number declines (Schnell, Schneider et al. 1997). The infiltration of peripheral leukocytes, together with activated glial cells (astrocytes and microglia) at the injured site results in an inflammatory response in the spinal cord. Elevated messenger RNA and protein levels of pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, have been found in the spinal cord following injury (Kumamaru, Saiwai et al., Bethea, Nagashima et al. 1999). The immune response at the lesion site contribute to both neuroprotective and neurodestructive processes after SCI (Bethea and Dietrich 2002).

It has been shown that the blood brain-barrier (BBB) is disrupted following SCI (Popovich, Horner et al. 1996, Bilgen, Dogan et al. 2002, Whetstone, Hsu et al. 2003, Cohen, Patel et al. 2009). Several studies have analyzed the integrity of BBB around the injure site at different time point following SCI and demonstrated that the BBB is not
fully restored at the chronic phase of SCI. Dr. Popovich’s study assessed the BBB permeability of both gray matter and white matter around the lesion epicenter of the cord. They show that BBB permeability increases at day 3 post-SCI and declines between day 3 and day 7. The restoration of BBB in gray matter continues between day 3 and day 28, with a secondary elevation of BBB permeability in the white matter between day 14 and day 28 (Popovich, Horner et al. 1996). Both Cohen’s study and Patel’s study demonstrated that the BBB is still compromised at day 56 after SCI using the method of dynamic contrast-enhanced MRI (Cohen, Patel et al. 2009, Patel, Cohen et al. 2009). There is also recent evidence from Schwartz’s group showing that the macrophage can also enter the central nervous system through choroid plexus after SCI (Shechter, Miller et al. 2013). BBB disruption contributes to the infiltration of circulating leukocyte into the cord.

1.1.4 Spinal cord injury and the autonomic nervous system

Traumatic injury to the spinal cord causes damage to neurons in the cord and dysregulation of descending and ascending pathways, leading to dysfunction of sensory, motor and autonomic nervous system. Since the autonomic nervous system plays an important role in controlling visceral function of peripheral organs, alteration in the function of autonomic nervous system following chronic SCI may participate in the secondary complications in multiple body systems of SCI patients.

The autonomic nervous system consists of sympathetic nervous system (SNS) and parasympathetic nervous system (PSNS). The cell bodies of preganglionic sympathetic neuron, which is part of SNS, reside in the intermediolateral (IML) zone within the gray matter at level T1 to L2 in the spinal cord. Preganglionic sympathetic neuron receives
synaptic inputs from brain stem, diencephalon or spinal interneurons. Preganglionic sympathetic neurons synapse on the postganglionic sympathetic neurons at the paravertebral ganglia in the sympathetic trunk or prevertebral ganglia (i.e. celiac ganglia, superior mesenteric ganglia and inferior mesenteric ganglia). The postganglionic neurons innervate and regulate target organs. All the preganglionic sympathetic neurons are cholinergic, whereas the neurotransmitter released by postganglionic sympathetic terminals is norepinephrine. The preganglionic neurons of PSNS originate from the brain stem (vagal preganglionic neurons) or the S2-S4 segments of the spinal cord (sacral preganglionic neurons). The vagal preganglionic neurons innervate many organs including heart, lungs, liver, pancreas, gall bladder and the gastrointestinal tract. Sacral preganglionic neurons, of which the cell bodies reside in the laminae V-VII of the spinal cord, innervate the low gastrointestinal and the urogenital organs. The neurotransmitter PSNS is acetylcholine (Llewellyn-Smith and Verberne 2011).

Autonomic dysreflexia is a life threatening problem that develops in patients following chronic spinal cord injury at level T6 and above. In autonomic dysreflexia, the sympathetic overactivity is often triggered by stimulation of the pelvic viscera, and causes vasoconstriction and hypertension. The high blood pressure stimulates the baroreceptor reflex, which results in parasympathetic response including flushing, profuse sweating and piloerection above the level of injury and bradycardia (Baguley 2008). Autonomic dysreflexia is due to loss of superspinal control of the preganglionic sympathetic neurons as well as neural reorganization within the cord below the level of injury (Rabchevsky 2006). Several animal studies have demonstrated plasticity of synaptic inputs on preganglionic sympathetic neurons, reorganized neurocircuits in the

Autonomic dysreflexia together with other chronic change in the SNS activity following SCI may participate in the dysregulation of peripheral organs and the chronic complications of SCI. For example, experimental induction of autonomic dysreflexia has been shown to impair the peripheral immune function (Zhang, Guan et al. 2013).

The sympathetic innervation of peripheral immune organs was investigated by multiple groups (discussed later). Specifically, retrograde tracing studies indicates that the cell bodies of preganglionic sympathetic neurons that innervate with splenic nerves are located in the IML zone of spinal cord at level T3 to T12 bilaterally (Cano, Sved et al. 2001). Mice with chronic thoracic SCI exhibit lesion in the gray matter and some of white matter within the spinal cord around the injury epicenter. The damage of preganglionic sympathetic neurons in the IML zone at thoracic level changes the activation and function of splenic sympathetic neurons, leading to dysfunction of the immune cells in the spleen (Nishi, Liu et al. 2007).

1.2 Immune response overview

1.2.1 Innate immune response

The innate immune response is the first-line of defense against pathogen infection. It occurs almost immediately after the entry of foreign organisms and the level of response is not improved after repetitive exposures to the same foreign organisms. It broadly recognizes pathogens by the interaction between pattern recognition receptors
(PRRs) expressed on the innate immune cell and the pathogen-associated molecular pattern (PAMP). The PRRs that have been identified include toll-like receptor (TLR), RIG-I-like receptor (RLR), NOD-like receptor (NLR) and DNA receptor. Lipopolysaccharide (LPS) is the most studied and utilized PAMP, which is the ligand for TLR4. LPS is the component of cell wall of gram-negative bacteria. Other PAMPs includes lipoarabinomannan, flagellin, diacyl or triacyl lipopeptides, and unmethylated CpG from the bacterial or viral genomic DNA, etc. (Kumar, Kawai et al. 2011). The cell components of innate immune system include neutrophils, macrophages, dendritic cells (DC), mast cells and natural killer (NK) cells.

Neutrophils are the first cell responder that migrates to the site of infection or injury and begin to phagocytose microbes or cell debris and release reactive oxygen products. Neutrophils only live for a few hours and upon death they expel the nuclear DNA complex to form neutrophil extracellular trap (NET) which captures the pathogen particles (Mocsai 2013).

Macrophages are activated by TLR or NLR signaling and by cytokines produced by T-cells or NK cells, such as IFN-γ. The activation of complement and antibody receptors on macrophage also participates in macrophage activation and phagocytosis (van Lookeren Campagne, Wiesmann et al. 2007). Upon activation, macrophage can kill the microbes by phagocytosis, release of reactive oxygen products and nitrogen mediates. Activated macrophage also produces cytokines including TNF, IL-1, IL-6 and IL-12 to regulate inflammation and adaptive immunity.

Dendritic cell, which is the antigen-presenting cell that connects innate immunity and adaptive immunity, enters the site of infection or injury between a few hours to
several days after the infiltration of neutrophils. Dendritic cells endocytosis the pathogen in the tissue and migrate to lymph node or spleen to present the antigen peptide with MHC class I or II molecules to activate T-cells and further to induce adaptive immunity.

NK cell is important in the immunity against intracellular pathogens. NK cells can kill the stressed self-cells, viral infected cells and tumor cells through a similar mechanism used by cytotoxic T-cells, which will be described later. NK cell also produces IFN-γ, which is the activator of macrophage and inflammation. The activation of NK cells is regulated by the balance between the function of activating receptors and inhibitory receptors on NK cells (Abbas, Lichtman et al. 2014).

### 1.2.2 Humoral immune response

Humoral immune response refers to the adaptive immune response mediated by the binding of antibody to neutralize the pathogen. Antibody, also known as immunoglobulin (Ig), was first discovered in 1890. It has a “Y” shape composed of two heavy chains and two light chains. The regions at the tip of “Y” shape are responsible for recognizing specific epitope of the antigen. There are different classes of antibodies known as IgA, IgD, IgE, IgM and IgG. The isotype of the heavy chain determines the classification of the antibody. Which antibody isotype is produced by the B-cell is dependent on the cytokine profile of the microenvironment. Different antibody isotype has different biological functions. IgM is the first isotype of antibody expressed on B-cells during its development. It is responsible for the antibody response at acute stage of primary pathogen infection. IgD level is very low in the serum. It participates in the activation in the basophils and mast cells (Chen, Xu et al. 2009). IgG is the major isotype of antibody in the body and has long half-life. IgA is critical antibody for the immunity at
mucosal surface. It is also found in breast milk. IgE is associated with allergy reaction and immunity to parasites (Schroeder and Cavacini 2010).

Naïve B-cells are activated by either ways: T-cell independent or T-cell dependent signals. T independent activation refers to the activation of B-cell by carbohydrate epitope, lipid and other nonprotein molecules without the help from T-cell. The T-independent antigen crosslinks the B-cell receptors (BCR) and initiates the activation. Secondary signaling from TLR activation is also required for complete activation of B-cells. T-dependent B cell activation is stimulated by protein antigen and requires the interaction between T-cell and B-cell in the lymphoid organ. The T-dependent antibody response occurs in a series of steps: 1. The BCR of B-cell recognizes the epitope of antigen initiated the B-cell activation. The B-cell becomes an antigen presenting cell (APC), which presents the antigen peptide with MHCII molecules after internalizing the antigen and increases expression of costimulatory molecule CD80/CD86; 2. The B-cell and antigen-primed T-cell migrates towards each other and interact at the interface between T-zone and the follicle. The TCR of T-cell recognizes the antigen peptide presented by the B-cells; 3. The T-cell expresses CD40L and secretes cytokines to promote the proliferation and differentiation of B-cells. 4. The B-cells differentiates into plasma cells in two pathways: germinal center pathway which occurs in the follicles and extrafollicular pathway which occurs at the extrafollicular foci of the spleen or medullary cord of the lymph node (MacLennan, Toellner et al. 2003). In the germinal center, the B-cells receive signals from follicular helper T-cells (T_{FH}) and undergo class switching of heavy chain isotype (from IgM or IgD to IgA, IgE or IgG) and somatic hypermutation. Finally the B-cell producing high affinity antibody is selected.
and becomes long-live plasma cells. The B-cells in the extrafollicular pathway also have some degree of isotype class switching with the help from T-cells (Abbas, Lichtman et al. 2014).

There are three major subsets of mature B-cells: B-1 cells, marginal zone (MZ) B-cells and follicular (FO) B-cells. B-1 cells are found mostly in the mucosal tissues and peritoneal cavities. It responds to nonprotein antigen and produce mainly IgM isotype. MZ B-cells locate in the interface between red pulp and white pulp in the spleen and responds to blood-borne pathogen. B-1 cells and MZ B-cells are involved mostly in the T-independent antibody response. FO B-cell resides in the lymphoid follicle of the spleen white pulp or the lymph node cortex. It is the classical B-cell subset that participates in the T-dependent antibody response and is differentiated to long-live plasma cells that produces high-affinity antibody (Swanson, Pelanda et al. 2013, Abbas, Lichtman et al. 2014).

1.2.3 T-cell mediated immune response

T-cells have important functions in host adaptive immunity against a variety of pathogens, including virus, some types of bacteria, fungi and parasites. T-cells are developed in the thymus and characterized by the expression of T-cell receptor (TCR). The classical role of T-cell function is inducing cytotoxic immune response that kills the infected cell directly, which is now known as the function of CD8+ T-cells. The cytotoxic immune response is responsible for clearance of intracellular pathogen, such as intracellular bacteria, virus and tumor cells. CD4+ T-cell, also known as T helper cells, is another major subset of T-cells. CD4+ T-cells participates in the antibody production by B-cells, as well as cytotoxic immune response by CD8+ T-cells. CD4+ T-cells also secret
cytokines, which promotes macrophage effector function and regulates the inflammatory response. Regulatory T-cells (Treg), one subset of CD4⁺ T-cells, is important for immune tolerance. The classification, activation and effector function of T-cell are described below.

a) T-cell activation and effector function

Both CD4⁺ T-cells and CD8⁺ T-cells express the T-cell receptor (TCR) specific for the antigen peptide-MHC complex. The antigen presenting cell (APC), such as dendritic cells, macrophages and B-cells, ingests the pathogen and present the antigen peptide to MHC class I or MHC class II molecules on the cell surface. The antigen-specific T-cell recognizes the peptide presented by MHC when interacting with the APC. Costimulatory signal from the same APC is also required for the T-cell activation. The CD80 and CD86 molecules expressed on the APC bind to CD28 on the T-cells and lead to costimulation. The TCR is associate with the CD3 subunits, including εγ dimer, εδ dimer and ζζ dimer. Upon TCR ligation, the immunoreceptor tyrosine-based activation motifs (ITAMs) of CD3 subunits are phosphorylated and numerous T-cell activation cascades are initiated (Guy and Vignali 2009, Huang, Meyer et al. 2012). The signalling pathways involved in the TCR activation include PKC-θ/NFκB pathway, PI3K/Akt pathway, Calcium/NFAT pathway and MAPK pathway (Abbas, Lichtman et al. 2014). The costimulatory molecule CD28 participates in the activation of PI3K/Akt pathway and the recruitment of PKC-θ and Ras (Chen and Flies 2013).

The CD4⁺ T-cell has four major subsets—Th1 cells, Th2 cells, Th17 cells and regulatory T-cells (Tregs). The four subsets are characterized by the transcription factor they express and the profile of cytokines they produce. The cytokines in the
microenvironment where naïve CD4$^+$ T-cells are activated determines which subset it will be differentiated into (Fig. 1.1). Briefly, naïve CD4$^+$ T-cell differentiated to Th1 cells when exposed to the IL-12 secreted by APC. Th1 cells, characterized by the production of IFN-$\gamma$ and IL-2, are important for macrophage activation and the clearance of intracellular pathogen. IL-4 drives Th2 cell development. Th2 effector cells produce multiple cytokines including IL-4, IL-5 and IL13 and participate in the parasite immunity, antibody response and allergic response (Dong and Flavell 2000, Hirahara, Poholek et al. 2013). Th17 cells are generated from naïve CD4$^+$ T-cells in the presence of TGF-$\beta$, IL-6 and IL13. They produce IL-17 and IL-23, which are important cytokines in multiple sclerosis, extracellular pathogen clearance and gut immunity. TGF-$\beta$ combined with TCR stimulation induced the differentiation of naïve CD4$^+$ T-cells to regulatory T-cells (Tregs), which exhibit suppressive functions for immune tolerance(Chen, Jin et al. 2003).

**Figure 1.1** The differentiation of T helper cell subsets. Naïve CD4$^+$ T-cells are activated and differentiated into different T helper cells depending on the cytokines in the microenvironment. The four T helper cell subsets are characterized by the transcription factor expression and the profile of cytokines they produce.
Many studies in the last decade challenge the traditional view of T helper cell classification and suggest that the differentiation of the effector T-cell subsets is plastic and very complicated. These studies including the discovery of follicular T helper cell (T\textsubscript{FH}), which promotes antibody response and express a wide range of cytokines under different conditions(Hirahara, Poholek et al. 2013). The phenotype and function of T\textsubscript{FH} cell will be discussed in the next section.

The activated CD8\textsuperscript{+} T-cell kills the target cell by secreting cytotoxic granules, which contains granzyme B, that activates the cell apoptosis and perforin, which forming a pore on the target cell to help the delivery of granules between cells. Effector CD8\textsuperscript{+} T-cell also expresses Fas and TRAIL, which induces apoptosis of target cells through Fas/FasL interaction and TRAIL/DR5 interaction. In addition, the virus-specific CD8\textsuperscript{+} T-cells have been demonstrated to have the ability to produce proinflammatory cytokines including IFN-\textgamma, TNF and IL-2 to regulate the viral clearance(Olson, Russ et al. 2010).

\textit{b) Interaction between T helper cells and B-cells}

T helper cell is the key player in B-cell antibody response to T-dependent antigen. Upon pathogen infection, the dendritic cells endocytose the antigen and move from site of infection to the lymph node. The dendritic cells process the antigen and present the antigen peptide to the cell surface with MHC II molecules. It also expresses CD80 and CD86 that costimulates the T-cells through CD28 molecule on the T-cell surface. The resting T helper cell which has the specific TCR for the MHCII-peptide gets activated by the dendritic cells that present the antigen peptide, and then moves to the interface between T-cell zone and B-cell zone in the lymph node and interact with B-cells. The B-cell is also an antigen-presenting cell when getting activated. B-cell also presents the
antigen peptide with MHCII and up-regulates the expression to costimulate the antigen-specific T-cells. After recognizing the antigen-specific B-cell, the T-cell increases CD40L expression to interact with the CD40 on B-cell and produces cytokines that regulating B-cell activation and differentiation. Th2 cell was considered the T helper cells that regulate antibody response. However, nowadays it’s believed that Th1, Th2 and Tfh cells are all involved in the production of antibody. The B-cell differentiates in two pathways with T-cell help: the extrafollicular pathways and the follicular pathways. Th1 and Th2 regulate the extrafollicular pathway of B-cell differentiation, which usually generates short-live plasma cells and produces low-affinity antibody. Tfh is the helper for the B-cell differentiation in the follicular pathway, in which it undergoes proliferation, somatic hypermutation and class switching of immunoglobulin in the germinal center to become long-live plasma cells or memory B-cells. The cytokines produced by T helper cell guide the immunoglobulin class-switching of B-cells. For example, IFN-γ promotes the class switching to IgG2, when IL-4 promotes the class switching to IgE.

c) T-cell exhaustion

During the chronic infection and cancer progression, T-cells become “exhausted” as it exhibits poor effector function and expresses inhibitory receptors(Wherry 2011). T-cell exhaustion was first described in a lymphocytic choriomeningitis virus (LCMV) chronic infection mouse model as the virus-specific CD8+ T-cell population during the chronic infection cannot elaborate efficient antiviral effectors’ function and even get deleted eventually (Zajac, Blattman et al. 1998). In the last decade, T-cell exhaustion has been investigated in multiple animal models including chronic viral infection, parasite infection, cancer and aging(Zajac, Blattman et al. 1998, Trautmann, Janbazian et al.)
2006, Radziewicz, Ibegbu et al. 2007, Channappanavar, Twardy et al. 2009, Lages, Lewkowich et al. 2010, Bhadra, Gigley et al. 2012, Topalian, Drake et al. 2012, Tzeng, Tsai et al. 2012). The impairment of CD8\(^+\) T-cell effector function occurs in a hierarchy manner during exhaustion. IL-2 production and the *in vitro* cell lysis ability are lost first, followed by decreased production of TNF. Severe T-cell exhaustion eventually leads compromised production of IFN-\(\gamma\) (Wherry, Blattman et al. 2003). Higher viral load, persistent infection and lack of CD4\(^+\) T-cell help are considered to correlate with T-cell exhaustion (Wherry 2011).

Increased expression of inhibitory receptors on the cell surface is a key feature of T-cell exhaustion (Wherry 2011). These exhaustion markers include PD-1, CTLA-4, TIM-3 and LAG-3, etc. Among those, program cell death protein-1 (PD-1) is the most widely studied inhibitory receptors in T-cell exhaustion. PD-1 is a 288 amino acid transmembrane protein that consists of an extracellular Immunoglobulin domain, a transmembrane domain and an intracellular domain containing immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) (Chemnitz, Parry et al. 2004, Keir, Butte et al. 2008). Interaction between PD-1 and its ligands—PD-L1 and PD-L2—initiates the PD-1 signaling. The PD-1 engagement induces the recruitment of phosphatases SHP1 and SHP2 to the TCR and downregulates the TCR signaling (Chemnitz, Parry et al. 2004). PD-1 is not expressed on naïve murine T-cells and expressed at low level following T-cell activation. Exhausted T-cells show prolonged and high expression of PD-1 (Yamazaki, Akiba et al. 2002, Keir, Butte et al. 2008, Wherry 2011). Higher PD-1 expression, which correlates with CD8\(^+\) T-cell dysfunction, have been characterized in many disease models including HIV, HCV and
HBV infection, cancer as well as aging (Trautmann, Janbazian et al. 2006, Penna, Pilli et al. 2007, Radziewicz, Ibegbu et al. 2007, Channappanavar, Twardy et al. 2009, Topalian, Drake et al. 2012, Tzeng, Tsai et al. 2012). Antagonist antibody to PD-1 has been studied in cancer clinical trials to enhance the T-cell immunity against tumor. Recently some groups have begun to study the transcriptional pathways that regulating PD-1 expression on T-cells. They found that B lymphocyte-induced maturation protein 1 (Blimp-1), nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) and Notch signaling are regulators of PD-1 gene expression, whereas high expression of T-bet suppresses PD-1 expression (Oestreich, Yoon et al. 2008, Shin, Blackburn et al. 2009, Kao, Oestreich et al. 2011, Mathieu, Cotta-Grand et al. 2013).

1.3 Spinal cord injury-induced immunodpression

1.3.1 Causes of rehospitalization and death in chronic patients

Several research groups have performed follow-up studies on individuals with SCI to analyze the causes of rehospitalization and death. In the 2012 annual statistical report by the National Spinal Cord Injury Statistical Center in United States, the leading cause of death in patients with chronic SCI is respiratory system diseases including pneumonia. Infective and parasitic disease including septicemia and AIDS is the second leading cause of death. The high rate of septicemia associates with decubitus ulcers, urinary tract infections and respiratory infections. Neoplasm ranks the third, followed by hypertensive and ischemic heart disease. The major causes of rehospitalization years post SCI are diseases of the genitourinary system, diseases of the skin and diseases of the respiratory system (National Spinal Cord Injury Statistical Center.). A retrospective study
in Norway found that the leading causes of death of Norwegian patients with years of SCI are pneumonia or influenza, ischemic heart diseases and urogenital diseases (Lidal, Snekkevik et al. 2007). A similar study performed in SCI patients in Denmark indicates that the major causes of death 40 years after injury are lung diseases (particularly pneumonia), suicide and ischemic heart diseases (Hartkopp, Bronnum-Hansen et al. 1997). The group in Australia showed that the leading causes of death in SCI patients are pneumonia or influenza, septicemia and cancer (Soden, Walsh et al. 2000). Although the order of the causes of death is different across countries, pneumonia and influenza infections are predominant causes of death in every country.

1.3.2 Spinal cord injury-induced immunodepression in human

Compared with the general population, SCI patients have higher mortality due to complications associated with infections, including pneumonia and septicemia. It suggests that the capacity to combat pathogen infections is compromised in patients with chronic SCI, leading to the concept of spinal cord injury-induced immunodepression (SCI-IDS). The immunodepression also occurs in patients with other forms of central nervous system (CNS) trauma, such as stroke and traumatic brain injury (TBI). Pneumonia is the major cause of death at post-acute phase of stroke. The mortality of pneumonia is much higher in stroke patients than in controls (Graham and Braciale 1997, Lund, Partida-Sanchez et al. 2002, Meisel, Schwab et al. 2005). In TBI patients, the rate and mortality of infection complications including pneumonia and septicemia is much higher than the general population as well as patients with other trauma or injury in intensive-care units (Palladino, Mozdzanowska et al. 1995, Graham and Braciale 1997, Mozdzanowska, Furchner et al. 1997, La Gruta, Turner et al. 2004). Taken together, CNS
trauma induces higher susceptibility to infection in the patients, suggesting CNS-injury induced immune deficiency syndrome (CIDS).

Several studies have analyzed the peripheral immune system in SCI patients and compared it with age-matched controls. The results from those studies suggest that the patients have a dramatic reduction in the number of monocytes, T-cells, B-cells and MHC II⁺ cells in their peripheral blood within the first weeks after SCI. The reduction in immune cell number is transient and recovers up to similar levels with control patients two weeks after injury (Riegger, Conrad et al. 2009). However, the patients’ immune cell function is weakened during the chronic phase of SCI. Chronic SCI impairs the cytotoxicity of NK cells and the bactericidal ability of neutrophils (Cruse, Lewis et al. 1992, Nash 1994, Campagnolo, Bartlett et al. 2000, Cruse, Lewis et al. 2000, Kanyilmaz, Hepguler et al. 2013). T-cell activation in the peripheral blood is also reduced in the patients with chronic SCI (Cruse, Lewis et al. 1992, Cruse, Lewis et al. 2000).

1.3.3 Spinal cord injury-induced immunodepression in rodents

Animal studies using rat or mouse SCI models are useful to further understand the characteristics and mechanisms of spinal cord injury-induced immunodepression. Recent studies have begun investigating the alterations in the peripheral immune system following acute and chronic SCI. Similar to human studies, rats have decreased numbers of monocytes, T-cells, B-cells and MHC II⁺ cells in the peripheral blood acutely post injury (Lucin, Sanders et al. 2007, Riegger, Conrad et al. 2007). When infected with mouse hepatitis virus (MHV) at 1 week after thoracic SCI, the injured mice show higher mortality and impaired viral clearance in the liver after MHV infection. Moreover, the proliferation and IFN-γ production of virus-specific CD4⁺ T-cells are both reduced in SCI
mice, with a decrease in the activation of antigen presenting cells (Held, Steward et al. 2010). For the chronic phase (>4 weeks after injury), the effect of SCI on the peripheral immune cell numbers is dependent on the injury level. Mice injured at thoracic level T3 have reduced numbers of macrophages, dendritic cells and B-cells in the spleen whereas mice with injury at T9 only have reduced numbers of macrophages and dendritic cells, with a comparable number of B-cells and T-cells at chronic phase of SCI (Held, Steward et al. 2010, Zhang, Guan et al. 2013). The antibody responses to thymus-independent and thymus-dependent antigens are impaired in the animals with SCI at both T3 and T9 level, when the B cell genesis in the bone marrow is intact (Ibarra, Jimenez et al. 2007, Oropallo, Held et al. 2012). In this study, we investigate for the first time the immune response against pathogen infection and the T-cell function in chronic spinal cord injured animals.

1.4 Sympathetic nervous system and immunity

1.4.1 Sympathetic innervation of immune organs

The sympathetic nervous system is disrupted following traumatic injury to the spinal cord. The immune organs are highly innervated and regulated with sympathetic nerve terminals. To explore the mechanisms of SCI-induced immunodepression, it is important to understand the sympathetic innervation of immune organs.

Rodent animal studies have characterized that primary and secondary immune organs are innervated by sympathetic postganglionic neurons. The locations where those sympathetic nervous pathways originate from have been identified by retrograde tracing methods. The postganglionic sympathetic nerves in the spleen are projected from celiac
ganglia as well as the paravertebral ganglia at the thoracic level of the sympathetic chain (Scherle, Palladino et al. 1992, Kedzierska, Venturi et al. 2006) The preganglionic sympathetic neurons that innervate these splenic sympathetic nerves are originated from the IML zone of spinal cord from T3 to T12 level bilaterally (Cano, Sved et al. 2001). For thymus innervation, injections of wheatgerm agglutinin-horseradish peroxidase (WGA-HRP) into the thymus results in cell labeling in the sympathetic chain from the superior cervical ganglia to the T3 ganglia (Nance, Hopkins et al. 1987). PRV injection into the thymus induces infection of cell bodies that reside in the IML zone of spinal cord from level T1 to T7 (Trotter, Stornetta et al. 2007). There are few studies about the autonomic innervations of the bone marrow. In the male rats received PRV injections into the femoral bone marrow, infected cells appear in ganglia of the paravertebral chain and the IML zone of lower thoracic spinal cord (Denes, Boldogkoi et al. 2005).

Tyrosine hydroxylase (TH) is the rate-limiting enzyme for catecholamine synthesis. TH has been widely used to identify catecholamine containing noradrenergic terminals in the spleen. Histological studies on rat and mouse show that the TH⁺ nerves enter the spleen in association with the blood vessels. TH⁺ innervation can be observed at the central artery in the white pulp and the periarteriolar lymphatic sheath. Some of the sympathetic nerves even pass through the white pulp and innervate the marginal zone, the red pulp and the venous sinus of spleen (Felten and Olschowka 1987, Madden, Bellinger et al. 1997, Lorton, Lubahn et al. 2005). Some of the TH⁺ terminals are adjacent to the T-cells in the white pulp (Felten and Olschowka 1987). It has been demonstrated that the splenic nerve ending forms a synapses-like structure with the acetylcholine-synthesizing T-cells in the white pulp of the spleen (Rosas-Ballina, Olofsson et al.). The results from
these studies provide the anatomical evidence that sympathetic innervation in the spleen regulates the T-cell function and immune response.

### 1.4.2 Norepinephrine regulation of immune cells

Norepinephrine (NE), which is released from sympathetic nerve terminals in the immune organs, has been shown to affect immune cell functions. Adrenergic receptors (AR), primarily the $\beta_2$AR subtype, are expressed in many immune cells including macrophages, B cells and T-cells. Naïve CD4$^+$ T-cells and Th1 CD4$^+$ T-cells express $\beta_2$AR, while Th2 CD4$^+$ T-cells do not. Some groups reported the expression of $\alpha_1$AR subtype on peripheral blood mononuclear cell (PMBC), monocyte, microglia and NK cells. However, the results regarding $\alpha_1$AR expression on immune cells is conflicting and depends on RT-PCR assay, which may involve contamination of other cell type (Harling-McNabb, Deliyannis et al. 1999). For the innate immune cells, NE has been shown to inhibit macrophage production of TNF-α in response to LPS (Ignatowski, Gallant et al. 1996, Meltzer, MacNeil et al. 2004). The sympathetic innervation associated with lymphatic vessels participates in directing dendritic cell (DC) migration from the site of inflammation to regional lymph nodes (Maestroni 2000). For the adaptive immune system, NE effects on the T-cell function is complex and dependent on its differentiation and activation stage. Naïve CD4$^+$ T-cell exposed to NE has higher IFN-γ secretion per cell when differentiated to Th1 cell (Swanson, Lee et al. 2001). When exposed to NE or $\beta_2$AR during, before or after activation, Th1 effector cells show similar, lower or higher IFN-γ production respectively compared with control cells. $\beta_2$AR stimulation results in reduced IL-2 production in both naïve CD4$^+$ T-cells and Th1 effector cells (Ramer-Quinn, Baker et al. 1997, Ramer-Quinn, Swanson et al. 2000, Nance and Sanders 2007, Sanders
When CD8+ T-cell differentiates into effector cytotoxic T-cells, β2AR stimulation will increases the generation of its lytic activity. However, if NE were added at the effector stage, the CTL activity and the TNF production of CD8+ T-cells is suppressed (Hatfield, Petersen et al. 1986, Kalinichenko, Mokyr et al. 1999, Nance and Sanders 2007). β2AR stimulation participates in CD86 expression as well as IgG production by B-cell. It also increases the IgE secreted by B-cell in the presence of IL-4 (Sanders 2012). In the SCI mouse model, increasing NE level during the acute phase after SCI participates in lymphocyte apoptosis (Lucin, Sanders et al. 2009). Splenic NE accumulation caused by experimental induction of autonomic dysreflexia in chronic SCI mice has also been demonstrated to contribute to the functional deficiency of immune cells (Zhang, Guan et al. 2013).

1.5 Study objective

Our study objective is to explore the effects of chronic SCI on the peripheral immune system using a mouse model. The hypothesis is that chronic thoracic SCI in mice impairs the immunity against pathogen infection, such as influenza virus, by altering the phenotype and function of the peripheral immune cells. Particularly, we focus on the chronic effects of SCI on peripheral T-cell immunity, including the cell number, subset distribution, cytokine production and exhaustion phenotype. We also determine whether the disruption of sympathetic nervous system by chronic SCI contributes to the functional deficiency of peripheral immune cells.

The results from this study will help us understand the mechanisms underlying chronic SCI-induced immune depression and will shed light on the development of therapeutic strategies to reduce the high rehospitalization and death rates from infection-
related complications in chronic SCI patients. New information from this study will also increase our knowledge of the interaction between the autonomic nervous system and immune system under pathological condition.
Chapter 2: Chronic thoracic spinal cord injury impairs T-cell function by up-regulating PD-1 expression

2.1 Overview

Recent studies using rodent SCI models have begun to investigate the effect of chronic SCI (> 4 weeks) on adaptive immunity. Chronic SCI impairs the antibody response in both mice and rats (Ibarra, Jimenez et al. 2007, Oropallo, Held et al. 2012, Zhang, Guan et al. 2013). The germinal center B cell number and function are also impaired by chronic SCI (Oropallo, Held et al. 2012). Importantly, T-cells have a key role in both antibody mediated and cytotoxic immune response to viral infection such as influenza (Damjanovic, Small et al. 2012). It has been shown that CD4+ T-cell effector function to mouse hepatitis virus (MHV) is suppressed following SCI in the acute phase (Held, Steward et al. 2010). However, whether or not SCI alters T-cell function at a chronic time point has yet to be explored.

exposure to antigen and loss of CD4+ T-cell correlates with T-cell exhaustion (Wherry 2011). It is unclear whether T-cell exhaustion is responsible for chronic SCI-induced immunodepression and whether changes in SNS activity affects T-cell exhaustion.

In the present study, we investigated the impact of chronic SCI on the peripheral T-cell immunity. We provide evidence that cytokine production by CD4+ and CD8+ T-cells from chronically injured mice was impaired and that this impairment was due to increased expression of PD-1 exhaustion marker on splenic T-cells. Blocking PD-1 rescued the functional defects of T-cells isolated from chronic SCI mice. We also demonstrated that increased levels of splenic norepinephrine following SCI may contribute to increased PD-1 expression on T-cells as we showed in vitro that PD-1 expression is increased on T-cells in the presence of sustained levels of NE. Collectively, these findings suggest that deregulation of splenic sympathetic activity by chronic SCI induces T-cell exhaustion, which in turn results in T-cell dysfunction and immune depression.

2.2 Materials and methods

2.2.1 Animals

Age-matched female C57BL/6 mice were purchased from The Jackson Laboratory or bred in the Animal Facility of the Miami Project to Cure Paralysis. All mice used for the experiments were 4-7 months old when sacrificed. All animal protocols were approved by the University of Miami Institutional Animal Care and Use Committee (IACUC) and are in accordance with National Research Council guidelines for the care and use of laboratory animals.
2.2.2 Spinal cord injury

Severe spinal contusion injury was induced using the Infinite Horizon Impactor (Precision Systems and Instrumentation, LLC). Briefly, mice were anesthetized by intraperitoneal injection of Ketamine (100mg/kg) and Xylazine (10mg/kg). A laminectomy was performed at vertebrae thoracic level 9 (T9). The underlying spinal cord was exposed and injured by the tip of the contusion device at a predetermined impact force of 70 kDynes (severe injury). After surgery, mice were housed separately and received daily subcutaneous injections of lactated Ringer’s solution to prevent fluid loss and Gentamycin (40mg/kg) to prevent urinary tract infections. Manual bladder expression (twice daily) was performed until mice regained bladder function.

2.2.3 Splenocyte isolation and T-cell enrichment

Mice were anesthetized and a laparotomy was performed to expose and excise the spleen. Single cell suspensions of individual spleens were prepared by mashing the spleens through a 100-µm nylon mesh strainer. Strainers were washed with HBSS (Gibco). Red blood cells were lysed with ACK lysing buffer (Gibco). For flow cytometry staining, splenocytes were washed with HBSS, resuspended in FACS staining buffer (HBSS, 1% BSA, 0.05% Sodium azide). For ex vivo stimulation assay, splenocytes were washed with cRPMI (RPMI 1640, 5% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin). The number of live cells was determined by trypan blue exclusion staining.

T-cell enrichment was obtained by plating splenocytes on tissue culture dishes coated with 100µg/mL goat anti-mouse IgG + IgM (H+L) (Jackson ImmunoResearch
Laboratories) for 1 hour at 37°C, 5% CO₂. The non-adherent T-cells were collected and washed with cRPMI before use in stimulation assays.

2.2.4 Flow cytometry

Prior to staining, all samples were incubated with 5µg/mL FcR block (anti-mouse CD16/32, Biolegend) for 5 min on ice to prevent nonspecific staining. Cells were stained for surface markers by adding the following conjugated Abs: APC/Cy7-anti-CD4 (1:200), Alexa Fluor 488-anti-CD8a (1:200) and PE/Cy7-anti-PD-1 (1:200) from Biolegend; PE/Cy7-anti-CD45 (1:10000), Alexa Fluor 488-anti-CD3e (1:200), efluor 450-anti-CD3 (1:200), PE-anti-CD4 (1:200) and APC-anti-CD8a (1:200) from eBioscience. For surface antibody staining, cells were then fixed overnight with FACS buffer containing 1% paraformaldehyde and resuspended in FACS buffer. For intracellular antibody staining, cells are fixed, permeabлизed and stained using Foxp3 staining Kit (eBioscience) according to the manufacture’s protocol. Intracellular marker expression was quantified using the following conjugated Abs: efluor 450-anti-IFN-γ (1:100) and PerCP-eFluor 710-anti-TNFα (1:100) from eBioscience. All antibody incubations were performed for 20 min at 4°C. Cells were analyzed using either LSRII or LSR Fortessa flow cytometers and were quantified using FACS-Diva Version 6.1.3 software (BD Biosciences).

2.2.5 T-cell stimulation

Splenocytes were isolated and counted as above. 10⁶ splenocytes were cultured in 1mL of cRPMI in a 24-well plate with 50 ng/mL PMA (Sigma), 0.75 µg/mL ionomycin calcium salt (Sigma) and 1 µL GolgiPlug protein transport inhibitor (BD Biosciences) for
4 hours at 37°C, 5% CO₂. For the PD-1 blockade assay, 10 µg/mL anti-PD-1 blocking antibody (Biolegend) or 10 µg/mL rat IgG2a, κ isotype control (Biolegend) was added to the cRPMI along with PMA and ionomycin.

### 2.2.6 Norepinephrine and T-cell exhaustion

To measure the effects of NE on T-cell exhaustion, T-cells were enriched and counted as described above. Prior to cell culture, a fresh stock solution of 100 mM NE was prepared by dissolving 50 mg of (-)-norepinephrine (Sigma) in 2.96 mL of 0.4 N perchloric acid containing 5 mM glutathione. Enriched T-cells (2 x 10⁵) were cultured in 200 µL of cRPMI with either NE diluted to a final concentration of 10 µM or an equivalent volume of perchloric acid/ glutathione solution as vehicle control. Cells were cultured for 1d, 2d or 3d at 37°C, 5% CO₂ and harvested for flow cytometry analysis. Culture medium with NE or vehicle was changed daily.

### 2.2.7 Protein extraction and western blotting

Spleens were harvested and homogenized in RIPA lysis buffer (10 mM Na-phosphate pH7.2, 150 mM NaCl, 1% Igepal CA-630, 1% Na-deoxycholate, 0.1% SDS, 2 mM EDTA) containing complete protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 3 (Sigma). The samples were mixed end-over-end at 4°C for 20 min and lysates were centrifuged at 4°C for 15 min at 14,000 rpm. The protein concentrations in the supernatant were quantified using DC Protein Assay Kit (Bio-Rad). Protein samples (20 µg) and protein prestained standards (Precision Plus) were resolved on a 10% SDS-PAGE and then transferred to nitrocellulose membrane (Bio-Rad). Membranes were blocked for 1 hour at room temperature in TBST containing 5% non-fat dry milk
followed by incubation overnight at 4°C with rabbit anti-tyrosine hydroxylase antibody (1:1000, Calbiochem,). After extensive washes in TBST, a conjugated HRP anti-rabbit secondary antibody was then applied for 30 min at room temperature. Immunoreactive signals were visualized using ECL Western blotting detection reagents (Amersham). The protein bands were quantified with Quantity One software (Bio-Rad) and normalized to β-actin.

### 2.2.8 Measurement of splenic norepinephrine

Spleens were snap frozen in liquid nitrogen and grinded to powder taking care of avoiding any thawing. Glutathione/PCA solution (0.4N perchloric acid with 5 mM glutathione) was added to the powder at 1mL per 100mg of tissue. Following homogenization, the samples were centrifuged for 15 min at 14,000 rpm and 4°C and the supernatants were stored at -80°C. Concentrations of norepinephrine in the supernatant were measured by the Hormone Assay & Analytical Services Core at Vanderbilt University School of Medicine.

### 2.2.9 Statistical analysis

All experimental data are expressed as mean ± standard error of the mean (SEM). Student’s t-test was applied for comparison between two groups. One-way ANOVA followed by Tukey’s post-hoc test was performed to compare means of three groups. Graphpad Prism (Graphpad Software) was used for statistical analysis. P<0.05 was considered as significant.
2.3 Results

2.3.1 Chronic SCI alters the function of CD4$^+$ and CD8$^+$ T-cells but not their numbers.

Several studies have shown that following acute SCI (<7 days post injury) there was a dramatic reduction in the number of splenic T-cells (Popovich, Stuckman et al. 2001, Lucin, Sanders et al. 2007, Riegger, Conrad et al. 2007). To evaluate the impact of spinal cord injury on spleen function at a chronic time point namely more than 4 weeks post-SCI, we first assessed the cell numbers with a focus on T-cells. As shown in figure 2.1A, the spleen weight was not significantly different between the uninjured and chronic SCI groups (uninjured: 77.1 ± 2.1 mg; chronic SCI: 70.6 ± 5.3 mg; $p=0.27$) nor was the total spleen cell number (uninjured: 86.5 ± 8.4 x 10$^6$; chronic SCI: 89.4 ± 9.2 x 10$^6$; $p=0.82$) (Fig 2.1B). Furthermore, the spleens of uninjured and chronic SCI mice contained similar numbers of T-cells (uninjured: 27.0 ± 1.9 x 10$^6$; chronic SCI: 22.6 ± 2.4 x 10$^6$; $p=0.16$) (Fig 2.1C, D) with no significant differences in the numbers of CD4$^+$ T-cells (uninjured: 12.4 ± 1.0 x 10$^6$; chronic SCI: 10.0 ± 0.7 x 10$^6$; $p=0.06$) or CD8$^+$ T-cells (uninjured: 13.5 ± 1.4 x 10$^6$; chronic SCI: 12.9 ± 2.5 x 10$^6$; $p=0.84$) (Fig 2.1C, E) between groups.
Figure 2.1  The number of splenocytes and splenic T-cells are not changed during chronic SCI.

(A) Bar graph represents the mean ± SEM spleen weights of uninjured mice (CT) and T9-SCI mice at 5-7 weeks after injury (SCI). n = 12 mice/group. Data are pooled across three independent experiments. (B) Bar graph represents the mean ± SEM of total splenocyte numbers for CT and SCI mice. n = 17 for CT, n = 19 for SCI. Data were pooled across four independent experiments. (C) Representative dot plots show the percentage of T-cells (CD45+CD3+) in live splenocytes (upper panels), as well as the percentages of CD4+ T-cells (CD4+CD8−, bottom right quadrant) and CD8+ T-cells (CD4−CD8+, upper left quadrant) in gated T-cells (bottom panels). (D) Bar graph represents the mean ± SEM number of splenic T-cells in CT and SCI mice. (E) Bar graph show the mean ± SEM numbers of splenic CD4+ T-cells and CD8+ T-cells in CT and SCI mice. n = 12 mice/group. Data are pooled across three independent experiments. No statistical difference was detected between the two groups. p>0.05, two-tailed Student’s t-test.

Effective T-cell response against pathogen requires the production of cytokines such as Interferon gamma (IFN-γ) and tumor necrosis factor (TNF, formally known as TNF-α) (Kuwano, Kawashima et al. 1993, Slifka and Whitton 2000, Schoenborn and
Wilson 2007, Chavez-Galan, Arenas-Del Angel et al. 2009). To evaluate the function of these T-cells, we isolated splenocytes from the spleens of uninjured and chronic SCI mice and stimulated them ex vivo with PMA/ionomycin. As shown in figure 2.2A and 2.2C, a significantly smaller percentage of CD4\(^+\) T-cells produced IFN-\(\gamma\) following stimulation (uninjured: 8.3 ± 0.5 %; chronic SCI: 5.8 ± 0.4 %; \(p=0.0005\)) also correlating with a significantly reduced number of IFN-\(\gamma\)^+CD4\(^+\) T-cells in chronically injured mice compared to controls (uninjured: 1.0 ± 0.1 x 10\(^6\); chronic SCI: 0.7 ± 0.1 x 10\(^6\); \(p=0.02\)).

The percentage or number of CD4\(^+\) T-cell expressing TNF (Fig. 2.2A, C) following stimulation was not significantly different between chronic SCI and control (percentage: uninjured: 9.5 ± 0.6 %; chronic SCI: 9.1 ± 0.8 %; \(p=0.37\); cell number: uninjured: 1.3 ± 0.1 x 10\(^6\); chronic SCI: 1.4 ± 0.2 x 10\(^6\); \(p=0.38\)). However, the percentage and number of CD8\(^+\) T-cell expressing TNF was significantly reduced following chronic SCI (percentage: uninjured: 6.3 ± 0.6 %; chronic SCI: 4.9 ± 0.3 %; \(p=0.02\); cell number: uninjured: 0.68 ± 0.06 x 10\(^6\); chronic SCI: 0.52 ± 0.06 x 10\(^6\); \(p=0.03\)) (Fig. 2.2B, D), when neither the percentage nor the number of IFN-\(\gamma\)^+CD8\(^+\) T-cells was changed by chronic SCI (percentage: uninjured: 20.1 ± 1.1 %; chronic SCI: 18.5 ± 0.9 %; \(p=0.14\); cell number: uninjured: 2.2 ± 0.2 x 10\(^6\); chronic SCI: 1.9 ± 0.2 x 10\(^6\); \(p=0.17\)) (Fig. 2.2B, D).

Collectively, we showed that both the CD4\(^+\) and CD8\(^+\) T-cells isolated from chronic SCI mice have defects in cytokine production, which may contribute to the chronic SCI induced immunodeficiency.
Isolated splenocytes (1 x 10^6) from uninjured (CT) or T9-SCI mice at 5-7 weeks after injury (SCI) were stimulated ex vivo with PMA/ionomycin in the presence of brefeldin A for 4 hours, then processed for flow cytometry analysis. (A) Representative dot plots show the percentage of IFN-γ^+ cells and TNF^+ cells in gated CD4^+ T-cells. (B) Representative dot plots show the percentage of IFN-γ^+ cells and TNF^+ cells in gated CD8^+ T-cells. (C) Bar graph represents the mean ± SEM percentages and the numbers of cytokine producing CD4^+ T-cells. (D) Bar graph represents the mean ± SEM percentages and the numbers of cytokine producing CD8^+ T-cells. n = 14 for CT, n = 12 for SCI. Data are pooled across four independent experiments. *p<0.05, ***p<0.001, one-tailed Student’s t-test.
2.3.2 Increased expression of exhaustion marker PD-1 on T-cells isolated from chronic SCI mice

T-cell exhaustion indicated by increased expression of exhaustion markers such as PD-1, cytotoxic T-lymphocyte antigen 4 (CTLA-4), T-cell immunoglobulin mucin-3 (TIM-3) and lymphocyte activation gene-3 (LAG-3) was shown to correlate with T-cell dysfunction in chronic viral infection models and aging animals (Day, Kaufmann et al. 2006, Urbani, Amadei et al. 2006, Blackburn, Shin et al. 2009, Nakamoto, Cho et al. 2009, Jin, Anderson et al. 2010, Lages, Lewkowich et al. 2010, Wherry 2011). To examine whether the T-cell impairment in cytokine production observed in chronic SCI mice was associated with T-cell exhaustion, we measured the expression of exhaustion markers on both CD4+ T-cells and CD8+ T-cells. The percentage of PD-1 expressing cells in both CD4+ T-cells and CD8+ T-cells was significantly higher in the spleen of chronic SCI mice compared with uninjured controls (CD4+PD1+ T-cells: uninjured: 12.9 ± 1.3 %; chronic SCI: 18.0 ± 1.7 %; p=0.02. CD8+PD1+ T-cells: uninjured: 3.4 ± 0.2 %; chronic SCI: 5.0 ± 0.4 %; p=0.003) (Fig. 2.3A, B). The number of splenic CD8+PD-1+ cells was also significantly increased in the chronic SCI mice (uninjured: 0.43 ± 0.05 x 10^6; chronic SCI: 0.59 ± 0.04 x 10^6; p=0.01) (Fig. 2.3C). However, the number of splenic CD4+PD-1+ cells was not significantly changed by chronic SCI (uninjured: 1.9 ± 0.4 x 10^6; chronic SCI: 2.7 ± 0.3 x 10^6; p=0.07) (Fig. 2.3C). The expression of other exhaustion markers including CTLA-4, TIM-3 and LAG-3 on T-cells was not increased by chronic SCI (data not shown).
Figure 2.3  Increased expression of exhaustion marker PD-1 on T-cells isolated from chronic SCI mice.

(A) Representative dot plots show the percentage of PD-1+ cells in gated CD4+ T-cells and CD8+ T-cells from uninjured (CT) and T9-SCI mice at 5-7 weeks after injury (SCI). (B) Bar graphs show the percentage of PD-1+ cells in CD4+ T-cells and CD8+ T-cells. (C) Bar graphs show the mean ± SEM numbers of PD-1 expressing CD4+ T-cells and CD8+ T-cells. n= 9 for CT mice, n= 11 for SCI mice. Data have been pooled across two independent experiments. n= 9 for CT mice, n= 11 for SCI mice. Data have been pooled across two independent experiments. *p<0.05, **p<0.01, one-tailed Student’s t-test.

2.3.3  Blocking PD-1 restores cytokine production by CD8+ T-cells

We next asked whether inhibiting PD-1 signaling will restore the subset-dependent functional defects of cytokine production observed on the splenic T-cells isolated from chronic SCI mice. Splenocytes from uninjured mice and chronic SCI mice were stimulated with PMA/ionomycin in the presence of either anti-PD-1 or an isotype
control antibody. Compared with splenocytes from uninjured mice, the splenocytes from chronic SCI mice showed a significant reduction in the percentage of IFN-γ-expressing CD4+ T-cells (uninjured: 7.8 ± 0.9 %; chronic SCI + Isotype: 4.0 ± 0.7 %; \( p=0.006 \)) (Fig. 2.4A, B) and TNF-expressing CD8+ T-cells (uninjured: 7.2 ± 1.2 %; chronic SCI + Isotype: 3.1 ± 0.7 %; \( p=0.044 \)) (Fig. 2.4 C, D) after PMA/ionomycin restimulation in presence of isotype control antibodies. Blocking PD-1 restored the percentage of CD8+ T-cells expressing TNF (chronic SCI + anti-PD-1: 5.6 ± 1.2 %; \( p=0.57 \) compared with uninjured) (Fig. 2.4 C, D). However, IFN-γ production by CD4+ T-cells from chronic SCI mice was not restored (chronic SCI + anti-PD-1: 3.7 ± 0.6 %; \( p=0.004 \) compared with uninjured) (Fig. 2.4 A, B).

### 2.3.4 Chronic SCI increases PD-1 expression by altering sympathetic activity

To understand how chronic SCI induces T-cell exhaustion, we investigated whether the activity of the sympathetic nervous system is altered by SCI. As a surrogate marker of SNS activity we measured the protein expression level of tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine (CA) synthesis, in the spleen extract from both control and SCI mice. Compared to uninjured control, TH expression levels in the spleen of injured mice was significantly elevated (Fig. 2.5A) and correlated with significantly higher norepinephrine concentrations in the spleen of those injured mice (uninjured: 0.26 ± 0.03 NE /mg spleen; chronic SCI: 0.38 ± 0.05 ng NE /mg spleen, \( p=0.04 \)) (Fig. 2.5B). These results led us to hypothesize that higher splenic NE levels in injured mice contributed to T-cell exhaustion.
Figure 2.4  Blockade of PD-1 pathway \textit{in vitro} rescues the TNF production by CD8$^+$ T-cells from chronic SCI mice.

Splenocytes (1 x 10$^6$) isolated from uninjured mice were stimulated \textit{ex vivo} with PMA/ionomycin in the presence of brefeldin A for 4 hours (group: CT). Splenocytes (1 x 10$^6$) isolated from chronic SCI (≥ 5 weeks after injury) mice were also stimulated \textit{ex vivo} with the same condition as the CT group, except that 10 µg/mL anti-PD-1 blocking antibody (group: SCI + αPD-1) or 10 µg/mL rat IgG2a, κ isotype (group: SCI + Isotype) were added to the culture. (A) Representative dot plots show the percentage of IFN-γ$^+$ cells in gated CD4$^+$ T-cells. (B) Bar graph represents the mean ± SEM percentage of IFN-γ$^+$ cells in CD4$^+$ T-cells. $n = 6$ mice/group. (C) Representative dot plots show the percentage of TNF$^+$ cells in gated CD8$^+$ T-cells. (D) Bar graph represents the mean ± SEM percentage of TNF$^+$ cells in CD8$^+$ T-cells. $n = 6$ for CT, $n = 7$ /group for SCI + Isotype and SCI + αPD-1. *$p<0.05$, **$p<0.01$, one-way ANOVA with post-hoc Tukey’s test.
Figure 2.5  Chronic SCI increases the sympathetic activity in the spleen.

(A) Western blot quantification of tyrosine hydroxylase (TH) in the spleens of uninjured mice (CT) and chronic SCI mice (SCI). Data are normalized to the expression of β-actin. Bar graph represents the mean ± SEM of TH expression in the spleen protein extract and are expressed as arbitrary units (AU). n = 4 mice/group. Data represents two independent experiments. *p<0.05, two-tailed Student’s t-test. (B) Bar graph represents the mean ± SEM of norepinephrine concentration (ng/mg) in the spleen homogenates from CT and SCI mice. n = 5 mice/group. *p<0.05, one-tailed Student’s t-test.

To test this hypothesis, we incubated enriched T-cells from uninjured mice with 10µM NE and measured PD-1 expression at 1, 2 and 3 days of in vitro culture. We showed that NE stimulation significantly up-regulated the percentage of PD-1+ cells in both CD4+ T-cells (Fig. 2.6A, B; Vehicle: 12.6 ± 1.5%; NE: 19.6 ± 2.3%; p=0.02) and CD8+ T-cells (Fig. 2.6C, D; Vehicle: 4.0 ± 0.2%; NE: 7.7 ± 0.5%; p=0.01) after 3 days of continuous stimulation. These data strongly suggest that sustained elevated levels of splenic NE following chronic SCI can induce T-cell exhaustion.
Figure 2.6  Norepinephrine up-regulates PD-1 expression on T-cells.

Enriched splenic T-cells (10^6 cells/ml) were stimulated with 10µM norepinephrine (NE) or its vehicle (Vehicle) in vitro for 1-3 days. PD-1 expression was analyzed by flow cytometry. (A) Representative dot plots show the percentage of PD-1^+ cells in gated CD4^+ T-cells. (B) Bar graph represents the mean ± SEM percentage of PD-1^+ cells in gated CD4^+ T-cells at day 1, day 2 and day 3 of Vehicle or NE stimulation. (C) Representative dot plots show the percentage of PD-1^+ cells in gated CD8^+ T-cells. (D) Bar graph represents the mean ± SEM percentage of PD-1^+ cells in gated CD8^+ T-cells at day 1, day 2 and day 3 of Vehicle or NE stimulation. n= 3 experiments/group. Data are pooled across three independent experiments with triplicate wells in each condition. *p<0.05, two-tailed paired Student's t-test.
2.4 Perspective

In the present work, we assessed the impact of chronic SCI on the peripheral immune system and explored the mechanisms by which traumatic injury to the spinal cord induces T-cell dysfunction. Using a severe spinal cord contusion model at thoracic level T9, we demonstrate that although there was no change in splenic T-cell numbers at the chronic phase following injury, their function was significantly altered as assessed by ex vivo stimulation with PMA/ionomycin. IFN-γ production by CD4+ T-cells and TNF production by CD8+ T-cells were significantly reduced in the cells isolated from chronically spinal cord injured mice. We provide evidence that T-cell exhaustion contributes to SCI-induced T-cell dysfunction. T-cell expression levels of exhaustion marker PD-1 was significantly increased by chronic SCI, while in vitro blockade of PD-1 restored CD8+ T-cell function. To our knowledge, this is the first report showing that chronic SCI alters T-cell function and increases T-cell exhaustion. Furthermore, the norepinephrine level in the spleen is higher in injured mice. Long-term in vitro exposure to norepinephrine increased PD-1 expression on T-cells. This finding suggests that alterations in the SNS output is involved in the mechanism by which chronic SCI induces T-cell exhaustion, and highlights the importance of the SNS in the regulation of T-cell function.

Previous studies in SCI animal models have characterized changes in the peripheral immune system occurring in the acute phase. T-cell loss in the spleen was reported at 1-3 days after injury (Lucin, Sanders et al. 2007, Riegger, Conrad et al. 2007). Increased level of glucocorticoids and NE at acute phase after SCI induces lymphocyte apoptosis and results in T-cell decrease (Lucin, Sanders et al. 2009). However, no
significant reduction in the T-cell number was observed at later time points such as day 7, day 14 and day 28 post-injury (Riegger, Conrad et al. 2007, Held, Steward et al. 2010). Consistent with those studies, we did not find any significant difference in the number of either CD4⁺ or CD8⁺ T-cell between uninjured and chronic SCI mice (5-7 weeks post injury). These results indicate that the temporary T-cell loss in the acute phase after SCI does not persist in the chronic phase. Therefore the SCI-induce immunedepression in the chronic phase is more likely due to functional defects of immune cells rather than their number.

T-cells regulate host immunity against pathogen infection by secreting cytokines upon activation (Slifka and Whitton 2000). We used *ex vivo* PMA/ionomycin stimulation, which resembles the T-cell receptor signaling activation and costimulation (Macian, Garcia-Cozar et al. 2002), to investigate the effect of chronic SCI on T-cell cytokine production as an indicator of T-cell effector function. In this study, chronic SCI mice showed a reduction in IFN-γ production by CD4⁺ T-cells as well as TNF production by CD8⁺ T-cells. These defects could be associated with SCI-induced immune depression. IFN-γ is a crucial modulator in multiple immune responses, including macrophage activation, major histocompatibility complex (MHC) I and MHC II antigen presentation up-regulation, lymphocyte recruitment, CD4⁺ T helper 1 response and inhibition of viral replication. The deregulation of IFN-γ production by CD4⁺ T-cell may contribute to the impaired capacity to combat infection in chronic SCI patients (Schoenborn and Wilson 2007). TNF plays a key role in both host inflammatory and cytotoxic responses against pathogens (Bradley 2008). Specifically, during viral infection, TNF produced by cytotoxic T-cells can induce apoptosis and lysis of virus-infected cells (Kuwano,
insufficient TNF production by CD8⁺ T-cells is a potential cause of SCI-induced immunodeficiency. Interestingly, some studies have suggested a role for TNF in forming germinal center and generating humoral responses (Pasparakis, Alexopoulou et al. 1996, Marino, Dunn et al. 1997, Gelinck, van der Bijl et al. 2008). Alteration in TNF production by CD8⁺ T-cell in injured mice may contribute to their defects in mounting an appropriate antibody response.

T-cell exhaustion has been well studied in the last decade for its role in T-cell dysfunction and immunodeficiency. It was first described in a lymphocytic choriomeningitis virus (LCMV) chronic infection mouse model as a virus-specific CD8⁺ T-cell population which cannot elaborate efficient antiviral effectors’ function (Zajac, Blattman et al. 1998). Since then, T-cell exhaustion has been investigated in many chronic viral infections including human immunodeficiency virus (HIV), hepatitis B virus and hepatitis C virus, cancer as well as aging models (Trautmann, Janbazian et al. 2006, Radziewicz, Ibegbu et al. 2007, Streeck, Brumme et al. 2008, Channappanavar, Twardy et al. 2009, Nakamoto, Cho et al. 2009, Lages, Lewkowich et al. 2010, Topalian, Drake et al. 2012, Tzeng, Tsai et al. 2012). To our knowledge, our study is the first to show T-cell exhaustion in a SCI model. Previous studies have demonstrated a correlation between higher PD-1 expression and reduced cytokine production in CD8⁺ T-cells (Barber, Wherry et al. 2006, Trautmann, Janbazian et al. 2006, Penna, Pilli et al. 2007). Herein we showed that our chronic SCI mice have a significantly higher number of CD8⁺ T-cells expressing PD-1. Moreover, the production of TNF by CD8⁺ T-cells was restored in vitro by blocking PD-1 signaling. These results suggest that higher PD-1 expression
contributes to SCI-induced CD8+ T-cell dysfunction. However, the number of CD4+ T-cells expressing PD-1 was not significantly up-regulated by chronic SCI and blocking PD-1 failed to restore IFN-γ production by CD4+ T-cells suggesting that other mechanisms are involved in SCI-induced CD4+ T-cell dysfunction.

We next explored how chronic SCI increases PD-1 expression on CD8+ T-cells. Since the spleen is innervated and modulated by the SNS, disruption of the sympathetic preganglionic neurons at the injury level may lead to altered SNS output to the spleen. Post-acute phase SCI causes reorganization of synapses on the sympathetic preganglionic neurons and reinnervation of the sympathetic terminals at the target organs (Weaver, Cassam et al. 1997, Llewellyn-Smith and Weaver 2001, Lujan, Palani et al.). Our results showed higher levels of TH in the splenic protein extract from injured animals compared to uninjured controls. As the rate-limiting enzyme for catecholamine synthesis, TH has been used to identify catecholamine containing noradrenergic terminals in the spleen (Felten and Olschowka 1987, Madden, Bellinger et al. 1997). Elevated TH levels in the spleen of chronic SCI mice correlated with higher splenic NE levels. These data could be explained by increased sympathetic innervation, higher catecholamine levels per cell or more endogenous catecholamine produced by lymphocytes in the spleen (Qiu, Peng et al. 2004, Laukova, Vargovic et al. 2013). NE has been reported to regulate the functions of immune cells (Nance and Sanders 2007). Particularly, experimental induction of autonomic dysreflexia in chronic SCI mice causes splenic NE accumulation, which is involved in the impaired immune function (Zhang, Guan et al. 2013). We hypothesized that higher NE levels in the spleen following chronic SCI were responsible for the increased T-cell exhaustion. Consistent with this hypothesis, we found that the expression
of exhaustion marker PD-1 on T-cells was increased after prolonged exposure to NE in vitro. While it is unclear how NE regulates PD-1 expression, several transcription factor pathways have been demonstrated to play a role in regulating T-cell exhaustion. Specifically, B lymphocyte-induced maturation protein 1 (Blimp-1), nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) and Notch signaling are regulators of PD-1 gene expression, whereas high expression of T-bet suppresses PD-1 expression (Oestreich, Yoon et al. 2008, Shin, Blackburn et al. 2009, Kao, Oestreich et al. 2011, Mathieu, Cotta-Grand et al. 2013). There is no direct evidence that NE regulates these transcription pathways in murine splenic T-cells. However, NE was reported to increase NFATc1 activity in primary neonatal cardiomyocyte culture (Lunde, Kvaloy et al. 2011), suggesting that NE stimulation may up-regulate PD-1 expression by activating NFATc1. Interestingly, NE stimulation of T-lineage cells increases the phosphorylation of phosphor 38 mitogen-activated protein kinase (p38 MAPK), which is involved in the mechanisms by which HIV-1 Nef protein induces PD-1 expression (Muthumani, Choo et al. 2008, Lajevic, Suleiman et al. 2011). The activation of the p38 MAPK pathway may also contribute to the higher PD-1 expression in our model.

In conclusion, we demonstrated that chronic SCI impaired T-cell function by inducing T-cell exhaustion. Alterations in the SNS contributed to the exhausted phenotype of T-cells from injured mice. These findings highlight the role of the nervous system and neurotransmitters in regulating peripheral immunity. Our study also shed light on the development of therapeutic strategies to reduce re-hospitalization and death rate from infection in chronic SCI patients. For example, clinical trials using antagonist antibodies to PD-1 are now ongoing for cancer treatment (Sznol and Chen 2013). Our
research provides evidence for the clinical application of PD-1 antibodies in SCI-induced immune depression treatment. In addition, adrenergic receptor antagonists, which have been widely used in cardiovascular diseases, constitute also a potential treatment to restore immunity in chronic SCI patients.

2.5 Summary

Chronic spinal cord injury (SCI) induces immune depression in patients, which contributes to their higher risk of developing infections. While defects in humoral immunity have been reported, complications in T-cell immunity during the chronic phase of SCI have not been explored yet.

To assess the impact of chronic SCI on peripheral T-cell number and function we used a mouse model of severe spinal cord contusion at thoracic level T9 and performed flow cytometry analysis on the spleen for T-cell markers along with intracellular cytokine staining. Furthermore we identified alterations in sympathetic activity in the spleen of chronic SCI mice by measuring splenic levels of tyrosine hydroxylase (TH) and norepinephrine (NE). To gain insight into the neurogenic mechanism leading to T-cell dysfunction we performed in vitro NE stimulation of T-cells followed by flow cytometry analysis for T-cell exhaustion marker.

We found that Chronic SCI impaired both CD4⁺ and CD8⁺ T-cell cytokine production. The observed T-cell dysfunction correlated with increased expression of programmed cell death 1 (PD-1) exhaustion marker on these cells. Blocking PD-1 signaling in vitro restored the CD8⁺ T-cell functional defect. In addition, we showed that chronic SCI mice had higher levels of splenic NE, which may contribute to the T-cell
exhaustion phenotype, as PD-1 expression on both CD4+ and CD8+ T-cells was up-regulated following sustained exposure to NE \textit{in vitro}.

These studies indicate that alteration of sympathetic activity following chronic SCI induces T-cell exhaustion, which in turn impairs T-cell function and contributes to immune depression. Inhibition of the exhaustion pathway should be considered as a new therapeutic strategy for chronic SCI-induced immune depression.
Chapter 3: Chronic thoracic spinal cord injury attenuates influenza virus specific antiviral immunity

3.1 Overview

Central nervous system (CNS) injury (e.g., SCI, TBI and stroke) disrupts the normal balance between the CNS and immune system resulting in a syndrome called “CNS Injury-Induced Immunodepression” (CIDS) resulting in infection, worse neurological outcome and often times, death. In fact, infections account for more than 50% of deaths following SCI (Soden, Walsh et al. 2000, Meisel, Schwab et al. 2005). Therefore it is critical to better understand mechanisms through which SCI mediates systemic immunodepression so that complications arising from secondary infections (e.g. chronic hospitalization, worse neurological outcome and death) can be reduced or alleviated altogether. Two independent research teams in addition to ours have begun to investigate the effects of SCI on peripheral immune function and have established a foundation for our studies.

Studies by the Lucin et al have shown that high level thoracic (T3) injury disrupts B-cell function and antibody synthesis through sympathetic nervous system dysregulation whereas low level (T9) thoracic injury has no effect on B-cell function or dysregulation of the sympathetic nervous system(Lucin, Sanders et al. 2007). This group also determined that SCI induces lymphocyte apoptosis within the spleen as early as 3d post-injury(Lucin, Sanders et al. 2009). Interestingly, studies by Held et al showed that SCI disrupts immune function at one week after injury, independent of injury level(Held, Steward et al. 2010). Furthermore, these studies showed a reduction in spleen size irrespective of injury location; however changes in T and B cell percentages were only detected in T3 injured mice and not in T9 injured mice. Finally, this group showed that
mice injured at T3 and T9 levels had a reduced ability to control a mouse hepatitis virus infection and that was attributed to reduction in proliferation and virus specific virus specific CD4+ T-cells.

These studies are very compelling in that they clearly demonstrate that SCI attenuates peripheral immune function and reduces the ability of the SCI mouse to effectively mount an anti-viral immune response. Immune responses in both patients and murine models of SCI injury has been primarily studied using acute phase models where both innate and adaptive are severely compromised. Recent studies in chronic patients also points to functional defects in innate immune responses(Campagnolo, Bartlett et al. 2000, Campagnolo, Dixon et al. 2008). A literature search reveals that almost nothing is known about how chronic SCI alters the various components of the immune system to combat virus-specific immunity. Thus the goal of our study was to establish whether chronic thoracic SCI alters the function of the peripheral immune system, such that it impairs immunity to elicit an appropriate immune response to relevant human pathogen infectious agents such as influenza.

Influenza A virus is a major respiratory pathogen and causes high morbidity and accounts for the majority of deaths in the elderly and younger population every year(Lu, Santibanez et al. 2013). SCI patients are at a high risk of developing complications of influenza infection followed by secondary pneumonia due to their reduced respiratory function and mobility after injury(DeVivo, Black et al. 1993, DeVivo, Krause et al. 1999). Furthermore emergence of pandemic flu strains in the past decade have heightened the awareness that immune-compromised patients such those suffering from SCI are most susceptible to new viruses(Louie, Acosta et al. 2009). To develop better
insight into the consequences of SCI-induced immune dysfunction we are using a well-established murine model of influenza virus infection (Matsuoka, Lamirande et al. 2009). Intranasal inoculation of influenza virus in inbred mice results in lower respiratory tract infection accompanied by weight loss. The effective clearance of the virus from the site of infection in the lung requires both virus specific antibodies and effector T-cell response to terminate infection (Kreijtz, Fouchier et al. 2011). Thus, this model offers a well-defined immunological system to track the underlying parameters of protective immunity in SCI. Because of the complex nature of SCI and how high level injury may influence immune function through dysregulation of the sympathetic nervous system, we chose to investigate SCI-induced immune dysfunction using a low level (T9) thoracic contusion injury model. Injury at the T9 level partially disrupts central sympathetic regulation to the peripheral lymphoid organs (Lucin, Sanders et al. 2007). These studies will have broad application to our mechanistic understanding to CIDS and therapeutic strategies to improve neurological outcome and reduce death to immune mediated complications following injury to the CNS.

3.2 Materials and methods

3.2.1 Mice and spinal cord injury

Adult C57Bl/6 female mice (3-4 month-old) purchased from Jackson Laboratory were subjected to spinal cord injury (SCI) at thoracic level T9 using the Infinite Horizon Impactor at a predetermined force of 70 kDynes resulting in a severe contusion injury. Immediately after injury mice were sutured and injected subcutaneously with 1 ml Lactated Ringer’s solution to prevent dehydration and gentamycin (40 mg/Kg) to prevent
urinary tract infection. The prophylactic antibiotic treatment was continued for 7 days post-injury. Bladder expression was performed twice daily until recovery of function. By 7 weeks post-SCI, mice have usually regained their initial body weight. Subsequently they were transferred to biosafety level 2 housing for influenza virus infections. All animal protocols were approved by the University of Miami Institutional Animal Care and Use Committee (IACUC) and are in accordance with National Research Council guidelines for the care and use of laboratory animals.

### 3.2.2 Influenza virus infection

Influenza virus infection was performed 6-7 weeks post SCI injury. Spinal cord injured and age matched control mice were anesthetized by intraperitoneal injection of a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) before intranasal infection with egg derived H3N2 subtype influenza virus, X31. This virus is a reassorted virus, which carries the hemaglutinin (HA) and neuraminidase (NA) genes of A/Hong Kong/1/1968 (H3N2) in the background of lab adapted A/Puerto Rico/8/1934 (H1N1) [PR8] (Kilbourne 1969). X31 has intermediate virulence in mice compared to PR8 via intranasal route and results in complete clearance of the virus by day 10 post-infection (Bouvier and Lowen 2010). Mice were monitored daily for weight loss and morbidity.

### 3.2.3 Virus titer

Mice were sacrificed at various times post infection and lungs were harvested. They were snap frozen in liquid nitrogen and stored in -80°C until assayed. Viral titers were determined from homogenized lungs using the 50% tissue culture infectious dose (TCID50) method (Flano, Jewell et al. 2009). Briefly, confluent Madine-Darby canine
kidney (MDCK) cultured in MEM (Invitrogen) containing 2 mM L-glutamine, 10 μg/ml gentamicin, and 5% FBS were grown in flat-bottom 96-well tissue culture plates. They were infected with serial dilution of lung homogenate and incubated at 37°C for 2 hours to allow the virus to adsorb to the cells. The inoculum was washed and replaced with media containing 2 μg/ml of TPCK-trypsin and incubated for 72h. Viral infection was scored by observing cytopathic effect (CPE) in infected wells after staining with crystal violet and was confirmed by hemagglutination inhibition assay with chicken red blood cells (RBC).

### 3.2.4 RNA extraction and real-time PCR

Lungs were homogenized in TRIZOL and total RNA isolated following manufacturer’s protocol. An aliquot of 20 μg of total RNA was further purified using the RNeasy mini kit (Qiagen) with on-column DNase digestion to remove any trace of genomic DNA contamination. cDNA were obtained by reverse transcription of 1 μg of purified RNA using the Omniscript RT kit (Qiagen) and analyzed by quantitative real-time PCR. For each gene a standard curve was obtained by diluting defined amounts of the target PCR product. All the data obtained were normalized to β-actin gene expression levels.

### 3.2.5 Flow cytometry of broncholaveolar lavage (BAL)

Mice were sacrificed and BAL cells were collected by flushing the exposed trachea after cannulation with a 18-gauge, ½ inch cannula connected to a 1-ml syringe. The lungs were flushed once with 0.5 ml of RPMI supplemented with 10% FBS and L-glutamine and spun at 1000 rpm for 5 min. The fluid was removed from the cell pellet
and frozen for virus-specific antibody assays. The remaining BAL cells were resuspended for staining and flow cytometric analysis. Cells were first blocked with anti-CD16/32 (FcR block, eBioscience) to prevent non-specific staining and then stained with anti-F4/80-APCcy7 (clone BM8), anti-CD11b-PEcy7 (clone M1/70), anti-CD11c-APC (clone N418) and anti-Gr1-FITC (clone RB6-8C5) and gated according to Hall et al. (Hall, Woolard et al. 2008). Briefly, cells were first gated as F4/80^+ or F4/80^- cells. F4/80^+ cells that are CD11b^high were referred to as macrophages and the one that are CD11c^high/CD11b^low as alveolar macrophages. DCs were gated as F40/80^low CD11c^high and monocytes as F40/80^low CD11c^low CD11b^mid. Finally neutrophils were gated as F40/80^low CD11c^low CD11b^high and Gr1^high. Data were collected using the LSRII flow cytometer and analyzed with FACSDiva 6.1 software (BD Biosciences).

### 3.2.6 ELISA for virus-specific antibody

Sera were collected on day 5 and 7 post infection. Influenza-specific Ab levels in sera and lung washes were determined by ELISA (Webby, Andreansky et al. 2003). A purified whole influenza HKx31, A/Aichi/68 (H3N2) virus (Charles River Breeding Laboratories) was used to coat microtiter plates overnight at 4°C. The virus was disrupted with buffer containing detergent, diluted to 10 μg/ml and aliquoted at 50 μl per well. Next day, the plates were washed with 0.05% Tween 20 (Sigma) and blocked with 1% BSA in PBS for an hour at room temperature. BAL and serum samples were added to wells and incubated for 2 h at room temperature. After washing, influenza-specific IgG, IgM or IgA were detected with a goat anti-mouse horse-radish peroxidase conjugate (Southern Biotechnology Associates) diluted 1:1,000 in 1% BSA for another hour. TMB (3,3',5,5'-tetramethylbenzidine) substrate (Sigma) was added and plates were further incubated for
60 min at room temperature for color development. The reaction was terminated with 2N sulfuric acid stop solution and absorbance was determined at 405 nm in an ELISA reader (Molecular Devices).

### 3.2.7 Virus neutralization assay

Madin-Darby canine kidney (MDCK) cells were grown in 96-well tissue culture plates. Influenza A virus HKx31 was diluted in MEM containing 5% BSA and 1 μg/ml TPCK-trypsin (Sigma-Aldrich) to give $1 \times 10^2$ 50% tissue culture infectious dose/ml, and then mixed with equal volumes of serum samples diluted 2-fold. After incubation for 2 h at 37°C in a 5% CO₂ atmosphere, the virus/sample mixtures were added to wash MDCK monolayers in the 96-well plates. Plates were incubated for 2 hrs, washed and incubated for 72h. Positive wells were identified by hemagglutination of chicken RBCs.

### 3.2.8 Tetramer and intracellular cytokine assay

The kinetics and magnitude of the virus-specific CD8⁺ T-cell responses were analyzed by flow cytometry. Mice were sacrificed and spleen and bronchoalveolar lavage (BAL) were harvested and processed according to published methods (Andreansky, Stambas et al. 2005). Lymphocytes were isolated from spleens after dissociation and subjected to red blood lysis with ACK buffer (Invitrogen). Lymphocytes were isolated from the pneumonic lung by bronchoalveolar lavage and macrophages were removed by incubating on plastic for 1 h at 37°C. Virus specific CD8⁺ T-cells were quantified by staining cells for 1 h at room temperature with tetramers (Altman, Moss et al. 1996) conjugated to phycoerythrin labeled DbNP366, ASNENMETM (Townsend, Rothbard et al. 1986) or DbPA224, SSLENFRAYV (Belz, Xie et al. 2000) obtained from NIH
Tetramer Facility which are restricted for H-2Db MHC Class I in C57BL/6 mice. The cells were washed extensively with FACS buffer (PSA/BSA/azide) and stained simultaneously with fluorescein conjugated CD8α (clone 53-6.7) and unlabeled anti-CD16/CD32 (clone 2.4G2) to block nonspecific Fc receptor-mediated binding (all antibodies in this study were from BD Pharmingen). For intracellular cytokine assay, lymphocytes were cultured in 96-well round-bottom plates for 6 h at 37°C in the presence of 1 μM NP366 or PA224 peptide in the presence of brefeldin A (Enzo LifeSciences). The cells were washed, stained with CD8-FITC for 30 min, fixed, and permeabilized and then stained with antibodies against allophycoerythrin labeled TNF (clone MP6-XT22), and phycoerythrin labeled IFN-γ (clone XMG1.2). After staining, cells were resuspended in FACS Buffer and detected using LSR II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

3.2.9 Statistical analysis

Student's t test was used to compare at each time point within each group. Two-way ANOVA, with Bonferroni post-test, was used to compare differences between groups. Statistical significance was inferred when p ≤ 0.05. Log-rank test was used to compare the survival distributions.

3.3 Results

We hypothesized that chronically injured mice have a significantly compromised immune system. To date most studies investigating rodent models of SCI-induced immune dysfunction have studied the post-injury interval of 1 week or less (Lucin, Sanders et al. 2009, Held, Steward et al. 2010). However since most patients with SCI
live for years and represent an understudied chronic SCI survival group we determined what effect SCI has on the antiviral immunity in chronically injured mice to better understand immune dysfunction in chronically injured humans.

### 3.3.1 Impaired viral clearance in chronically injured mice

In our primary infection model, age-matched naïve and injured mice were infected i.n. with H3N2 virus (1x10⁴ PFU), 7 weeks post SCI (Fig. 3.1A). Lungs were collected at days 5 and 10 post-infection, homogenized and assayed on canine kidney cells. Viruses were confirmed by hemagglutination assay and expressed as tissue culture dose required to-infect 50% of the culture (TCID50/ml). The data (Fig. 3.1B) demonstrates that viral infection was established in lungs of both uninjured and injured groups 5 days post infection. However, on day 10 post-infection there was significant deficit in viral clearance in injured mice. All uninjured mice were able to clear live virus from their lungs. Out of infected SCI mice (n=10), 7 had very high viral titers in their lungs on day 10 and two died on day 9 and were not included in our titer analysis. Survival of mice (n=15) after virus infection was also monitored in a second experiment for 10 days and 38.3% of SCI mice succumbed to infection (Fig. 3.1C). These data suggest that chronic SCI significantly inhibits viral immunity.
Figure 3.1  Impaired viral clearance in spinal cord injured mice.

(A) Schematic of the experiment. Mice received a spinal cord injury (SCI) at thoracic level T9 and 7 weeks later were intranasally infected with $1 \times 10^4$ H3N2 (x31) influenza particles. Lungs were harvested 5 or 10 days post-infection for virus titer. (B) Viral titers were determined from lung homogenates isolated on day 5 and 10 post-infection. While all the control uninjured mice cleared the virus, spinal cord injured mice still had active virus in their lungs (mean ± SEM, 3 mice/group d5 and 10 mice/group d10, two-tailed Student’s $t$-test, ***$p < 0.001$) (C) Survival was monitored daily for 10 days post infection. 15 mice/group. Log-rank test, $p < 0.01$.

3.3.2 Maintenance of innate response in injured mice

The innate immune system is considered as the first line of defense against viral infections. At the initial stages of infection, innate immune mechanisms in the lungs initiate antiviral responses to the binding of viral particles to pattern recognition receptors (Swain, McKinstry et al. 2012). The release of proinflammatory mediators recruits a
rapid influx of PMNs (polymorphonuclear leukocytes), primarily neutrophils, natural killer (NK) cells, and macrophages to the site of infection. This response next mobilizes cells of the adaptive immune system to promote viral clearance through the production of neutralizing antibodies and cytotoxic T-cells (Swain, McKinstry et al. 2012). To begin investigating innate immunity in the lung we examined the expression of IFNβ, CXCL10, CCL4, CCL2 and IRF9 at 0, 2 and 4 days post-infection by quantitative PCR in non-injured-infected and SCI-infected groups. There was no difference in the level of cytokines/chemokine in either group, at the time points examined (Fig. 3.2A). We also show that the virus specific gene M1 is expressed in lungs of both naïve and injured mice indicating there was no difference in the establishment of virus infection on day 2 and 4. These data, while not complete, suggest that innate immunity within lungs of chronically injured mice is not compromised.

The composition of innate immune cells was also assessed on day 2 and 4 post infection from individual mice to demonstrate that the cytokine and chemokine gene patterns in the lungs elicited the influx of innate effector cells at the site of infection. BAL cells were initially gated by size and side-scatter and then stained for various cell types as described in Materials and Methods and the data as average values of four individual mice. We report that in both uninjured and injured mice there was an increase in the appearance of innate immune effector cells such as neutrophils, alveolar macrophages and myeloid derived dendritic cells (DC) into the airway as reflected in the numbers of cells present in the BAL over time (Fig. 3.2B). By far the largest numbers of cells were neutrophils and monocytes (CD11b+CD11c+) followed by the conventional DC (CD11+CD11c+) and macrophages (F4/80+ and CD11bhigh) and alveolar macrophages
(CD11c^{high}/CD11b^{low}). The ability of all of these cell types to control viral replication and the development of adaptive immunity is well established (McGill, Heusel et al. 2009).

**Figure 3.2 Early response to influenza in the lungs of spinal cord injured mice is not altered.**

(A) Expression of proinflammatory cytokines (CCL2, CXCL10, CCL4) and antiviral genes (IFNβ and IRF9) was examined in the lungs of SCI and control mice prior to infection (d0) and 2 and 4 days post infection using quantitative real-time PCR (qPCR). Data are expressed as percent of uninjured control at d0 (mean ± SEM, 4 mice/group). In addition we assessed the levels of virus using qPCR for the matrix 1 (M1) influenza gene at d2 and d4 post-infection. Data represents mean ± SEM (4 mice/group). (B) Innate immune response in the BAL of naive and SCI H3N2-infected mice was assessed by flow cytometry using the differential expression of the markers F4/80, CD11b, CD11c and Gr1 according to Hall et al., 2008. Bars represent the mean ± SEM (4 mice/group).
Figure 3.3 Impaired antibody response to influenza in SCI mice.

(A) The Influenza virus-specific antibody response was assessed by ELISA using H3N2 virus-coated plates and diluted sera from SCI and CT mice 5 or 7 days post-infection. Mean ± SEM, $n = 5$ mice/group, except $n = 4$ for uninjured group at d7. (B) Virus-specific IgA response was measured in the BAL of infected mice on day 5 and day 7. Mean ± SEM, $n = 5$ mice/group, except $n = 4$ for SCI group at d7. (C) The ability of the antibody to neutralize the virus was tested using serial dilution of the serum from uninjured and SCI mice. one-tailed Student’s $t$-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

3.3.3 Reduction in virus-specific antibodies in injured mice

Evidence from both animal models and humans indicates an adaptive immune response such as B cells is important to the control of influenza virus replication in the lungs and clearance of virus from infected lungs (Maines, Szretter et al. 2008). To determine if SCI altered B cell response, we investigated the production of virus-specific antibodies in the serum and lungs of chronically injured mice using ELISA. Data was
normalized after subtracting the values from uninfected mice from both the uninjured and injured groups at day 0 prior to infection. There was a significant reduction in virus-specific IgM on day 5 (p< 0.01) and day 7 (p<0.001) post-infection and significant reductions in IgG and IgA at 7 days post infection in serum (Fig. 3.3A) and IgA in BAL (Fig. 3.3B). Total serum was therefore assayed to test whether these virus-specific antibodies were able to block virus infection in a tissue culture assay. Serum was diluted 2-fold and incubated with 100 TCID50 of X31 for one hour prior to infection of canine kidney cells. The production of virus was analyzed by hemagglutination assay. We observed that there were negligible antibody titers to block virus infection in both groups on day 5 post infection. However at day 10, all of the mice from the uninjured group had a virus neutralization titer of 1:40, whereas none of the sera isolated from SCI mice were able to neutralize virus. In the SCI group neutralization was not detected at a serum dilution of 1:10, which is the limit of detection for this assay (Fig. 3.3C).

![Figure 3.4](image)

**Figure 3.4 Spinal cord injured mice produce significantly less virus-specific CD8^+ T-cells than naïve mice.**

(A) Splenocytes from naïve and SCI mice isolated 7 days post-infection were analyzed by flow cytometry using tetrameric complexes of either the nucleoprotein peptide (NP_366-374) or the polymerase 2 protein peptide (PA_224-233) presented by H-2D^b (mean ± SEM, 5 mice/group, *P<0.05). (B) In vitro cytokine assay revealed a significant decrease in the number of cells producing either IFN-γ or both IFN-γ and TNF-α following stimulation with the peptide D^bPA224 (mean ± SEM, 5 mice/group, two-way ANOVA, *p<0.05).
3.3.4 Decrease in virus-specific CD8$^+$ T-cells in injured mice

CD8$^+$ T-cells (cytotoxic T lymphocytes, CTL) plays an important role in antiviral immunity by destroying influenza-infected cells via perforin/granzyme-dependent granule exocytosis and also by FasL/Fas-mediated apoptosis following TCR engagement in the infected lungs (Topham, Tripp et al. 1997). Pro-inflammatory cytokines (e.g., IFN-γ and TNF-α) are hallmarks of these CTLs which contributes to the recruitment and activation of innate inflammatory cells, in particular, inflammatory CD11c$^{hi}$ DC and pDC (La Gruta, Kedzierska et al. 2007). Knowing that viral specific CD8$^+$ T-cells play an important role in influenza specific anti-viral immunity, we therefore quantitated the numbers of virus specific CD8$^+$ T-cells and their ability to produce intracellular cytokine IFN-γ and TNF-α. Lymphocytes were isolated from spleen and BAL on day 10 after primary infection to correlate with viral titers (Fig. 3.1B). Virus-specific CD8$^+$ T-cells were quantified using flowcytometry by MHC Class I tetramers (NIH Tetramer Facility, Emory University) specific for two immunodominant CD8$^+$ T-cell peptides derived from internal proteins, nucleoprotein (D$^b$NP366$^{CD8^+}$) and acid polymerase (D$^b$PA224$^{CD8^+}$). Figure 3.4A demonstrates that compared to infected controls, SCI mice had a significant (p<0.05, Student’s t-test) reduction of virus-specific CD8$^+$ T-cells in spleens. The ability of these CD8$^+$ T-cells to secrete antiviral cytokines was analyzed as a qualitative measure of virus specific CD8$^+$ T-cell function. Isolated lymphocytes from spleen and BALF were cultured with virus specific MHC Class I restricted peptides (e.g., NP366-374 from viral nucleoprotein and PA224-236 from acid polymerase) in the presence of Brefeldin A for 6h and then stained with antibodies to CD8, IFN-γ and TNFα. The number of CD8$^+$ T-cells producing IFN-γ and TNF-α were calculated after
determining the percentage of cytokine producing cells by flow analysis. SCI mice show a dramatic reduction in the numbers of CD8^+ T-cells expressing IFN-γ, TNF-α or IFN-γ^+TNF-α^+ (Fig. 3.4B).

### 3.3.5 No change in the B-cell subset phenotype and function

In the previous section, we found a significant reduction in antibody response to influenza infection in the chronically spinal cord injured mice (Fig. 3.3). It is possible that chronic SCI changes the B cell subset phenotype and affect their intrinsic functions prior to influenza infection. We first isolated the splenocytes from the uninjured and injured mice and quantify the percentage and number of mature B-cell subsets by staining cells with different cell surface markers. We found no change in the percentages (Fig. 3.5A) and the numbers (Fig. 3.5B) of the follicular zone (FO) B cells, marginal zone (MZ) B cells, intermediate (Int.) B cells and age-associated B cells (ABC) in the mice with chronic SCI. We next measured the class-switching function of B cells by measuring the expression of activation-induced deaminase (AID), which is an important enzyme for B cell class switching following LPS stimulation. No change was found in the AID expression between injured mice and control mice (Fig. 3.5C). Taken together, we did not detect any intrinsic defects in the mouse splenic cells following chronic SCI.
Fig 3.5 B-cells isolated from SCI mice are not functionally impaired.

(A) Representative dot plots show the percentage of four different subsets in mature B cells from uninjured mice and SCI mice. (B) Bars represent the mean ± SEM cell numbers of follicular (FO) B-cell, Marginal Zone (MZ) B-cell, Intermediate (Int.) B-cell and age-associated B-cell (ABC) in the spleen of uninjured and SCI 4 mice/group. (C) Splenic B cells were isolated from uninjured and SCI mice and stimulated with LPS (1mg/10^6 cells). At d5 and d7 cells were collected and RNA isolated and reverse transcribed. Quantitative real-time PCR was used to determine AID expression relative to GAPDH using the ΔΔCT method. Bars represent mean ± SEM, 4 mice/group. No statistical difference was detected by two-tailed Student’s t-test, p>0.05.
3.4 Perspective

Crosstalk between the nervous system and the immune system plays a pivotal role in maintaining homeostasis of the host. This coordination is achieved via the sympathetic nervous system (SNS) and the hypothalamic-pituitary-axis (HPA), which regulates a variety of cytokines, hormones and neurotransmitters (Meisel, Schwab et al. 2005). Thus direct injury to the neural pathways that originated from the spinal cord contributes to functional deficit in the peripheral immune response and renders patients with SCI more susceptible to viral and bacterial infections (Meisel, Schwab et al. 2005, Lucin, Sanders et al. 2009). Indeed, patients with longer duration of SCI have higher frequency of respiratory infections, which increases in older individuals (Hartkopp, Bronnum-Hansen et al. 1997, Soden, Walsh et al. 2000, Lidal, Snekkevik et al. 2007). We report here for the first time that chronic SCI alters the adaptive immune response to a relevant human respiratory virus such as influenza in an experimental mouse model. In order to minimally disrupt the innervations from preganglionic sympathetic neurons to lymphoid organs, a lower thoracic level T9 injury was chosen for the SCI.

We demonstrate that infection of injured mice at the T9 level post 7 weeks SCI with influenza A virus causes severe morbidity and mortality. Approximately forty percent of injured mice succumbed to infection and sixty percent of the mice had high viral titers in lungs on day 10 post-infection when compared to uninjured controls. Comparison of viral titers on day 5 in both groups demonstrated that the similar onset of virus replication, which is indicative of SCI injury did not exacerbate the viral infection per se. This was confirmed by the ability of the SCI mice to mount innate immune response on day 2 and 4 as demonstrated by quantitative analysis of IFNβ, CXCL10,
CCL4, CCL2 and IRF9 genes. In addition influx of innate immune effector cells such as neutrophils, alveolar macrophages and myeloid derived dendritic cells (DC) into the airway were not compromised in both groups.

The adaptive system comprising of humoral and cell mediated immunity plays a vital role in controlling respiratory virus infections such as influenza and is mediated respectively by virus-specific antibodies and T-cells. The virus specific antibodies are targeted to two outer surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) both of which predict correlates of protection (Gerhard 2001). The main antibody isotypes produced during influenza infection are IgA, IgM and IgG in serum with secretary IgA being required in protection from viral infection at the local site of replication (Kreijtz, Fouchier et al. 2011) Since SCI injured mice were not able to clear viral infection, a detailed analysis of virus-specific isotypes on day 5 and 7 were performed.

Although there were no differences in IgA and IgG production early during infection (day 5), a significant deficit in the generation of all isotypes were observed in serum on day 7 post-infection in injured animals. The production of serum specific IgM isotype was impaired on both days. Influenza-specific IgM initiate neutralization of the virus by complement mediated system and therefore may contribute to the deficit in viral clearance on day 10. Furthermore, decrease in IgG isotype on day 7 reflects that the injured mice were not able to generate long-lived protective titers. This was verified using the serum-binding assay to neutralize the virus where the uninjured mice had a four-fold increase in antibodies in the serum compared to the SCI mice. Further analysis of mucosal IgA isotype in lung fluids of infected injured mice reflected the decrease in IgA in the serum. IgA is not only required at the site of local inflammation, it can
neutralize the virus intracellularly. Thus, taken together virus-specific antibody response was blocked in injured mice.

The importance of virus-specific CD8$^+$ T-cells during influenza infection is well documented (La Gruta, Turner et al. 2004, Kedzierska, Venturi et al. 2006). Activated CD8$^+$ T-cells traverse to the site of infection and eliminate virus-infected targets by various mechanisms. Quantitative assays using tetrameric complexes facilitates the detection of antigen-specific CD8$^+$ T-cells of antigen-specific immune responses. Our result demonstrate that the numbers of CD8$^+$ T-cells specific to DbNP366 and DbPA224 peptides in C57bl/6 mice which are characterized CTL epitopes from internal viral such as NP (nucleoprotein) and PA (acid polymerase) proteins were decreased in spleen. These are also analogous to human CTLs, which are targeted to NP, M1 (matrix), PB2 and PA proteins (Gotch, McMichael et al. 1987). These cells were functionally defective in the SCI mice as they produced decreased TNF$\alpha$ and IFN$\gamma$ cytokines. Taken together we conclude that the virus-specific adaptive immune response is severely hampered in chronically spinal cord injured mice. Future research on understanding the effectors and cell types mediating these deficits are warranted.

3.5 Summary

Individuals suffering from spinal cord injury (SCI) are at an extremely higher risk for respiratory related infections such as influenza virus due to their impaired respiratory functions after injury. Investigation on the effect of SCI on the peripheral immune system in murine models demonstrates that injury determines the outcome of adaptive immune response. Thus the goal of our study was to establish how the immune system is affected by chronic SCI using a well-established influenza virus mouse model. We used a
contusion model of SCI at thoracic level 9 and infected them intranasally with a H3N2 influenza-A virus seven to six weeks post injury. Virus specific immunity was analyzed at different times post-infection in comparison to uninjured controls. We report that chronic SCI injury impairs the ability of the animals to mount antiviral immunity. While all the control mice cleared the virus from the lungs 10 days post-infection, significant number of SCI mice did not clear the virus. This was attributed to severe deficit in both virus-specific antibody and CD8$^+$ T-cell response in injured mice. Taken together our data demonstrate that the immune system of chronically spinal cord injured mice is compromised. Our objective is to better understand the mechanisms of spinal cord injury induced immune-depression with the goal of developing more effective therapies and reduce mortality due to complications from influenza and other infections.
Chapter 4: Discussion

These studies systematically investigated how chronic SCI affects the peripheral immune system using a SCI mouse model. We investigated the peripheral immunity in mice during the chronic phase after thoracic SCI, with a focus on the T-cell immunity. We also challenged the SCI mice with influenza virus and assessed whether the immune response to the viral infection is impaired in the chronic SCI mouse.

We first analyzed how the phenotype and function of peripheral T-cells are altered during the chronic phase of SCI in the pathogen-free condition. We demonstrated that although the splenic T-cell number is not changed during the chronic SCI, their function as measured in cytokine production, upon ex vivo stimulation, is diminished. We further explored the mechanisms of SCI-induced T-cell dysfunction and demonstrated that chronic SCI increases sympathetic nervous system (SNS) activity and elevates norepinephrine levels in the spleen, which correlates with a T-cell exhaustion phenotype as shown by higher expression of exhaustion marker PD-1 on T-cells. The up-regulation of PD-1 expression may contribute to the T-cell functional deficiency in chronic SCI mice.

We next infected the uninjured mice and chronic SCI mice with influenza virus H3N2(x31) and measured their immune responses following infection. The results indicate that the chronic SCI mice are not able to mount an appropriate adaptive immune response against the virus, leading to increased mortality and higher virus titer. The deficiencies in adaptive immunity to influenza virus include impaired antibody response and reduced number of virus peptide-specific CD8\(^+\) T-cells. To our knowledge, this is the first report on how the immunity against viral infection is altered in the mice with chronic SCI.
Taken together, our results highlight the important role of central nervous system and neurotransmitters in regulation of immune cell function and provide insights on developing therapeutic strategies to restore immunity in patients with chronic SCI.

4.1 High infection risk induced by SCI is due to impaired host adaptive immunity

Respiratory system diseases from pneumonia and influenza infections are the leading causes of death in chronically spinal cord injured patients. We infected the mice with H3N2 subtype of influenza virus to understand the immunological mechanisms of higher mortality following influenza infection in individuals with chronic SCI. In our mouse model of H3N2 influenza infection, 30% of the mice with chronic SCI died at day 10 following influenza infection, with all the uninjured control mice alive. This result is consistent with the higher death rate from influenza infection in chronic SCI patients. Compared with uninjured mice, the chronic SCI mice have impaired viral clearance in the lung, as shown by the higher virus titer in the lung at day 10 after influenza infection. To better understand the mechanisms of SCI-induced immune dysfunction following injury, we first analyzed the innate immunity. Neutrophils, alveolar macrophages and DCs as well as the type I IFN in the lung contributes to early innate immune response to influenza. We did not find any difference between uninjured control and chronic SCI mice regarding the numbers of innate immune cells and type I IFN expression in the lung at day 2 and day 4 after influenza infection. In addition, chronic SCI mice did not show any changes in the expression of CCL2, CXCL10 and CCL4 mRNA within the lungs at day 2 and day 4 after infection. In addition, uninjured controls and chronic SCI mice have
comparable mRNA levels of influenza gene M1 in the lung at day 2 and day 4 following infection. These data are consistent with the results that chronic SCI mice have similar virus titers in the lung with uninjured controls at day 5 after infection. Together these results suggest that the innate immune response to influenza is intact within the lungs of chronically injured mice. These data suggest the higher mortality and impaired viral clearance in the lung following influenza infection may result from the deficiency in adaptive immunity against influenza.

To investigate this, we examined the adaptive immunity including antibody response and CD8$^+$ T-cell response in uninjured control and chronic SCI mice following influenza infection. The antibody response to influenza is important for viral clearance, as B-cell deficient mice exhibit higher mortality and delayed viral clearance in the lung (Graham and Braciale 1997). Furthermore, treating SCID mice with virus-neutralizing antibody eliminates the influenza virus infection (Palladino, Mozdzanowska et al. 1995, Mozdzanowska, Furchner et al. 1997). Previous studies have shown that antibody-secreting cells in the draining lymph nodes can be detected between 4 and 5 days after infection and reaches the peak between 7 and 10 days (Harling-McNabb, Deliyannis et al. 1999). In the serum, IgM is generated first and dominates the antibody response acutely (day 5 to day 7), while IgA and IgG are generated in the later phase of infection (day 7 and later) (Harling-McNabb, Deliyannis et al. 1999, Lund, Partida-Sanchez et al. 2002). We found that in chronically injured mice the production of all virus-specific immunoglobulin isotypes (IgA, IgM and IgG) is reduced. Specifically, injured mice have reduced serum levels of virus-specific IgM antibody compared with uninjured control at both day 5 and day 7 post infection. At day 7 post infection, chronic SCI mice produce
significantly less virus-specific IgA and IgG levels compared with uninjured control. Reduced level of IgA in BALF suggests that the mucosal humoral immunity to influenza within the lung is impaired by chronic SCI. IgM is the primary isotype during the early phase (~day 5) of influenza infection and defects of virus-specific IgM production at day 5 post-infection in chronic SCI mice may lead to impaired viral clearance at that time point and in turn result in higher lung virus titer. IgG is produced by a T-dependent antibody response in the germinal center following isotype switching of heavy chain and somatic hypermutation, and participates in the long-term protection and secondary humoral response to influenza infection. A lower level of virus-specific IgG in the chronic SCI mice contributes to the loss of neutralizing antibody to influenza as well as the inability to clear virus at day 10 post-infection. It also suggests that there may be deficits in T-cell mediated B-cell antibody response (discussed later). Moreover, the virus neutralizing activity of serum from chronic SCI mice is significantly lower at day 10 after influenza infection, suggesting that the injured mice cannot produce enough antibodies that binds to virus to prevent the attachment of virus to host cell, and leads to more virus spreading in the lung which results in the higher virus titer in the lung.

In naïve C57BL/6 mice, anti-viral CD8+ T-cell responses are initiated at approximately day 5, peaking at day 10 after influenza infection (La Gruta, Turner et al. 2004, Kedzierska, Venturi et al. 2006). DbNP366-374 epitope-specific CD8+ T-cells and DbPA224-233 epitope-specific CD8+ T-cells are the two major populations of CD8+ T-cells generated following primary influenza infection and reach similar number at day 10 post-infection (Kedzierska, Venturi et al. 2006). We used the tetramer assay to measure the number of CD8+ T-cells specific for these two epitopes following influenza infection and
found that they are both significantly decreased in chronic SCI mice at day 7 post-infection. In parallel, we isolated the splenic CD8+ T-cells at day 7 post-infection and measured the cytokine production following ex vivo stimulation with D\textsuperscript{b}PA\textsubscript{224-233} and D\textsuperscript{b}NP\textsubscript{366-374}. Consistent with the results from the tetramer assay, there is a significant reduction in the number of IFN-\(\gamma\)^+CD8+ and IFN-\(\gamma\)^+TNF+CD8+ T-cell following viral peptide stimulation. CD8+ T-cell responses are crucial for the clearance of influenza virus and host recovery. Although antibody response can mediate recovery from influenza infection, the viral clearance is delayed in the absence of CD8+ T-cell(Scherle, Palladino et al. 1992). Virus-specific CD8+ T-cells can kill infected cells directly by secreting granzyme B and express Fas to induce apoptosis of infected cells(Topham, Tripp et al. 1997). The decreasing number of virus-specific CD8+ T-cells following influenza infection in chronic SCI mice may cause the inefficient killing of virus-infected cells and in turn lead to impaired viral clearance. Moreover, we demonstrate that there are less splenic CD8+ T-cells producing cytokine upon viral peptide stimulation in the chronic SCI mice. The IFN-\(\gamma\) and TNF produced by influenza virus-specific cytotoxic T-cells have an antiviral effect, which includes mediating the lysis and apoptosis of influenza-infected cell(Kuwano, Kawashima et al. 1993). IFN-\(\gamma\) also participates in other immune functions, such as macrophage activation, MHC I and MHC II antigen presentation upregulation, antibody class switching and viral replication inhibition. Thus, insufficient cytokine producing CD8+ T-cells in chronic SCI mice following influenza infection may lead to defects in the killing of infected cells and reduced IgG antibody response. Taken together, chronic SCI mice have impaired host adaptive immunity against influenza virus, which increases the mortality and morbidity following infection.
4.2 Impaired T-cell effector function by chronic SCI contributes to immunodepression

The cytokine production by T-cell plays an important role in host immunity against infection (Slifka and Whitton 2000). Chronically injured mice show deficiency in the cytokine production by the splenic T-cells upon \textit{ex vivo} restimulation, as shown by a reduction in the number of IFN-\(\gamma\) producing CD4\(^+\) T-cells and TNF producing CD8\(^+\) T-cells.

IFN-\(\gamma\) is an important cytokine in the immunity. It was discovered in 1965 as a virus inhibitory protein (Wheelock and Sibley 1965, Zhang, Boisson-Dupuis et al. 2008). Deficits in IFN-\(\gamma\) production may directly lead to increased viral replication and impaired viral clearance after infection. Moreover, IFN-\(\gamma\) is the signature cytokine of Th1 cells. Reduced number of IFN-\(\gamma\) producing CD4\(^+\) T-cells suggest that the number of Th1 cells is decreased during the chronic phase of SCI. Th1 cells are derived from naïve CD4\(^+\) T-cell and differentiation is initiated and directed by IL-12 (Robinson and O'Garra 2002). Th1 cell participates in cell-mediated immunity. Th1 cell secreted IFN-\(\gamma\) when getting activated by recognizing the MHCII associated antigen peptide. IFN-\(\gamma\) promotes macrophage activation, as shown by production of microbicidal substances and secretion of pro-inflammatory cytokines. IFN-\(\gamma\) also increases the expression of MHC molecules on macrophage, in turn augmenting the T-cell response (Abbas, Lichtman et al. 2014).

Reduced number of CD4\(^+\) Th1 cells during chronic SCI causes inefficient activation and function of macrophage following infection, leading to SCI-induced higher mortality and morbidity of infection. Moreover, the activation of Th1 cells is also essential in mounting an optimal cytotoxic T-cell response. Th1 cell activation is required for the generation
and expansion of cytotoxic T-cells (Keene and Forman 1982, Cassell and Forman 1988, Buhlmann, Gonzalez et al. 1999). Activated Th1 cells increase the expression of 4-1BBL on APC, which triggers the co-stimulatory signaling of CD8+ T-cells by interacting with the 4-1BB molecule on the CD8+ T-cell (Ridge, Di Rosa et al. 1998, Diehl, van Mierlo et al. 2002). When chronic SCI mice lose the Th1 cells in the spleen, the cytotoxic T-cell response will also be impaired. It may explain the defects in influenza-specific CD8+ T-cell response that is observed in our chronic SCI model. IFN-γ producing CD4+ T-cell is also involved in the antibody response. Tfh cell is the T-cell player in the germinal center that provides help to antibody-producing B-cells. It has been demonstrated that a population of the CD4+CXCR5+ Tfh cells from human tonsils produces IFN-γ upon ex vivo stimulation (Schaerli, Willimann et al. 2000). Excessive IFN-γ signaling in T-cells results in Tfh cell accumulation in germinal center and autoantibody response of lupus development in mice (Lee, Silva et al. 2012). IFN-γ also induces antibody class switching to IgG2a, which is the predominant antibody isotype generated during the antiviral response in mice (Coutelier, van der Logt et al. 1987, Snapper and Paul 1987). Taken together, the downregulation of IFN-γ production in CD4+ T-cell in our chronic SCI mice may cause functional defects of Tfh cells in the germinal center and insufficient production of IgG2a antibody, leading to impaired antibody response during chronic phase of SCI, as shown by our influenza infection study and the results from other groups (Ibarra, Jimenez et al. 2007, Lucin, Sanders et al. 2007, Oropallo, Held et al. 2012).

We also show that the TNF production of CD8+ T-cells is reduced during the chronic phase of SCI. TNF plays a key role in both host inflammatory response and
cytotoxic response against antigen. TNF have multiple biological functions during infection, including increasing the permeability of vascular endothelium, regulating coagulation, inducing cell apoptosis, activating macrophage and promoting the maturation of dendritic cells, etc (Waters, Pober et al. 2013). During viral infection, TNF can inhibit viral replication directly (Mestan, Digel et al. 1986, Wong and Goeddel 1986) and the TNF secreted by cytotoxic T-cell can induce influenza-infected cell death (Kuwano, Kawashima et al. 1993). Thus, less TNF producing CD8+ T-cells in chronic SCI mice may disrupt the defense against pathogen infection and result in a higher death rate. Interestingly, antibody responses are impaired in both TNF-deficient mice and human patients receiving anti-TNF treatment (Pasparakis, Alexopoulou et al. 1996, Marino, Dunn et al. 1997, Gelinck, van der Bijl et al. 2008). It is possible that the disruption of TNF production of CD8+ T-cells in our chronic SCI mice participates in SCI-induced impairment of antibody response.

4.3 Up-regulated expression of T-cell exhaustion marker PD-1 by chronic SCI causes CD8+ T-cell functional defects

T-cell exhaustion has been well studied in the last decade for its role in T-cell dysfunction and immunodeficiency. T-cell exhaustion was first discovered in a lymphocytic choriomeningitis virus (LCMV) chronic infection mouse model. It was described as a virus-specific CD8+ T-cell population which cannot elaborate efficient antiviral effector function (Zajac, Blattman et al. 1998). Since then, T-cell exhaustion has been investigated in many chronic viral infections including HIV, HBV and HCV, cancer as well as aging models (Trautmann, Janbazian et al. 2006, Radziewicz, Ibegbu et al. 2007, Streeck, Brumme et al. 2008, Channappanavar, Twardy et al. 2009, Nakamoto,
Cho et al. 2009, Lages, Lewkowich et al. 2010, Topalian, Drake et al. 2012, Tzeng, Tsai et al. 2012). To our knowledge, our study is the first to show T-cell exhaustion in a SCI model. Previous studies have demonstrated a correlation between higher PD-1 expression and reduced cytokine production in CD8$^+$ T-cells (Barber, Wherry et al. 2006, Trautmann, Janbazian et al. 2006, Penna, Pilli et al. 2007). Herein we showed that our chronic SCI mice have a significantly higher number of CD8$^+$ T-cells expressing PD-1. Moreover, the production of TNF by CD8$^+$ T-cells was restored \textit{in vitro} by blocking PD-1 signaling using an antibody to PD-1. These results suggest that higher PD-1 expression contributes to SCI-induced CD8$^+$ T-cell dysfunction. However, the number of CD4$^+$ T-cells expressing PD-1 was not significantly changed by chronic SCI and blocking PD-1 failed to restore IFN-$\gamma$ production by CD4$^+$ T-cells suggesting that other mechanisms are involved in SCI-induced CD4$^+$ T-cell dysfunction. For example, IL-12 produced by APC is the cytokine that drives IFN-$\gamma$ production in CD4$^+$ T-cell(Robinson and O'Garra 2002). Our chronic SCI mice show reduced level of IL-12 in the serum (data not shown), which may result in the reduced number of IFN-$\gamma$ producing CD4$^+$ T-cells.

4.4 Increased sympathetic activity in chronic SCI mice correlates with up-regulated PD-1 expression on T-cell

To understand the link between chronic SCI and increasing PD-1 expression on CD8+ T-cells, we first investigated the activity of SNS in the spleen following chronic SCI. The spleen is innervated and modulated by the sympathetic nerve terminals that originate from preganglionic sympathetic neurons at levels T3 to T12, traumatic injury on the cord at T9 level damages the cell bodies of preganglionic sympathetic neurons in the IML zone, eventually causing partially disruption of sympathetic innervation in the spleen. It was shown that chronic SCI causes reorganization of synaptic input to the preganglionic sympathetic neurons within the cord and reinnervation of the sympathetic terminals within the target organs (Weaver, Cassam et al. 1997, Llewellyn-Smith and Weaver 2001, Lujan, Palani et al.). We analyzed the sympathetic nervous system in the spleen and showed higher levels of tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis, in the splenic protein extract from chronically injured animals. As the rate-limiting enzyme for catecholamine synthesis, TH has been used to identify catecholamine containing noradrenergic terminals in the spleen (Felten and Olschowka 1987, Madden, Bellinger et al. 1997). Consistent with the elevated TH levels in the spleen, splenic NE levels are also upregulated in chronic SCI mice. These data could be explained by increased sympathetic innervation, higher catecholamine levels per cell or more endogenous catecholamine produced by lymphocytes in the spleen (Qiu, Peng et al. 2004, Laukova, Vargovic et al. 2013). NE has been reported to regulate the function of immune cells (Nance and Sanders 2007). Thus, we hypothesized that higher NE levels in the spleen following chronic SCI could contribute to increased PD-1 expression on T-cells.
expression and T-cell exhaustion. Consistent with this hypothesis, we found that the expression of PD-1 on T-cells was increased after prolonged exposure to NE in vitro. Interestingly, Dr. Popovich’s group have shown that splenic NE accumulation induced by experimental induction of autonomic dysreflexia in chronic SCI mice causes impaired immune function such as defects in lymphocyte proliferation (Zhang, Guan et al. 2013). These results could be explained by NE-induced immune cell dysfunction.

4.5 Potential mechanisms by which norepinephrine up-regulating PD-1 expression

While it is unclear how NE regulates PD-1 expression, several transcription factor pathways have been demonstrated to play a role in regulating T-cell exhaustion. Specifically, B lymphocyte-induced maturation protein 1 (Blimp-1), nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) and Notch signaling are regulators of PD-1 gene expression, whereas high expression of T-bet suppresses PD-1 expression (Oestreich, Yoon et al. 2008, Shin, Blackburn et al. 2009, Kao, Oestreich et al. 2011, Mathieu, Cotta-Grand et al. 2013). There is no direct evidence that NE regulates these transcription pathways in murine splenic T-cells. However, NE was reported to increase NFATc1 activity in primary neonatal cardiomyocyte culture (Lunde, Kvaloy et al. 2011), suggesting that NE stimulation may up-regulate PD-1 expression by activating NFATc1. Interestingly, NE stimulation of T-lineage cells increases the phosphorylation of phosphor 38 mitogen-activated protein kinase (p38 MAPK), which is involved in the mechanisms by which HIV-1 Nef protein induces PD-1 expression (Muthumani, Choo et al. 2008, Lajevic, Suleiman et al. 2011). The activation of the p38 MAPK pathway may also contribute to the higher PD-1 expression in our model.
4.6 Blockade of PD-1 and norepinephrine signaling as therapeutic strategies to treat SCI-induced immunodepression

Our study demonstrate that chronic SCI-induced PD-1 upregulation on CD8+ T-cells results in T-cell dysfunction, leading to immunodepression. Blockade of PD-1 in vitro restore the CD8+ T-cell function as measured by cytokine production. These results suggest that administration of antagonist antibody to PD-1 is a potential treatment for SCI-induced immunodepression. There are three monoclonal antibodies to PD-1 under investigation in clinical trials for cancer treatment: Nivolumab (MDX-1106, BMS-936558), lambrolizumab (MK-3475) and Pidilizumab (CT-011). Each of these is well tolerated and safe, with a correlation to tumor regression in patients with different type of cancers, including non-small-cell lung cancer, melanoma, renal-cell cancer, advanced melanoma and advanced hematologic malignancies (Berger, Rotem-Yehudar et al. 2008, Topalian, Hodi et al. 2012, Hamid, Robert et al. 2013). Our research provides evidence for the clinical application of these PD-1 antibodies in the treatment of immunodepression in patients with chronic SCI.

Since elevated splenic sympathetic activity following chronic SCI is associated with the higher PD-1 expression on T-cells, blocking the effect of norepinephrine on immune cells is also an optional therapeutic strategy for SCI-induced immunodepression. As described in the introduction, β2AR is the major subtype of adrenergic receptor expressed on T-cells. It is highly possible that β2AR mediates the upregulation of PD-1 expression by norepinephrine. Nonselective antagonist to βAR (e.g. Propranolol) or selective antagonist to β2AR (e.g. Butoxamine) may inhibit the PD-1 upregulation on T-cell and prevent T-cell dysfunction. β1AR is widely expressed in cardiac muscle and the
activation of β1AR increases the contractility of cardiac muscle and the cardiac output. Thus, nonselective βAR antagonist is used in the clinical treatment of angina pectoris, hypertension, arrhythmia and heart failure (Wachter and Gilbert 2012). Butoxamine is the selective antagonist to β2AR and was used in the laboratory experiments to study the effect of β2AR activation and blockade. Although blocking β2AR may downregulate PD-1 expression on T-cells and restore T-cell function in patients with chronic SCI, we need to remain cautious on their adverse effect on the cardiovascular system. Particularly, β2AR participates in the smooth muscle relaxation. Blocking β2AR in the respiratory tract may result in smooth muscle constriction and asthma attack. Instead of systemic drug administration, the site-specific release of β2AR blocker in the spleen or lymph nodes is a potential therapeutic strategy for chronic SCI-induced immunodepression.

4.7 Model of chronic SCI-induced immunodepression

According to the results in this dissertation, we propose a model in which chronic thoracic SCI induce immunodepression by up-regulation of PD-1 expression on T-cells (Fig 4.1). Traumatic injury to the spinal cord at the level T9 leads to increased activity of the sympathetic nervous system within spleen. When exposed to elevated levels of norepinephrine, the T-cells in the spleen express higher PD-1 on the cell surface. Up-regulation of PD-1 signaling interferes with the downstream signaling of TCR activation and inhibits the cytokine production of T-cells. Impairment of T-cell cytokine production eventually leads to immunodepression during the chronic phase following SCI.
Figure 4.1 Chronic thoracic SCI induces T-cell dysfunction by up-regulating PD-1 expression.

The spleen is innervated by sympathetic nerves originated from T3 to T12 level in the spinal cord. SCI at T9 level results in increasing norepinephrine level in the spleen. Elevated norepinephrine level leads to up-regulation of PD-1 expression, which in turn inhibits the downstream signaling of TCR activation and impaired cytokine production.
Chapter 5: Summary and future directions

We investigated the chronic effect of thoracic SCI on peripheral immunity in mice. The mice with chronic SCI show deficits in T-cell function, as measured by cytokine production. We demonstrated that chronic SCI alters the activity of the sympathetic nervous system in the spleen, leading to elevated norepinephrine levels in the spleen. Exposure to norepinephrine up-regulates the expression of exhaustion marker PD-1 on T-cells, which contributes to T-cell dysfunction. We also showed that chronic SCI impairs the ability of the injured mice to mount an antiviral immune response against H3N2 influenza-A virus. Both virus-specific antibody response and CD8⁺ T-cell response in injured mice are compromised, leading to their higher mortality and reduced viral clearance following infection. Since T-cell is important in both antibody immune response and CD8⁺ T-cell immune response, the T-cell dysfunction prior to infection as shown in our study may contribute to the impaired adaptive immunity following influenza infection in the chronically injured mice. These results highlight the important role of spinal cord and neurotransmitters in regulation of immune cell function and provide insights on developing therapeutic strategies to restore immunity in patients with chronic SCI.

Our study raises a number of questions which can be studied more in the future: 1) Does the T-cell exhaustion occur following influenza infection in the chronic SCI mice? It can be addressed by measuring PD-1 expression on the virus-specific T-cells following influenza infection in our chronic SCI mice. 2) Can PD-1 antibody be used in vivo in our SCI mouse model to rescue the SCI-induced immunodepression? We showed that blocking PD-1 signaling by an antagonist antibody to PD-1 restores the T-cell
function *in vitro*. To further investigate the potential of using a PD-1 antibody to treat SCI-induced immunodepression, we can examine whether the chronic SCI-induced defects in immune response to influenza virus can be rescued by administration of PD-1 antibody in the mice. 3) How does chronic SCI up-regulate the activity of the sympathetic nervous system? We hypothesize that the sympathetic innervation in the spleen is increased during the chronic phase of SCI. Stereological analysis of sympathetic terminals in the spleen from uninjured and chronic SCI mice is needed. 3) What are the molecular mechanisms of norepinephrine-induced PD-1 up-regulation on T-cells? As we discussed in Chapter 4, multiple transcriptional pathways including NFATc1 and Notch may mediate norepinephrine-induced upregulation of PD-1 expression on T-cells. This hypothesis can be tested by quantifying the mRNA expression, the protein expression and the phosphorylation state of the molecule members in these pathways. Blocking these pathways by pharmaceutical antagonists is also a potential therapeutic strategy to treat the SCI-induced immunodepression.
Reference

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Appendix

We measured whether the T-cell number and T-cell function is affected by laminectomy only at T9 level during the chronic phase of surgery. No change was found in the spleen weight, splenocyte number or T-cell number. The cell numbers of CD4+ T-cell and CD8+ T-cell are similar between uninjured mice and mice with laminectomy (Fig A.1). Furthermore we measured the cytokine production of T-cells after *ex vivo* restimulation in the mice with laminectomy. Thoracic laminectomy does not impair the production of IFN-γ and TNF in T-cells (Fig A.2). The original purpose of using the laminectomy group was to test whether the T-cell dysfunction we found in the SCI mice was due to the surgery procedure and the injury of the vertebral bone. However, the mice in the laminectomy group were more active and nervous all the time during the experiments. The stress and extreme activity in the mice from the laminectomy group brings in another factor that could affect the peripheral immunity and metabolism. So it will be problematic if we compare the laminectomy group and SCI group and to state that the difference between these two groups is solely due to the traumatic injury to the cord. Moreover, comparison of the results between the uninjured group and the SCI group will be more clinical relevant, as there are no patients with laminectomy alone. Thus we did not include the laminectomy group in the data of Chapter 2 and Chapter 3.
Figure A.1  The number of splenocytes and splenic T-cells are not changed during chronic laminectomy.

(A) Bar graph represents the mean ± SEM spleen weights of uninjured mice and T9-laminectomy mice at 5-7 weeks after surgery (Lam). (B) Bar graph represents the mean ± SEM of total splenocyte numbers for uninjured and Lam mice. (C) Bar graph represents the mean ± SEM number of splenic T-cells (CD45^+CD3^+) in uninjured and Lam mice. (D) Bar graph show the mean ± SEM numbers of splenic CD4^+ T-cells (CD4^+CD8^-) and CD8^+ T-cells (CD4^+CD8^-) in uninjured and Lam mice. n = 3 for uninjured mice, n = 4 for uninjured mice. p>0.05, two-tailed Student’s t-test.

Figure A.2  Laminectomy does not impair the cytokine production of T-cells

Isolated splenocytes (1 x 10^6) from uninjured or T9-laminectomy mice at 5-7 weeks after surgery (Lam) were stimulated ex vivo with PMA/ionomycin in the presence of brefeldin A for 4 hours, then processed for flow cytometry analysis. (A) Bar graph represents the mean ± SEM percentages and the numbers of cytokine producing CD4^+ T-cells. (B) Bar graph represents the mean ± SEM percentages and the numbers of cytokine producing CD8^+ T-cells. n = 3 for Uninjured, n = 5 for Lam. p>0.05, two-tailed Student’s t-test.