



Research Paper

Chronic spinal cord injury impairs primary CD8 T cell antiviral immunity but does not affect generation or function of memory CD8 T cells



Diana M. Norden, Anas Qatanani, John R. Bethea*, Jiu Jiang*

Biology Department, Drexel University, Philadelphia, PA 19104, United States of America

ARTICLE INFO

Keywords:

Spinal cord injury
Infection
Influenza virus
CD8 T cells

ABSTRACT

Antiviral immunity is severely compromised following trauma to the central nervous system. In mice with chronic spinal cord injury (SCI), primary infection with influenza virus leads to high mortality rates due to impaired expansion of virus-specific CD8 T cells. One strategy to increase resistance to viral infections is to generate memory immune cells that protect from recurrent infections. However, it is unknown if chronic SCI also impairs secondary immune responses to influenza challenge as it does primary responses. Here, we used a mouse model of chronic SCI and a clinically relevant influenza A infection to investigate CD8 T cell response. As shown previously, chronic SCI mice had impaired primary antiviral responses with high mortality rates and decreased expansion of virus-specific CD8 T cells following intranasal infection. To investigate CD8 T cell memory, we used two strains of influenza A virus [PR8(H1N1) and X31(H3N2)] that share internal proteins but differ in surface antigens. Chronic SCI mice immunized with live X31 were able to generate memory CD8 T cells that secreted IFN γ upon stimulation with viral peptides *ex vivo*, which was comparable to immunized uninjured mice. Importantly, immunization prior to challenge with a lethal dose of PR8 resulted in no mortality and significant CD8 T cell recall responses in both uninjured and chronic SCI mice. In addition, memory CD8 T cells generated before SCI remained functional up to 8 weeks after injury. These pre-existing memory CD8 T cells provided full protection from lethal PR8 challenge given at the chronic timepoint following injury. Overall, this study shows that memory CD8 T cells generated either before or after chronic SCI still remain functional. These results highlight the need for proper immunization of SCI patients and show the potential of memory T cells to confer protection against not only influenza, but other viral infections as well.

1. Introduction

Yearly outbreaks of influenza A virus (IAV) are a continuous threat for immune compromised individuals, including individuals suffering from neurological disorders. Trauma to the central nervous system (CNS), including the spinal cord, leads to compromised neuro-immune communication and impaired immune responses to infections like influenza. Impaired immune function and its associated increased susceptibility to infections after CNS injury is known as “CNS Injury-Induced Immuno-depression” (CIDS) (Meisel et al., 2005). CIDS is a major concern after spinal cord injury (SCI). For instance, patients living with chronic SCI are at greater risk of serious life-threatening complications from IAV. Moreover, respiratory infections are the leading cause of re-hospitalization and mortality in SCI patients (Cardenas et al., 2004; Smith et al., 2007; Soden et al., 2000). Therefore, yearly outbreaks of IAV is of particular concern for SCI patients living with chronic injury throughout their life.

IAV, also known as the flu virus, is a major respiratory pathogen that is highly contagious and causes widespread outbreaks and disease. In immunocompetent individuals, IAV infection is cleared by humoral and cellular immune responses. B cells make antibodies that neutralize surface antigens to prevent infection and spreading of the virus between infected cells (Subbarao and Joseph, 2007). IAV expresses surface proteins hemagglutinin (HA) and neuraminidase (NA) of various subtypes. These surface proteins undergo frequent mutations and reassortments, therefore immunizations that promote induction of neutralizing antibodies do not provide optimal protection from subsequent infections (Schmidt and Varga, 2018). Cytotoxic CD8 T cells recognize internal peptides that are presented in MHC complexes by infected cells (Subbarao and Joseph, 2007). This antigen-specific CD8 T cell response is critical in mediating viral clearance after infection (Schmidt and Varga, 2018). Internal peptides nucleoprotein (NP) and polymerase (PA) recognized by CD8 T cells are highly conserved between IAV strains (Altenburg et al., 2015). Therefore, primary CD8 T cell

* Corresponding authors at: Department of Biology, Drexel University, 3245 Chestnut Street, Rm 415, Philadelphia, PA 19104, United States of America.
E-mail addresses: jrb445@drexel.edu (J.R. Bethea), jj73@drexel.edu (J. Jiang).

responses to IAV leads to formation of long-lived memory CD8 T cells that provide protection from not only reinfection but also subsequent infections from heterologous strains. SCI patients live for years following their injury and continue to be at risk of recurrent infections, thus it is essential to provide optimal immunization strategies to protect this vulnerable population from IAV.

Using mouse models, we recently reported deficits in CD8 T cell responses to viral infection in mice with both acute and chronic SCI (Bracchi-Ricard et al., 2016; Norden et al., 2018). Following infection with influenza virus X31, chronically injured mice had impaired viral clearance and increased mortality compared to uninjured controls. Importantly, the increased mortality was associated with impaired generation and effector function of virus specific CD8 T cells (Bracchi-Ricard et al., 2016). While these data show impaired primary CD8 T cell responses to influenza infection, it is unknown if secondary memory responses are impaired as well.

The goal of this study was to investigate the effect of chronic SCI on the CD8 T cell memory response to virus infection. We used a model of chronic SCI to mimic chronic immune depression as it would occur in patients and two strains of influenza A virus [PR8(H1N1) and X31(H3N2)] that share internal proteins but differ in surface antigens. In our previous report, we showed that immunization with inactivated X31 protected against homologous challenge but was not sufficient to induce protection from heterologous challenge with PR8. Vaccination with inactivated virus is designed to generate protection through neutralizing antibodies (Kirchenbaum and Ross, 2014), therefore, immunization with inactivated X31 was unlikely to induce protection from PR8 as the two viruses express different surface antigens. Here, we used an immunization paradigm where mice were immunized with a low dose of live X31 and then challenged with PR8 to investigate immunization strategies and the function of memory CD8 T cells in detail. This strategy allows for viral infection and intracellular processing which induces an active CD8 T cell response. Our findings indicate that memory CD8 T cell recall responses are not impaired following SCI. Immunization with live virus either before or after SCI provided complete protection from lethal challenge with a heterologous strain, indicating that CD8 memory T cell function remains intact following SCI. This study has broad clinical implications for SCI patients that are at risk of complications from not only influenza, but also other viral or bacterial infections.

2. Materials and methods

2.1. Mice and spinal cord injury

Experiments were conducted in accordance with Drexel University Institutional Laboratory Animal Care and Use Committee (IACUC). Adult (10 weeks old) female C57BL/6J wild-type (WT) mice were obtained from Jackson Laboratories. Contusion of the spinal cord was performed as previously described (Bracchi-Ricard et al., 2016). In brief, mice were anesthetized with a ketamine (100 mg/kg)-xylazine (10 mg/kg) cocktail. Using aseptic techniques, mice were subjected to laminectomy and subsequent contusion injury at thoracic level T9 using the Infinite Horizon Impactor (Precision Systems and Instrumentation) at a predetermined force of 70 kDynes. Mice were sutured and injected subcutaneously with 2-mL Lactated Ringer's solution to prevent dehydration and gentamicin (5 mg/kg) to prevent urinary tract infection. During recovery, mice received antibiotics and saline for 5 days and bladders were manually expressed twice per day (Hoschouer et al., 2010).

2.2. Influenza virus infection, immunization, and challenge

To mimic clinically relevant respiratory infections in SCI patients, nasal application of influenza virus was used. Influenza virus subtype A/HKx31 (HKx31, H3N2) (Charles River) and A-Puerto Rico/8/34

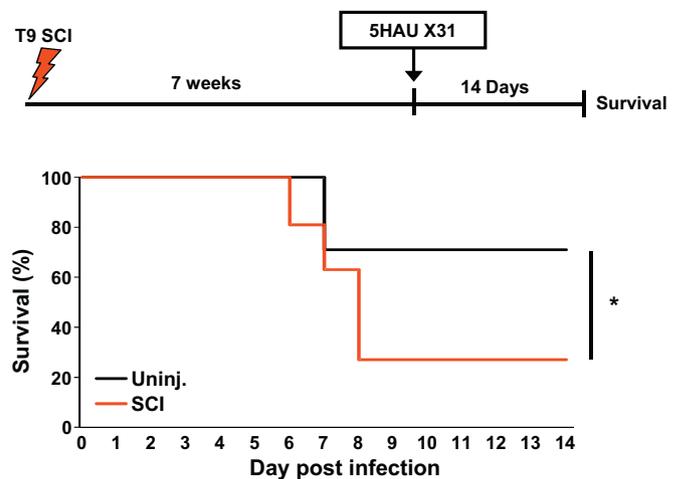


Fig. 1. Decreased survival of chronic SCI following primary infection. Uninjured naïve (Uninj.) or SCI mice (7 weeks after injury) were infected i.n. with 5 HAU X31. Body weight loss and mortality were monitored for 14 days. Data represent 12 mice per group. * $p < 0.04$.

(PR8, H1N1) were used for these studies. PR8 expresses the surface protein hemagglutinin (HA) and neuraminidase (NA) of H1N1 subtype, whereas the reassorted X31 virus expresses the H3N2 surface protein of A/Hong Kong/1/1968 on the background of PR8 (Bouvier and Lowen, 2010; Kilbourne, 1969). PR8 and X31 have six identical internal proteins, including nucleoprotein (NP) and polymerase acidic protein (PA), which can be specifically recognized by CD8 T cells (Bouvier and Lowen, 2010; Kilbourne, 1969). For primary infection studies, chronically injured (7 weeks post SCI) mice were anesthetized as described above and inoculated intranasally (i.n.) with 5 hemagglutination unit (HAU) X31 in 20 μ L sterile saline. For immunizations with X31, mice were inoculated i.n. with 0.25 HAU X31. This dose was chosen because uninjured mice lost minimal weight while still mounting an efficient CD8 T cell response. For challenges, immunized injured and uninjured and non-immunized naïve mice were inoculated i.n. with 1 HAU PR8. This dose was chosen because it resulted in 100% mortality in non-immunized mice. Mice were monitored daily up to 2 weeks for weight loss and euthanized if their body weight (BW) loss exceeded 25%.

2.3. Cell preparation

Mice were sacrificed by CO₂ asphyxiation followed by cervical dislocation and spleen and lungs were aseptically removed. Spleens were homogenized using the plunger of a 3 mL syringe (Becton Dickinson) through a 100 μ m strainer and washed with RPMI-1640 medium (GenDEPOT). Lungs were minced using sterile scissors and digested in 5 mL RPMI containing 3 mg/mL Collagenase A (Sigma) and 0.15 mg/mL DNase I (Sigma) at 37 °C for 1 h. Digested lungs were homogenized using the plunger of a 3 mL syringe (Becton Dickinson) and filtered through a 40 μ m strainer (EZFlow Cell Strainer, Foxx Life Sciences). Red blood cells were lysed using 0.83% ammonium chloride. Cells were washed and resuspended with complete RPMI (RPMI 1640, 5% FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin). The number of live cells was determined by trypan blue (0.4%) exclusion staining and absolute number of cell populations was calculated based on total number of cells after FACS.

2.4. Ex vivo treatments and peptide stimulation

Following virus infection, splenocytes and leukocytes from lungs were isolated and counted as above. Cells (10^6 cells) were cultured with influenza viral peptides D^b-PA_{224–233} or D^b-NP_{366–374} (0.001 mg/mL) (AnaSpec) in the presence of IL-2 (50 U/mL) and GolgiStop for 4 h. Cells

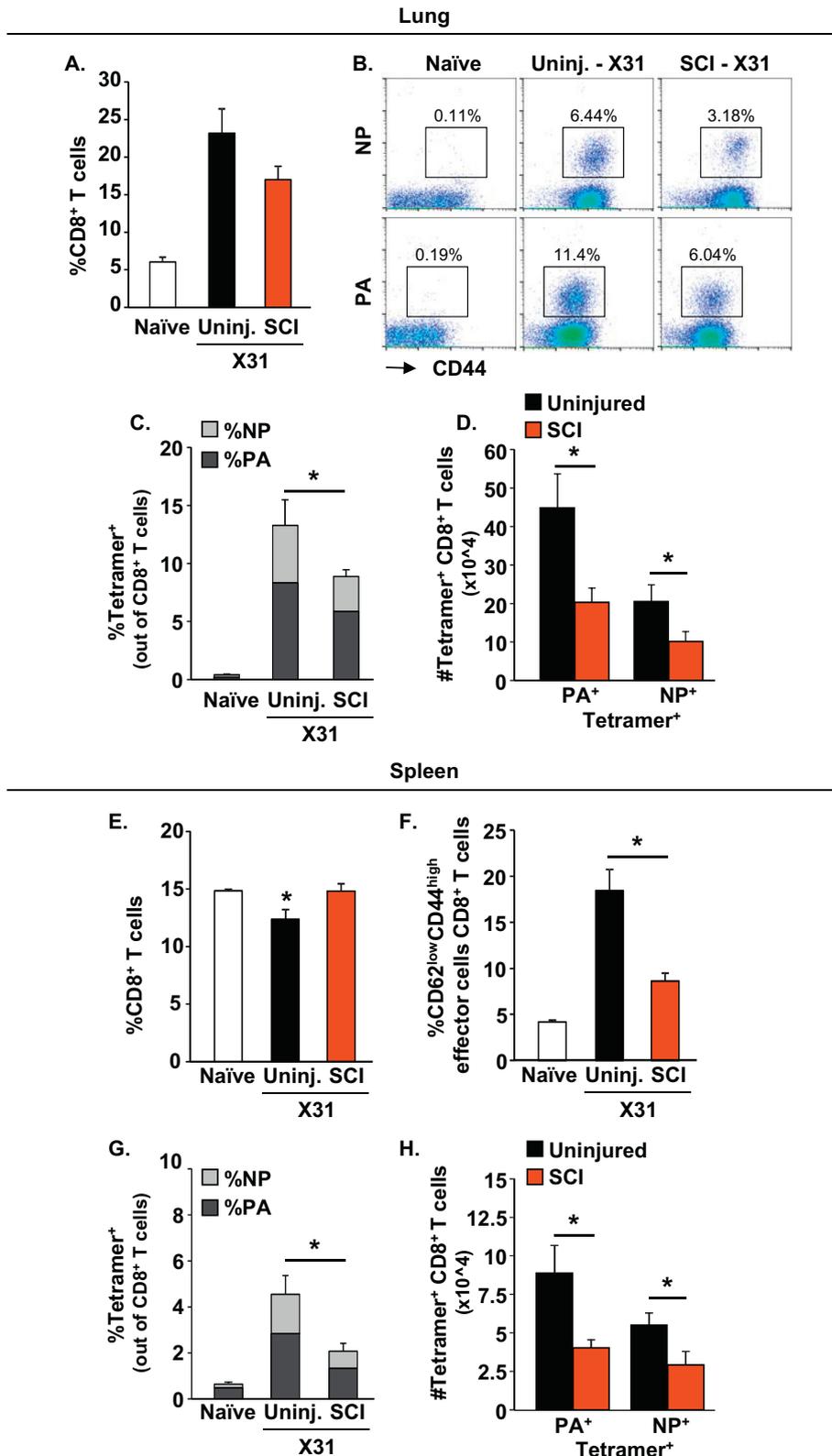


Fig. 2. Impaired primary CD8 T cell response to flu infection in chronic SCI mice. Uninjured naïve (Uninj.) or chronic SCI mice were infected i.n. with 5 HAU X31. Lungs were collected 7 days after infection and the percentage of CD8 T cells was determined (A). (B) Representative dot plots of NP and PA tetramer staining. Cells were gated for CD8⁺ T cells. The percentage (C) and absolute number (D) of NP and PA tetramer positive CD8 T cells in the lung were determined. In the spleen, percentage of total CD8 T cells (E) and proportion of effector CD8 T cells (F) was determined. The percentage (G) and absolute number (H) of NP and PA tetramer positive CD8 T cells in the spleen were determined. Data represent 5 mice per group. **p* < 0.05.

were then stained for surface markers and intracellular cytokines.

2.5. Flow cytometry

Cellular responses and the magnitude of the virus-specific CD8 T cell responses were analyzed by flow cytometry. Cell suspension aliquots were washed with staining buffer (1 × PBS, 1% BSA) and stained for the

following surface markers: CD8, CD4, B220, NK1.1, CD11b, CD11c, Ly6G, Ly6C, MHCII, CD44, and CD62L from BD Pharmingen or eBioscience. Virus-specific CD8 T cells were quantified using tetramers. H- 2D^b-PA_{224–233} and H- 2D^b-NP_{366–374} tetramers were obtained from NIAID MHC Tetramer Core Facility (Atlanta, GA). For surface antibodies and tetramer labeling, cells were incubated for 30 min at 4 °C and then washed and fixed using 1% paraformaldehyde. Intracellular

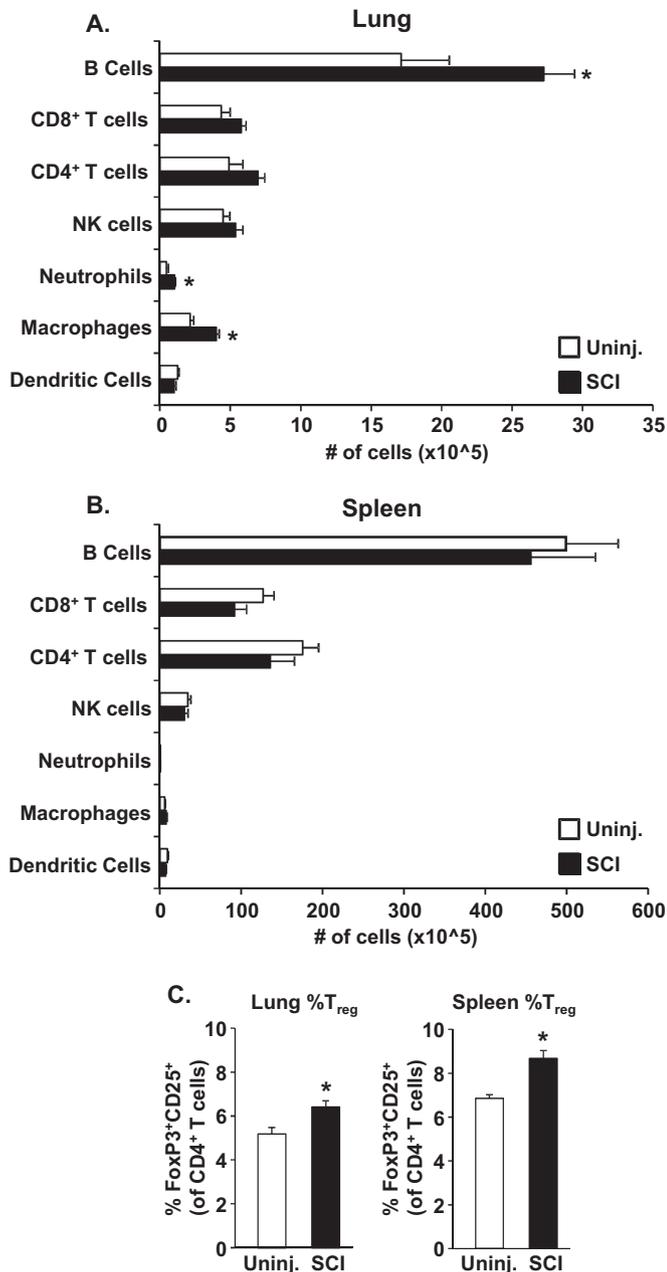


Fig. 3. Altered profile of immune cells in lung and spleen of chronic SCI mice. Mice were naïve (uninj.) or received SCI and 7 weeks later lungs and spleen were collected for immune cell analysis. (A) Lymphocyte counts in lung. (B) Lymphocyte counts in spleen. Foxp3 expression in CD4⁺ T cells was determined in (C) lung and (D) spleen. Data represent 6 mice per group. **p* < 0.05.

staining for IFN γ was performed using the Cytotfix/Cytoperm kit (BD Pharmingen). Foxp3 intracellular staining was performed using the mouse Foxp3 buffer set (BD Pharmingen) according to the manufacturer's protocol. Cells were analyzed using a FACSCanto (Becton Dickinson) and data was analyzed using FlowJo software v10 (FlowJo, LLC). The results were analyzed by an observer who was blinded to the experimental groups.

2.6. Statistics

Group comparisons were analyzed using one-way ANOVA with Tukey's *post hoc* testing or Student's unpaired *t*-test when appropriate (IBM SPSS). Comparison of survival curves was performed using the

log-rank test. Means and standard error of the mean (SEM) are reported throughout. Significance is set at *p* < 0.05.

3. Results

3.1. Decreased survival of chronic SCI mice following primary infection

To assess the primary response to influenza infection, mice were challenged i.n. with a high dose of influenza virus (X31) 7 weeks after T9 SCI and monitored for body weight loss and survival (mortality at < 25% BW loss). Here, the day of infection was used as the baseline for determining weight loss. It is important to note that SCI mice did not have reduced weight 7 weeks after injury compared to the uninjured mice. Following infection, injured mice had overall increased mortality (*p* < 0.04) (Fig. 1), consistent with our previous study (Bracchi-Ricard et al., 2016). Mortality was observed in 29% of uninjured mice, while 73% of injured mice succumbed to this high dose primary infection.

The cellular response was analyzed in a subset of the mice chosen at random at the peak of infection (day 7) to assess impaired functions that contribute to the increased mortality. We have previously shown decreased CD8 T cell activation and generation of specific CD8 T cells in the spleen following viral infection in SCI mice (Bracchi-Ricard et al., 2016). Here, we determined infiltration of CD8 T cells into the lungs which is the target tissue of viral replication following intranasal challenge. In both uninjured and injured mice, there was robust CD8 T cell recruitment to the lungs, and there was a trend for decreased recruitment in the injured mice (*p* = 0.056) (Fig. 2A). Importantly, SCI mice had attenuated generation of virus-specific NP⁺, PA⁺ CD8 T cells in the lungs (*p* < 0.05) (Fig. 2B,C). Moreover, the absolute number of NP⁺- and PA⁺- CD8 T cells was drastically decreased in injured mice compared to uninjured mice (NP: *p* < 0.03, PA: *p* < 0.02; Fig. 2D). Overall, these data show an impaired primary CD8 T cell response with diminished accumulation of virus-specific CD8 T cells in the lung of SCI mice following influenza infection.

We also analyzed the CD8 T cell response in the spleen, as CD8 T cells can be primed in lymphoid tissues, including the spleen (Turner et al., 2013), before potentially infiltrating the lung. There was a decrease in the percent of total CD8 T cells in the spleen of uninjured mice (*p* < 0.05), but no change in SCI mice (Fig. 2E). After infection, uninjured mice had a large expansion of effector CD8 T cells (CD62^{low}/CD44^{high}) while these cells were significantly decreased in injured mice (*p* < 0.01) (Fig. 2F). Similar to the lung, the generation of specific CD8 T cells was attenuated in the spleen of injured mice. Both the percentage (*p* < 0.01) and absolute number (*p* < 0.03) of specific CD8 T cells in the spleen were decreased in SCI mice compared to uninjured mice (Fig. 2G–H). Overall, these data show that generation of specific effector CD8 T cells is impaired in the spleen after chronic SCI.

3.2. Altered profile of immune cells in lung and spleen of chronic SCI mice

SCI induces massive inflammation locally and systemically (Sun et al., 2016) acutely after injury. While much of the inflammation subsides through time, some of these inflammatory changes remain, leading to chronic inflammation following injury (Brennan and Popovich, 2018). To evaluate possible SCI-induced changes in the immune system that could impair the response to virus, lung lymphocytes and splenocytes were profiled 7 weeks after injury (before influenza challenge). As shown in Fig. 3A, there was no change in the number of CD8 T cells, CD4 T cells, or NK cells in the lung following SCI. However, significantly increased accumulation of B cells (*p* < 0.04), neutrophils (*p* < 0.01), and macrophages (*p* < 0.01) was observed in the lung of SCI mice.

Previous studies have reported significant splenic atrophy following high-thoracic SCI (Lucin et al., 2007; Pruss et al., 2017; Zhang et al., 2013). Here, there was no difference in immune cell numbers in the spleen following chronic T9 SCI (Fig. 3B). Although there was no

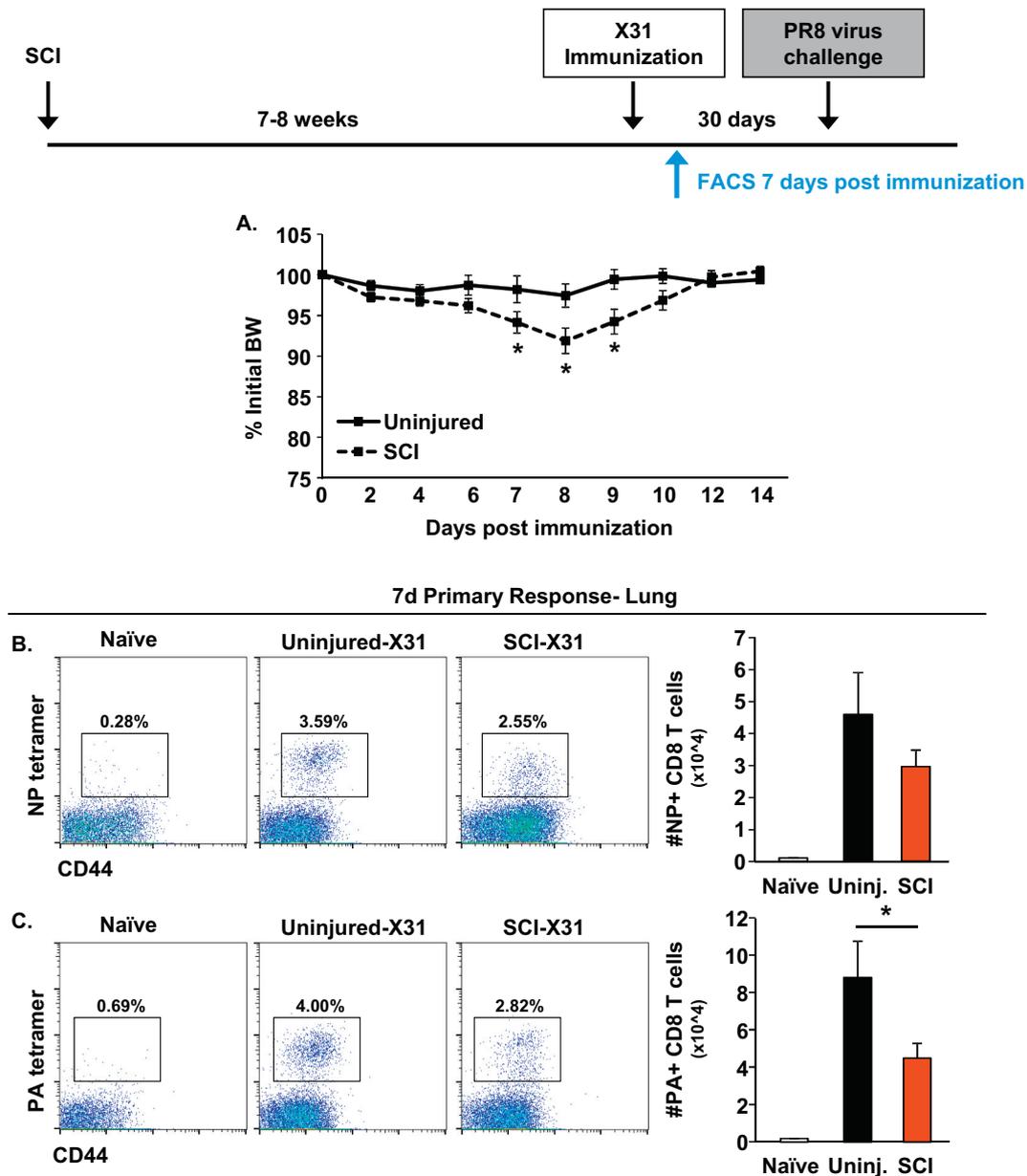


Fig. 4. Increased weight loss of chronic SCI mice following low dose immunization. Chronic SCI and age-matched uninjured (uninj.) mice were immunized with 0.25 HAU X31 i.n. (A) Body weight loss was monitored for 14 days. Data represent 10 mice per group. Seven days after immunization, lungs were collected and specific CD8 T cells were analyzed. The percentage and absolute number of (B) NP and (C) PA positive CD8 T cells was determined. Data represent 6 mice per group. * $p < 0.05$.

difference in cell counts, there were differences in the profiles of the immune cells. For example, we have previously shown increased expression of inhibitory PD1 on CD8 T cells following chronic SCI (Zha et al., 2014). Here, we characterized the proportion of regulatory T cells (Tregs) following SCI. Injured mice had increased percentage of CD4 T cells that were positive for intracellular Foxp3, indicating more Tregs generated in both spleen and lung after SCI ($p < 0.02$) (Fig. 3C–D).

3.3. Increased weight loss, but functional CD8 T cell memory formation, following immunization with a low dose of live virus in chronic SCI mice

Our data indicate an impaired CD8 T cell response and increased mortality following primary immune challenge after SCI. These data highlight the need for proper protection against influenza virus after SCI. Therefore, we investigated the effectiveness of immunization in protecting against secondary infections. Here, mice were immunized

i.n. with a low dose (0.25 HAU) of live virus (X31) to generate primary and memory CD8 T cell responses. Functional CD8 T cell memory was characterized following challenge with the heterologous influenza virus PR8. In this scenario, protection will be mediated by CD8 T cells that recognize both X31 and PR8 NP peptide (Crowe et al., 2006).

First, we determined the effectiveness of immunization that was provided during chronic SCI (7–8 weeks after injury) (Fig. 4). Chronic SCI mice lost body weight (10%, $p < 0.04$) following immunization while there was no body weight loss in uninjured mice. Immunization successfully induced a CD8 T cell response in both uninjured and SCI mice. There was a robust increase in NP- and PA- specific CD8 T cells in the lung following immunization (Fig. 4B,C). However, similar to primary high dose infection, there was decreased specific CD8 T cells in the lung of SCI mice (PA, $p < 0.05$) (Fig. 4C). Although there was no mortality in SCI mice infected with a low dose of virus, these data show increased sensitivity to low dose infection with weight loss and

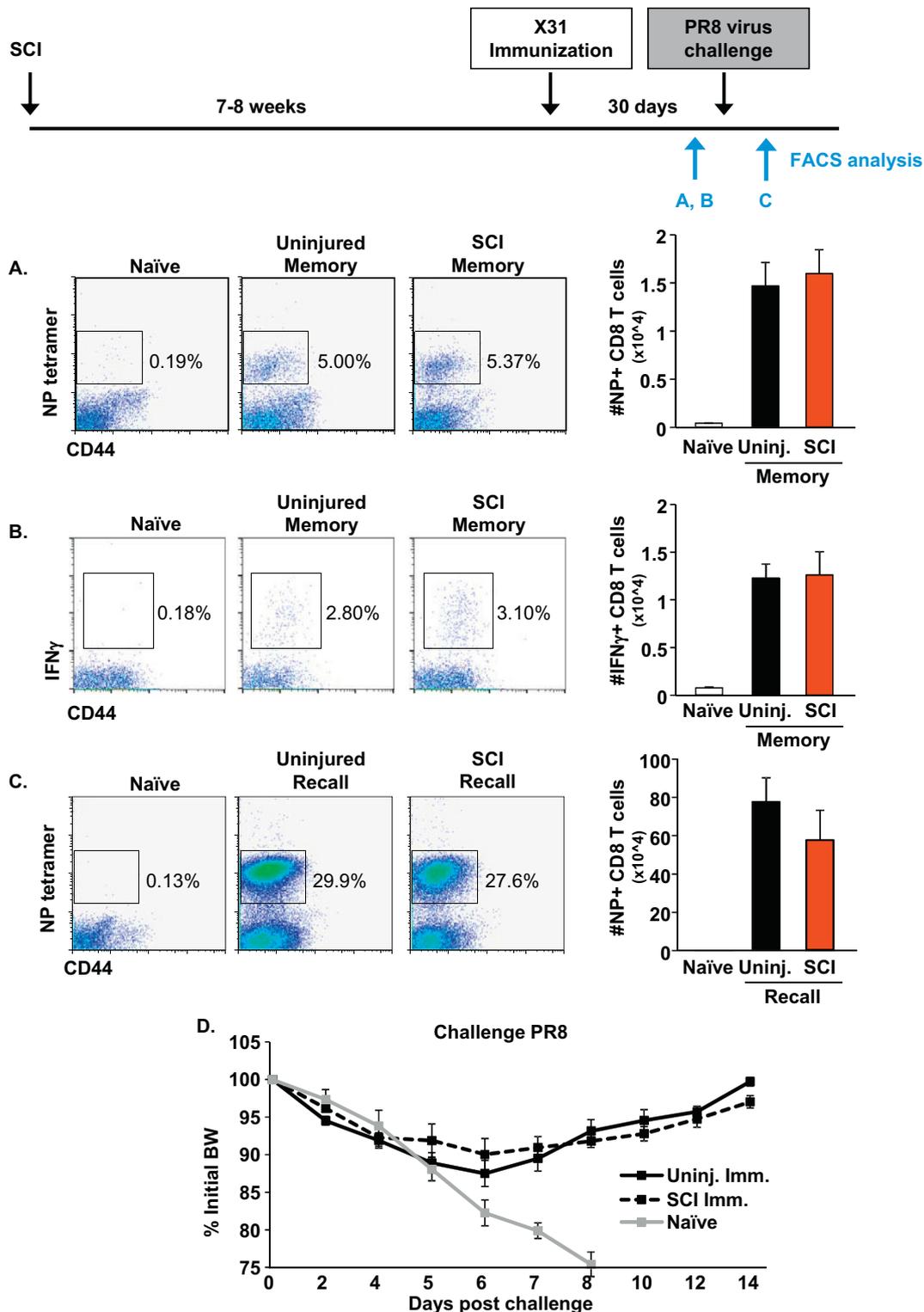


Fig. 5. Functional CD8 T cell memory formation in chronic SCI following immunization. Chronic SCI and age-matched uninjured (uninj.) mice were immunized with 0.25 HAU X31 i.n. Memory CD8 T cells in the lung were analyzed 30 days after immunization. (A) The percentage and absolute number of NP positive CD8 T cells was determined. (B) Cells were cultured *ex vivo* with NP peptide. After 4 h, IFN γ production was determined. Data represent 6 mice per group. Immunized SCI, immunized uninjured and naïve mice were challenged with 1 HAU PR8 i.n 35 days after immunization. (C) Expansion of NP positive CD8 T cells was determined in the lungs 5 days after challenge. Data represent 6 mice per group. (D) BW loss was monitored for 14 days after challenge. Data represent 10 mice per group.

attenuated CD8 T cell responses following immunization.

Next, we evaluated generation and function of memory CD8 T cells following immunization in SCI mice. Lungs were collected 30 days after immunization and NP⁺-specific CD8 T cells were analyzed since these

cells are able to confer protection against secondary challenge (Crowe et al., 2006). Surprisingly, there was no difference in the number of NP⁺-CD8 T cells between uninjured and SCI mice 30 days after immunization (Fig. 5A). To analyze the function of these memory CD8 T

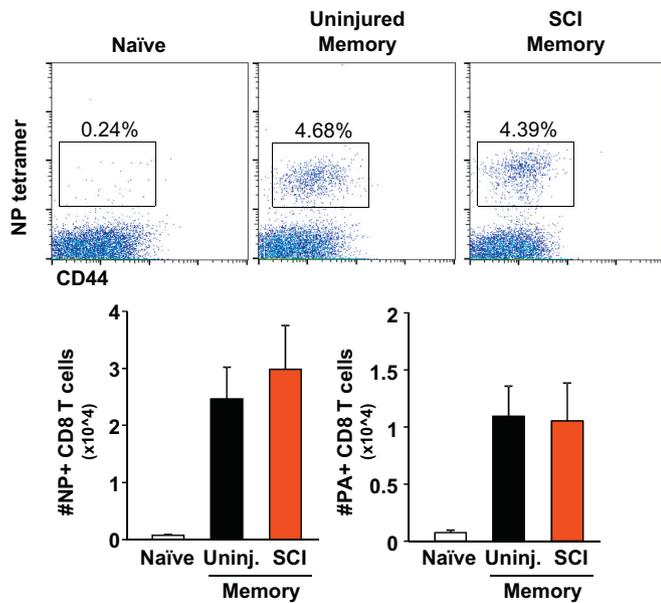


Fig. 6. Equal memory formation following primary infection at a high dose of flu virus in SCI and uninjured mice. Mice were given SCI or left uninjured (uninj.). After 7 weeks, mice were infected with 5 HAU X31. Number of memory CD8 T cells were determined in the lung of mice that survived the infection. Data represent 6 mice per group.

cells, leukocytes from lung were cultured *ex vivo* with NP peptide and IFN γ production was determined. Memory CD8 T cells from immunized mice treated with NP peptide *ex vivo* had increased IFN γ expression and there was no difference between uninjured and SCI mice (Fig. 5B). This was an unexpected finding because we surmised that the attenuated primary response would lead to decreased memory CD8 T cell formation. Therefore, we also analyzed memory CD8 T cell formation following high dose primary infection, when there is a drastic difference in the primary CD8 T cell response (Fig. 2). Lungs from mice that survived high primary infection were collected 30 days later. Similar to low dose immunization, there was no difference in the number of NP⁺-CD8 memory T cells 30 days after high dose primary infection (Fig. 6).

These data indicate that injured mice are able to generate similar memory CD8 T cell populations following immunization. Next, we investigated whether these antigen-specific memory cells were fully functional and would protect against a heterologous challenge with PR8. Immunized uninjured and injured mice were challenged with PR8 and expansion of resident NP⁺ memory CD8 T cells was determined 5 days later in the lung. There was a large expansion of specific CD8 T cells following challenge in both uninjured and injured mice, with 27–30% of all CD8 T cells staining positive for NP tetramer (Fig. 5C). There was no difference in either percentage or absolute number of NP⁺-specific CD8 T cells in the lungs of uninjured and SCI mice (Fig. 5C). Rapid expansion of NP⁺-CD8 T cells resulted in limited weight loss following challenge. Immunized uninjured and SCI mice lost 10–12% body weight maximum at day 6 and recovered back to baseline (Fig. 5D). As a comparison, nonimmunized naïve mice continued to lose weight and were euthanized at day 8 (> 25% body weight loss) (Fig. 5D). Thus, immunization with a low dose of X31 generated functional memory CD8 T cells that provided protection from heterologous challenge with PR8. Both uninjured and SCI mice had large expansion of NP⁺-specific CD8 T cells and limited weight loss following challenge. These data demonstrate that SCI mice can generate functional CD8 T cell memory comparable to uninjured mice.

3.4. Pre-existing CD8 T cell memory remains intact following chronic SCI

Our data show that CD8 T cell memory can be generated in mice after SCI. However, SCI patients may have received numerous vaccinations through their life prior to injury. Therefore, we also investigated whether pre-existing memory CD8 T cells remain functional following chronic SCI. Naïve mice were *i.n.* immunized with low dose live X31. Primary CD8 T cell response and memory formation were confirmed on days 7 and 30 post immunization, respectively. Following memory formation, SCI was induced in half the mice. Presence of memory CD8 T cells was determined 7 weeks after injury (Fig. 7). NP⁺-CD8 T cells remained in the lung of immunized mice (Fig. 7A). Similar to immunization after injury, there was no difference in the number of memory CD8 T cells between immunized uninjured and injured mice (Fig. 7A). In addition, following *ex vivo* NP peptide stimulation there was an equal number of IFN γ positive CD8 T cells with similar expression level (MFI) between uninjured and SCI mice (Fig. 7B). To determine protection potential of memory CD8 T cells, mice were challenged with PR8 virus. Significant expansion of specific NP⁺-CD8 T cells was observed 5 days after challenge and there was no difference between uninjured and SCI mice (Fig. 7C). These CD8 T cells provided protection from the challenge, as there was limited weight loss and no mortality in both immunized injured and uninjured mice (Fig. 7D). Overall, these data show that pre-existing memory CD8 T cells remain in the lung of SCI mice, and these cells are able to provide full protection from heterologous challenge.

4. Discussion

Spinal cord injury induced immune depression is a serious concern for SCI patients. Infections are the leading cause of death in people living with chronic SCI. These infections include respiratory infections like pneumonia and influenza. Influenza is of particular significance as there is a yearly outbreak and thus a reoccurring threat to SCI patients. The CDC (2005) has included SCI as a high-risk condition, and has recommended annual influenza immunization for the first time in history for this population (Goldstein et al., 2005). However, the mechanism by which SCI leads to impaired antiviral immunity remains unclear and it is unknown whether immunizations are fully protective and effective following injury. In this study, we used a mouse model of chronic SCI and heterologous viral challenge system to directly evaluate the protection mediated by CD8 memory T cells, as X31 and PR8 have conserved internal, but different surface, peptides and thus there is no protection mediated by antibodies.

Our data show that primary CD8 T cell responses were impaired following chronic SCI. Here, we confirm increased mortality in chronic SCI mice following high dose primary virus infection (Bracchi-Ricard et al., 2016) and also show increased sensitivity (weight loss) following low dose primary infection. Chronic SCI mice had attenuated recruitment of total and specific CD8 T cells to the lung following infection. In addition, there was decreased generation of specific CD8 T cells and decreased expansion of effector (CD44^{high}CD62L^{low}) CD8 T cells in the spleen of infected SCI mice. The mechanisms leading to impaired CD8 T cell response following SCI remain unknown but may be due to increased activation of regulatory pathways. We previously showed upregulation of the PD-1/PD-L1 immunomodulatory pathway in the spleen of injured mice (Zha et al., 2014). Here, we extend these findings and show that SCI mice also have an increased proportion of Tregs in both spleen and lung. These pathways are likely upregulated after SCI to counteract the systemic inflammatory response to limit further spread of inflammation at the injury site and also protect against autoimmunity (Sun et al., 2016). However, the PD-1/PD-L1 pathway and Tregs can interfere with antiviral immunity. Therefore, it is possible that the regulatory mechanisms set in motion following injury lead to attenuated responses to influenza challenge. In addition, we report an unbalanced immune profile of the lung following chronic SCI. SCI mice

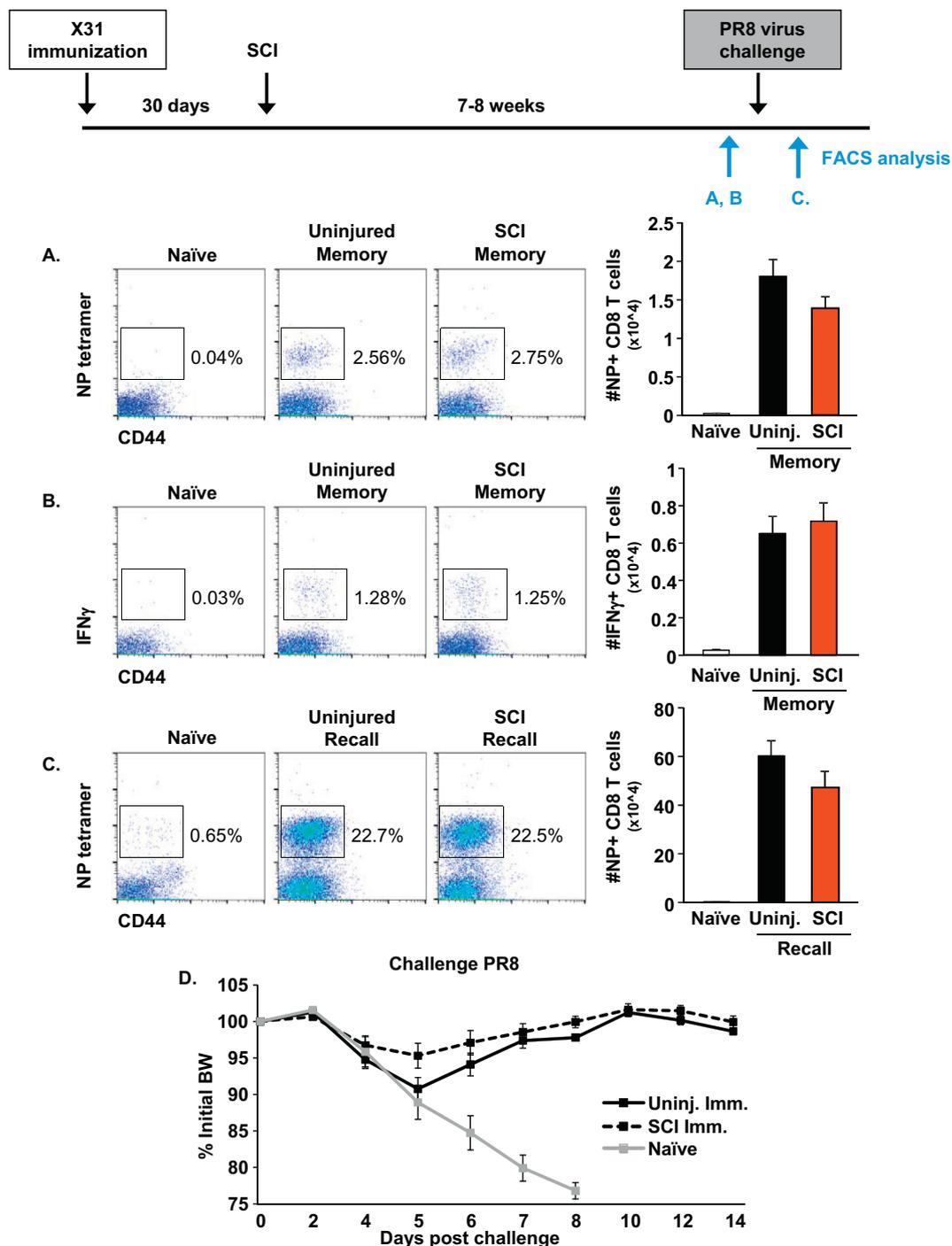


Fig. 7. Pre-existing CD8 T cell memory remains intact following chronic SCI. Uninjured mice were immunized i.n. with 0.25 HAU X31. After 35 days, half of the mice received SCI. Memory CD8 T cells were analyzed 7 weeks after SCI (11–12 weeks after immunization). (A) The percentage and absolute number of NP⁺-CD8 T cells was determined. (B) Cells were cultured *ex vivo* with NP peptide. After 4 h, IFN γ production was determined. Data represent 6 mice per group. Immunized SCI, immunized uninjured and naïve mice were challenged i.n. with PR8. (C) Expansion of NP⁺-CD8 T cells was determined 5 days after challenge. Data represent 6 mice per group. (D) BW loss was monitored for 14 days after challenge. Data represent 10 mice per group.

had an accumulation of B cells, macrophages, and neutrophils in the lung (before infection). This altered immune profile may inhibit proper antiviral responses, e.g., neutrophils can decrease CD8 T cell activation and proliferation (Coffelt et al., 2015; Leliefeld et al., 2015), and thus the exaggerated neutrophil accumulation in SCI mice may potentially impair viral clearance.

In contrast to primary CD8 T cell response, memory CD8 T cells generated in uninjured and SCI mice were comparable, not only in number but also in function. Following immunization with low dose

live X31 virus, SCI mice lost weight while there was no weight loss in uninjured mice. Injured mice also had an attenuated primary CD8 T cell response to the immunization with decreased expansion of specific CD8 T cells at 7 days post immunization. These data show an increased “sensitivity” to primary infection in SCI mice; this low dose of virus did not result in observed pathological changes in uninjured mice, while SCI mice lost significant weight. Given the differential primary response to immunization, we predicted that SCI mice would have attenuated number of memory CD8 T cells generated. However, this was not the

case as there was no difference in the number of memory CD8 T cells in the lung or spleen of uninjured and SCI mice 30 days after immunization. In addition, there was no difference in the number of memory CD8 T cells generated from a high dose primary challenge when the effector CD8 T cell response was drastically different. Most importantly, memory CD8 T cells expanded equally in uninjured and injured mice and provided full protection from lethal PR8 challenge in both groups. In mice immunized before injury, pre-existing memory CD8 T cells will receive significant input from inflammation caused by SCI and will be exposed to an “SCI environment” for a longer duration compared to memory CD8 T cells formed following injury. Therefore, we predicted that pre-existing memory cells may be more affected by SCI compared to those generated following injury. However, SCI mice immunized with X31 before chronic injury were also fully protected from PR8 challenge and had similar weight loss and CD8 T cell expansion as uninjured mice. Intranasal challenge with PR8 lead to rapid expansion of memory CD8 T cells in the lung, while there was limited expansion of memory CD8 T cells in the spleen suggesting that the recall response was mainly mediated by resident memory CD8 T cells (Van Braeckel-Budimir et al., 2018). Since intramuscular immunization with inactive X31 does not sufficiently mediate protection from intranasal PR8 challenge (Bracchi-Ricard et al., 2016), immunization strategies that fully engage the CD8 T cell response are necessary to provide protection from heterologous challenge during chronic SCI.

Our finding of comparable number of memory CD8 T cells generated after a differential primary response between SCI and uninjured mice is surprising. After viral clearance, effector cells undergo apoptosis (contraction) or differentiate into memory cells (Wherry and Ahmed, 2004). The magnitude of effector cell expansion has been implicated in the number of memory cells generated (Wherry et al., 2002). However, in our model of SCI, we report similar generation of memory CD8 T cells in injured mice despite decreased effector cell expansion. Similar to our results, another study showed equal number and function of memory CD8 T cells generated after low vs high doses of virus during immunization (Wang et al., 2016), showing that different primary responses can still lead to comparable memory formation. A similar mechanism of regulating number of memory cells generated may occur in our studies. In addition, it is possible that SCI mice have a continued or delayed effector response compared to uninjured mice that leads to high number of memory cells. Last, the mechanisms that determine memory CD8 T cell formation and function may be similar between uninjured and injured mice (e.g., IL-7 (Hand et al., 2007; Kaech et al., 2003) and IL-15 (Richer et al., 2015; Schluns et al., 2002) signaling).

The differential effect of SCI on primary and memory CD8 T cell function provides insight into the mechanisms of impaired CD8 responses following SCI. Why would SCI have an effect on primary, but not memory, CD8 T cells? SCI leads to activation or disruption of both the sympathetic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. Previous studies have shown increased norepinephrine and corticosterone during chronic SCI (Lucin et al., 2007; Popovich et al., 2001; Zha et al., 2014; Zhang et al., 2013), both of which have immunosuppressive effects (Coutinho and Chapman, 2011; Lucin et al., 2007). Naïve CD8 T cells and memory CD8 T cells might have differential expression or regulation of activation receptors, such as glucocorticoid receptors, making naïve CD8 T cells more affected by the SCI environment. Most likely, these differences can be explained by differences in antigen presentation and activation signals. The activation process and signals needed to activate naïve vs memory CD8 T cells are highly differentially regulated. Memory CD8 T cells respond quickly to secondary activation and undergo rapid proliferation compared to naïve CD8 T cells (Wherry and Ahmed, 2004). In addition, primary CD8 T responses to intranasal challenge may rely on both resident and recruited CD8 T cells (Turner et al., 2013) whereas secondary responses rely mainly on resident memory CD8 T cells (Van Braeckel-Budimir et al., 2018). Following primary infection, there was decreased generation of specific CD8 T cells in both spleen and lung. Following recall

challenge, however, the main expansion of memory CD8 T cells occurred in the lung with limited expansion in the spleen. Mid-thoracic SCI disrupts sympathetic regulation in the spleen (Zha et al., 2014) which may partially explain why resident memory CD8 T cells located in the lung are less influenced by the injury. Our data are also similar to previous studies of humoral immunity. During chronic SCI, primary B cell responses are impaired while B cell memory remains intact (Bracchi-Ricard et al., 2016; Oropallo et al., 2012), which may suggest that B cell responses are likewise affected by the injury.

In summary, our study shows impaired CD8 T cell antiviral responses to primary influenza virus infection following chronic SCI. However, memory CD8 T cell formation and function were not affected in SCI mice. This has significant clinical implications for SCI patients as cross-reactive memory T cells that are targeted to conserved internal proteins of influenza virus can provide cross protection between two different strains (Rimmelzwaan et al., 2007). In addition, our findings highlight the necessity of proper immunizations in SCI patients. Current standards for immunizations use trivalent inactivated influenza vaccines that aim to induce antibody responses against HA and NA. As mentioned, these vaccines are inefficient at inducing virus-specific CD8 T cell responses (He et al., 2006). Our findings indicate that immunizations that engage CD8 T cell responses are protective during chronic SCI. Therefore, SCI patients could benefit from live-attenuated influenza vaccines. Last, our results point to differential roles of the central nervous system in regulating primary vs secondary CD8 T cell immunity. Future studies investigating the differential effect of SCI on primary vs secondary responses will uncover mechanisms leading to immune depression following CNS trauma.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

We thank MHC Tetramer Core Facilities of NIH at Atlanta for kindly providing H-2D^b-PA₂₂₄₋₂₃₃ and H-2D^b-NP₃₆₆₋₃₇₄ tetramers. This work is funded by Craig Neilsen Foundation Grant (Grant 385706 to JJ). DMN was supported by an American Association for Immunologist Careers in Immunology Fellowship.

References

- Altenburg, A.F., Rimmelzwaan, G.F., de Vries, R.D., 2015. Virus-specific T cells as correlate of (cross-)protective immunity against influenza. *Vaccine* 33, 500–506.
- Bouvier, N.M., Lowen, A.C., 2010. Animal models for influenza virus pathogenesis and transmission. *Viruses* 2, 1530–1563.
- Bracchi-Ricard, V., Zha, J., Smith, A., Lopez-Rodriguez, D.M., Bethea, J.R., Andreansky, S., 2016. Chronic spinal cord injury attenuates influenza virus-specific antiviral immunity. *J. Neuroinflammation* 13, 125.
- Brennan, F.H., Popovich, P.G., 2018. Emerging targets for reprogramming the immune response to promote repair and recovery of function after spinal cord injury. *Curr. Opin. Neurol.* 33, 334–344.
- Cardenas, D.D., Hoffman, J.M., Kirshblum, S., McKinley, W., 2004. Etiology and incidence of rehospitalization after traumatic spinal cord injury: a multicenter analysis. *Arch. Phys. Med. Rehabil.* 85, 1757–1763.
- Coffelt, S.B., Kersten, K., Doornebal, C.W., Weiden, J., Vrijland, K., Hau, C.S., Versteeg, N.J., Ciampriotti, M., Hawinkels, L.J., Jonkers, J., et al., 2015. IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* 522, 345–348.
- Coutinho, A.E., Chapman, K.E., 2011. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Mol. Cell. Endocrinol.* 335, 2–13.
- Crowe, S.R., Miller, S.C., Woodland, D.L., 2006. Identification of protective and non-protective T cell epitopes in influenza. *Vaccine* 24, 452–456.
- Goldstein, B., Weaver, F.M., Hammond, M.C., 2005. New CDC recommendations: annual influenza vaccination recommended for individuals with spinal cord injuries. *J. Spinal Cord Med.* 28, 383–384.
- Hand, T.W., Morre, M., Kaech, S.M., 2007. Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection. *Proc. Natl. Acad. Sci. U. S. A.* 104, 11730–11735.
- He, X.S., Holmes, T.H., Zhang, C., Mahmood, K., Kemble, G.W., Lewis, D.B., Dekker, C.L.,

- Greenberg, H.B., Arvin, A.M., 2006. Cellular immune responses in children and adults receiving inactivated or live attenuated influenza vaccines. *J. Virol.* 80, 11756–11766.
- Hoschouer, E.L., Basso, D.M., Jakeman, L.B., 2010. Aberrant sensory responses are dependent on lesion severity after spinal cord contusion injury in mice. *Pain* 148, 328–342.
- Kaech, S.M., Tan, J.T., Wherry, E.J., Konieczny, B.T., Surh, C.D., Ahmed, R., 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* 4, 1191–1198.
- Kilbourne, E.D., 1969. Future influenza vaccines and the use of genetic recombinants. *Bull. World Health Organ.* 41, 643–645.
- Kirchenbaum, G.A., Ross, T.M., 2014. Eliciting broadly protective antibody responses against influenza. *Curr. Opin. Immunol.* 28, 71–76.
- Leliefeld, P.H., Koenderman, L., Pillay, J., 2015. How neutrophils shape adaptive immune responses. *Front. Immunol.* 6, 471.
- Lucin, K.M., Sanders, V.M., Jones, T.B., Malarkey, W.B., Popovich, P.G., 2007. Impaired antibody synthesis after spinal cord injury is level dependent and is due to sympathetic nervous system dysregulation. *Exp. Neurol.* 207, 75–84.
- Meisel, C., Schwab, J.M., Prass, K., Meisel, A., Dirnagl, U., 2005. Central nervous system injury-induced immune deficiency syndrome. *Nat. Rev. Neurosci.* 6, 775–786.
- Norden, D.M., Bethea, J.R., Jiang, J., 2018. Impaired CD8 T cell antiviral immunity following acute spinal cord injury. *J. Neuroinflammation* 15, 149.
- Oropallo, M.A., Held, K.S., Goenka, R., Ahmad, S.A., O'Neill, P.J., Steward, O., Lane, T.E., Cancro, M.P., 2012. Chronic spinal cord injury impairs primary antibody responses but spares existing humoral immunity in mice. *J. Immunol.* 188, 5257–5266.
- Popovich, P.G., Stuckman, S., Gienapp, I.E., Whitacre, C.C., 2001. Alterations in immune cell phenotype and function after experimental spinal cord injury. *J. Neurotrauma* 18, 957–966.
- Pruss, H., Tedeschi, A., Thiriot, A., Lynch, L., Loughhead, S.M., Stutte, S., Mazo, I.B., Kopp, M.A., Brommer, B., Blex, C., et al., 2017. Spinal cord injury-induced immunodeficiency is mediated by a sympathetic-neuroendocrine adrenal reflex. *Nat. Neurosci.* 20, 1549–1559.
- Richer, M.J., Pewe, L.L., Hancox, L.S., Hartwig, S.M., Varga, S.M., Harty, J.T., 2015. Inflammatory IL-15 is required for optimal memory T cell responses. *J. Clin. Invest.* 125, 3477–3490.
- Rimmelzwaan, G.F., Fouchier, R.A., Osterhaus, A.D., 2007. Influenza virus-specific cytotoxic T lymphocytes: a correlate of protection and a basis for vaccine development. *Curr. Opin. Biotechnol.* 18, 529–536.
- Schluns, K.S., Williams, K., Ma, A., Zheng, X.X., Lefrancois, L., 2002. Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. *J. Immunol.* 168, 4827–4831.
- Schmidt, M.E., Varga, S.M., 2018. The CD8 T cell response to respiratory virus infections. *Front. Immunol.* 9, 678.
- Smith, B.M., Evans, C.T., Kurichi, J.E., Weaver, F.M., Patel, N., Burns, S.P., 2007. Acute respiratory tract infection visits of veterans with spinal cord injuries and disorders: rates, trends, and risk factors. *J. Spinal Cord Med.* 30, 355–361.
- Soden, R.J., Walsh, J., Middleton, J.W., Craven, M.L., Rutkowski, S.B., Yeo, J.D., 2000. Causes of death after spinal cord injury. *Spinal Cord* 38, 604–610.
- Subbarao, K., Joseph, T., 2007. Scientific barriers to developing vaccines against avian influenza viruses. *Nat. Rev. Immunol.* 7, 267–278.
- Sun, X., Jones, Z.B., Chen, X.M., Zhou, L., So, K.F., Ren, Y., 2016. Multiple organ dysfunction and systemic inflammation after spinal cord injury: a complex relationship. *J. Neuroinflammation* 13, 260.
- Turner, D.L., Bickham, K.L., Farber, D.L., Lefrancois, L., 2013. Splenic priming of virus-specific CD8 T cells following influenza virus infection. *J. Virol.* 87, 4496–4506.
- Van Braeckel-Budimir, N., Varga, S.M., Badovinac, V.P., Harty, J.T., 2018. Repeated antigen exposure extends the durability of influenza-specific lung-resident memory CD8(+) T cells and heterosubtypic immunity. *Cell Rep.* 24, 3374–3382 e3373.
- Wang, Z., Kedzierski, L., Nuessing, S., Chua, B.Y., Quinones-Parra, S.M., Huber, V.C., Jackson, D.C., Thomas, P.G., Kedzierska, K., 2016. Establishment of memory CD8+ T cells with live attenuated influenza virus across different vaccination doses. *J. Gen. Virol.* 97, 3205–3214.
- Wherry, E.J., Ahmed, R., 2004. Memory CD8 T-cell differentiation during viral infection. *J. Virol.* 78, 5535–5545.
- Wherry, E.J., McElhaugh, M.J., Eisenlohr, L.C., 2002. Generation of CD8(+) T cell memory in response to low, high, and excessive levels of epitope. *J. Immunol.* 168, 4455–4461.
- Zha, J., Smith, A., Andreansky, S., Bracchi-Ricard, V., Bethea, J.R., 2014. Chronic thoracic spinal cord injury impairs CD8+ T-cell function by up-regulating programmed cell death-1 expression. *J. Neuroinflammation* 11, 65.
- Zhang, Y., Guan, Z., Reader, B., Shawler, T., Mandrekar-Colucci, S., Huang, K., Weil, Z., Bratasz, A., Wells, J., Powell, N.D., et al., 2013. Autonomic dysreflexia causes chronic immune suppression after spinal cord injury. *J. Neurosci.* 33, 12970–12981.