Chronic Spinal Cord Injury Impairs Primary Antibody Responses but Spares Existing Humoral Immunity in Mice

Michael A. Oropallo,*[,1] Katherine S. Held,[†,1] Radhika Goenka,*[,1] Sifat A. Ahmad,* Patrick J. O'Neill,* Oswald Steward,[‡,§] Thomas E. Lane,[†,¶,‖,#] and Michael P. Cancro*

Spinal cord injury (SCI) results in immune depression. To better understand how injury inhibits humoral immunity, the effects of chronic thoracic SCI on B cell development and immune responses to thymus-independent type 2 and thymus-dependent Ags were determined. Mice received complete crush injury or control laminectomy at either thoracic level 3, which disrupts descending autonomic control of the spleen, or at thoracic level 9, which conserves most splenic sympathetic activity. Although mature B cell numbers were only mildly reduced, bone marrow B cell production was transiently but profoundly depressed immediately after injury. Despite the return of normal B cell production 4 wk after SCI, mice receiving thoracic level 3 injury showed a significant reduction in their ability to mount primary thymus-independent type 2 or thymus-dependent immune responses. The latter were marked by decreases in germinal center B cells as well as class-switched high-affinity Ab-secreting cells. Importantly, injury did not affect affinity maturation per se, pre-existing B cell memory, or secondary humoral immune responses. Taken together, these findings show that chronic high thoracic SCI impairs the ability to mount optimal Ab responses to new antigenic challenges, but spares previously established humoral immunity. *The Journal of Immunology*, **2012, 188: 5257–5266.**

Bacterial infections are the leading cause of death among patients who survive spinal cord injury (SCI), reflecting generalized immune depression (1, 2). These observations suggest that SCI impairs humoral immunity via multiple mechanisms, including dysregulation of both the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS). For example, corticosteroids secreted by the HPA axis following stress or injury can diminish B cell lymphopoiesis (3). Furthermore, norepinephrine secreted by SNS nerves, which innervate lymphoid organs, can bind to B cells and influence their responsiveness (4–8). Accordingly, assessment of how SCI per se, as well as accompanying dysregulation of the HPA axis and/or SNS, contributes to these effects is of particular clinical interest.

Studies using murine models of SCI have begun to dissect the relative roles played by loss of splenic sympathetic regulation versus increased injury-induced stress hormones in perturbations of B cell homeostasis and function. Acute injury at thoracic level 3 (T3), which disrupts autonomic control of the spleen, results in fewer total splenic B cells and impaired thymus-dependent (TD) Ab responses (9, 10). Dysregulation of the SNS was implicated in these alterations, as blocking of SNS-derived norepinephrine signaling restored TD Ab responses in T3-injured mice and was intact in both laminectomy controls and mice injured at T9, a level at which most central sympathetic regulation to the spleen is conserved (9). Although these findings show that acute SCI disrupts primary TD humoral responses, the question remains whether these effects persist during chronic injury. Moreover, it is unclear whether these findings reflect generalized shifts in the numbers or functional capacities of all B lineage cells, or instead differentially impact particular B cell subsets and their associated functions. Furthermore, as patients are most often severely affected by pathogens that characteristically elicit thymus-independent (TI) humoral responses (2), it is essential to know how SCI affects primary TI responses. Finally, whether the processes required to generate high-affinity Abs during primary TD responses are intact, as well as whether pre-existing memory B cell numbers and responses are retained, is unknown.

Accordingly, to further understand how SCI affects B cell maintenance, responsiveness, and memory, we have conducted detailed assessments of B cell subsets and function in mice receiving complete crush SCI at either T3 or T9. We show that previously observed reductions in splenic B cells during acute SCI

*Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104; [†]Department of Molecular Biology and Biochemistry, School of Biological Sciences, University of California, Irvine, Irvine, CA 92697; [‡]Department of Anatomy and Neurobiology, Reeve–Irvine Research Center, School of Medicine, University of California, Irvine, Irvine, CA 92697; [§]Department of Neurosurgery, School of Medicine, University of California, Irvine, Irvine, CA 92697; [¶]Multiple Sclerosis Research Center, University of California, Irvine, Irvine, CA 92697; [‖]Institute for Immunology, University of California, Irvine, Irvine, CA 92697; and [#]Sue and Bill Gross Stem Cell Center, University of California, Irvine, Irvine, CA 92697

[1]M.A.O., K.S.H., and R.G. contributed equally to this work.

Received for publication June 30, 2011. Accepted for publication March 22, 2012.

This work was supported by the Craig H. Neilsen Foundation (to T.E.L. and M.P.C.), the Roman Reed Spinal Cord Injury Research Program of the State of California (Grant RR09-CY2009 to T.E.L.), National Institutes of Health Grant NS47718 (to O.S.), the Research for Cure, and a private donation to the Reeve–Irvine Research Center. K.S.H. was supported in part by National Institutes of Health Training Grant T32 NS045540-05. R.G. was supported by National Institutes of Health Training Grant T32 AI-055428.

M.A.O., R.G., K.S.H., P.J.O., and S.A.A. performed the experiments, M.P.C., T.E.L., and O.S. helped design the experiments and provided critical reagents, and M.A.O., R.G., K.S.H., M.P.C., and T.E.L. wrote the manuscript.

Address correspondence and reprint requests to Dr. Michael P. Cancro or Dr. Thomas E. Lane, Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, 284 John Morgan, Building 6082, 36th Street and Hamilton Walk, Philadelphia, PA 19104-8062 (M.P.C.) or Department of Molecular Biology and Biochemistry, University of California, Irvine, 3205 McGaugh Hall, Irvine, CA 92697-3900 (T.E.L.). E-mail addresses: cancro@mail.med.upenn.edu (M.P.C.) and tlane@uci.edu (T.E.L.)

The online version of this article contains supplemental material.

Abbreviations used in this article: ASC, Ab-secreting cell; BM, bone marrow; CGG, chicken gamma globulin; FO, follicular; GC, germinal center; HPA, hypothalamic-pituitary-adrenal; MZ, marginal zone; NP, 4-hydroxy-3-nitrophenyl acetyl; PNA, peanut agglutinin; SCI, spinal cord injury; SNS, sympathetic nervous system; T3, thoracic level 3; T9, thoracic level 9; TD, thymus-dependent; TI, thymus-independent; TR, transitional.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1101934

reflect cessation of B lymphopoiesis, since developing bone marrow (BM) B cell subsets and transitional (TR) B cells were profoundly reduced 8 d after SCI. Blunted B cell genesis is transient, as developing BM subsets were completely restored to preinjury levels after 28 d. Furthermore, mature follicular (FO) B cells, but not marginal zone (MZ) B cells, were reduced following injury. Evaluation of Ag-specific B cell responses during chronic injury revealed that the magnitude of both TI and primary TD responses were reduced in T3 injured mice. Finally, we show that SCI impacts neither memory B cell numbers nor the ability to mount anamnestic responses to Ags encountered prior to injury. Taken together, our findings reveal that the humoral immune system is dynamically altered following SCI, and that time after injury and the injury level per se are important considerations for future basic and translational investigations.

Materials and Methods

Mice and injury

Age-matched 5- to 7-wk-old female C57BL/6 mice were purchased from the National Cancer Institute (Bethesda, MD). All procedures were approved by the University of California at Irvine Institutional Animal Care and Use Committee. Mice were initially anesthetized with tribromoethanol (Avertin; 0.5 ml/20 g); when supplemental anesthesia was required, one-fourth of the original dose was given. Body temperature was maintained by placing mice on a water-circulating jacketed heating pad at 37 ± 0.5°C. The skin over the upper thoracic area was shaved and cleaned with a povidone-iodine (Betadine) solution. Using aseptic techniques, the skin was incised and connective and muscle tissues were bluntly dissected to expose vertebral bodies T3 or T9. A laminectomy of a single vertebral lamina was performed at T2–T3 or T9–T11 to expose the dorsal spinal cord. Experimental bilateral crush injury was performed using forceps (Dumont no. 5) placed on either side of exposed spinal cord following laminectomy. The points of the forceps were then brought together, held for 1 s, and released. A complete bilateral crush injury results in loss of motor function caudal to the injury site. The crush injury produces complete paralysis of the hindlimbs and mice do not recover the ability to walk (11). Therefore, hindlimb motor recovery behavioral assessment was not conducted for our studies. Incomplete lesions were identified on days 0 and 27 postinjury (following recovery from surgical anesthesia and prior to immunization, respectively) in mice displaying any degree of ankle, knee, and/or hip nonreflexive movement, and they were subsequently excluded from experimental analysis. After injury or laminectomy only, the muscles and skin were sutured separately and mice were given s.c. injections of lactated Ringer's solution (1 ml/20 g) for hydration, buprenorphine (Buprenex; 0.05 mg/kg) for analgesia, and enroflaxacin (Baytril; 2.5 mg/kg) for prophylaxis against urinary tract infections. Uninjured mice did not undergo any surgery, but were anesthetized. Mice were placed in cages with Alpha-Dri bedding (Newco Distributors) and warmed directly on water-jacketed heating pads at 37°C until they recovered from anesthesia. Thereafter, half of each cage was place on heating jacket for up to 3 d postsurgery until coat quality improved and mobility around the cage resumed. Postoperative care involved daily treatments of lactated Ringer's solution and enroflaxacin for the first 6 d postsurgery, and daily buprenorphine treatments for the first 3 d postsurgery. Postoperative care of injured mice also included manual bladder expression twice daily for the duration of experiments.

Injury induction, immunizations, mouse care, spinal column histology, and serum corticosterone analyses were performed at the University of California, Irvine. All mice were euthanized in a closed receptacle containing gauze soaked with isoflurane anesthetic, after which death occurred within 2 min. Following euthanasia and harvest, tissues were shipped to the University of Pennsylvania overnight on ice in DMEM containing 10% FBS for flow cytometry and 4-hydroxy-3-nitrophenyl acetyl (NP)-specific ELISPOT analyses. Serum was frozen and shipped on dry ice for quantification of NP-specific Abs.

Ags and immunizations

Primary immunizations with NP conjugated to protein or carbohydrate Ag were conducted after 28 d postinjury. For primary TI type 2 responses, mice were immunized i.p. with 50 μg NP_{50}-Ficoll (Biosearch Technologies) in PBS and analyzed at 4.5 d postimmunization. For primary TD responses, mice were immunized i.p. with 50 μg NP-conjugated to chicken gamma globulin (NP_{15}-CGG; Biosearch Technologies) precipitated in aluminum potassium sulfate and analyzed at 14 d after immunization. For secondary immunizations, mice were immunized i.p. with 50 μg NP_{15}-CGG 54 d prior to injury. Twenty-eight days postinjury, mice were either boosted with 50 μg NP_{15}-CGG i.p. or received no treatment. Organs were harvested 7 d after secondary immunization.

Corticosterone enzyme immunoassay

All blood collection by cardiac puncture occurred following euthanasia with anesthetic, and we consistently performed these procedures at the same time of day throughout these experiments. Previously it has been shown that postmortem blood collection does not influence corticosterone levels in other nonmanipulated mice sharing the same environment (12); however, as a precautionary measure to avoid variable stress responses, blood collection from individual experimental groups (beginning with uninjured control mice) was done in a procedure room separate from where animals were housed. Plasma was isolated using buffered citrate from blood collected from each mouse postmortem, and corticosterone levels were assessed by ELISA (Immunodiagnostic Systems).

Histological analysis

Spinal columns were dissected from experimentally injured mice and postfixed in 4% paraformaldehyde overnight. Prior to spinal cord extraction from column, the thoracic vertebral location of the laminectomized region was recorded. Spinal cords were cryoprotected in 20% sucrose and embedded in OCT (Tissue-Tek). Eight-micrometer-thick sagittal sections were stained with H&E and visualized using a bright field microscope. Spleens were immersed in OCT and flash frozen using 2-methylbutane cooled with liquid nitrogen. Seven-micrometer sections were fixed with cold acetone and stored at −20°C. Prior to staining, the sections were rehydrated in 1× PBS, blocked with 10% goat serum, and stained with murine-reactive Abs: AF647-anti-IgD (eBioscience), rhodamine-anti-IgM $F(ab')_2$ (SouthernBiotech), and AF647-anti-CD3ε (eBioscience). Sections were mounted using BioMeda Gel Mount (Electron Microscopy Sciences) and were visualized on an LSM-510 Meta confocal microscope (Zeiss).

Flow cytometry

Splenocytes and BM were harvested and stained using the following murine-reactive Abs: allophycocyanin-Cy7-anti-CD19 (BD Biosciences), FITC-anti-B220 (BD Biosciences), eFluor 450-anti-CD21/35 (eBioscience), PE-anti-CD23 (BD Biosciences), PE-Cy7-anti-IgM (BD Biosciences), Qdot 705-streptavidin (Invitrogen), allophycocyanin-anti-AA4.1 (eBioscience), AF700-anti-B220 (eBioscience), FITC-anti-Lambda (SouthernBiotech), PE-Cy5-anti-CD4 (BD Biosciences), PE-Cy5-anti-CD8 (BD Biosciences), PE-Cy5-anti-Gr1 (eBioscience), PE-Cy5-anti-F4/80 (eBioscience), peanut agglutinin (PNA) conjugated to FITC (Vector Laboratories), eFluor 450-anti-IgD (eBiosciences), PE-Cy7-anti-Fas (BD Biosciences). PE-Cy5.5-anti-CD21/35 was donated by D. Allman (University of Pennsylvania). NP was conjugated to allophycocyanin and Qdot 655 was conjugated to anti-Kappa (SouthernBiotech) in-house. For identification of NP-specific responders, intracellular staining was conducted. Live cells were stained for T ($CD4^+$, $CD8^+$) and myeloid cells ($F4/80^+$, $Gr1^+$) to gate out non-B cells. Then, the cells were fixed and permeabilized using a Fix and Perm kit (Caltag Laboratories) according to the manufacturer's protocol and stained with reagents to detect NP, Igλ, and Igκ. For identification of surface NP^+ germinal center (GC) B cells, we conducted staining on live nonpermeabilized splenocytes. Live/dead discrimination was assessed using either DAPI (Invitrogen) or Live/Dead Fixable Aqua (Invitrogen). Doublet discrimination was performed by forward scatter/side scatter width versus height analysis. Total cell numbers were calculated by multiplying the frequency of gated cells among live singlets by the total number of live cells harvested. Data were collected on a BD LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

NP-specific ELISA/ELISPOTs

Plates were coated with either 10 μg/ml NP_{33}-BSA or NP_4-BSA in 100 mM bicarbonate buffer and blocked with PBS containing 2% BSA. For ELISA, sera were incubated for 2 h at 37°C/5.5% CO_2. NP-specific IgG1 or IgM standard was a gift from Dr. Garnett Kelsoe (Duke University, Durham, NC). Detection was conducted using HRP-conjugated goat anti-mouse IgG_1 or IgM (SouthernBiotech) with a tetramethylbenzidine substrate kit (BD Biosciences) and color development was quantified using EMax (Molecular Devices). For ELISPOTs, cell suspensions containing 1×10^6 cells were incubated for 4 h at 37°C and developed with biotin-conjugated anti-mouse IgM or IgG_1 (SouthernBiotech) followed by ExtrAvidin-alkaline phosphatase using NBT/5-bromo-4-chloro-3-indolyl

phosphate substrate (Sigma-Aldrich). Color development was terminated with 1 M NaH_2PO_4 and spots were enumerated on a CTL ImmunoSpot reader (Cellular Technologies).

Data analyses and statistics

One-way ANOVA with a Dunnett post hoc analysis was performed with GraphPad Prism (GraphPad Software). Comparisons were made between injured groups and uninjured control groups, between SCI groups and their respective laminectomy control groups, and between T9 and T3 SCI groups.

Results

Splenic architecture is preserved after spinal cord injury

Most splenic sympathetic innervation derives from midthoracic levels, and thus injury at or above this region will result in loss of supraspinal control and decentralized sympathetic activity (13, 14) (depicted in Supplemental Fig. 1A). Accordingly, the effects of SCI on humoral immune function were assessed using a previously established model of injury above the site of splenic innervation, that is, T3 crush injury (9, 10). As controls, we used either uninjured mice, mice that received a crush injury at T9, a level at which splenic innervation is largely intact, or mice that received laminectomy surgery without crush injury. Postmortem histological analysis of thoracic vertebra from T3- and T9-injured mice was routinely performed to confirm injury location and complete crush (Supplemental Fig. 1B, 1C). The lesions in injured mice at 1 wk postinjury were qualitatively similar to previously described crush SCI in C57BL/6 mice (15). Importantly, crush injury results in no white matter sparing and produces complete paralysis of the hindlimbs and loss of the ability to walk (11). Additionally, T3 SCI was accompanied by a decrease in splenic weight as reported previously (9, 10). Immunohistologic evaluations at day 28 postinjury revealed that splenic microanatomy was preserved, including the T cell-rich periarterial lymphoid sheaths and adjacent IgD^+IgM^+ B cell follicles (Supplemental Fig. 1D). Thus, the general organization of the splenic white pulp is preserved following SCI.

B cell genesis transiently ceases following SCI

Despite the maintenance of lymphoid architecture, prior reports showed that T3 SCI adversely affects total splenic B cell numbers (9, 10, 16). Such reductions may reflect reduced B cell genesis, a direct loss of all or some mature B cell subsets, or both. To assess the relative contributions of these mechanisms, we examined developmental and mature B cell subsets in the BM (Fig. 1, Table I) and spleen (Fig. 2, Table II) during acute and chronic SCI. The gating strategies employed (17) resolve developmental and mature B cell subsets whose dynamics and functional characteristics are well established (reviewed in Refs. 18–20). Briefly, following Ig H chain and L chain gene rearrangements during the pro-B and pre-B cell stages, developing B cells in the BM enter the immature (IMM) B cell stage and then migrate to the periphery as TR B cells. All of these developmental subsets bear the AA4.1 marker and are further resolved according to the criteria shown in Figs. 1 and 2. In the BM, pre-B and pro-B cells lack surface IgM, whereas immature B cells are surface IgM^+ (Fig 1). Splenic TR cells are all $AA4.1^+$ and are further resolved into the TR1, TR2, and TR3 subsets based on differential CD23 and IgM expression levels (Fig. 2). Developing B cells fully transit these BM and splenic developmental stages during 4–6 d (17, 21–24), and thus all of

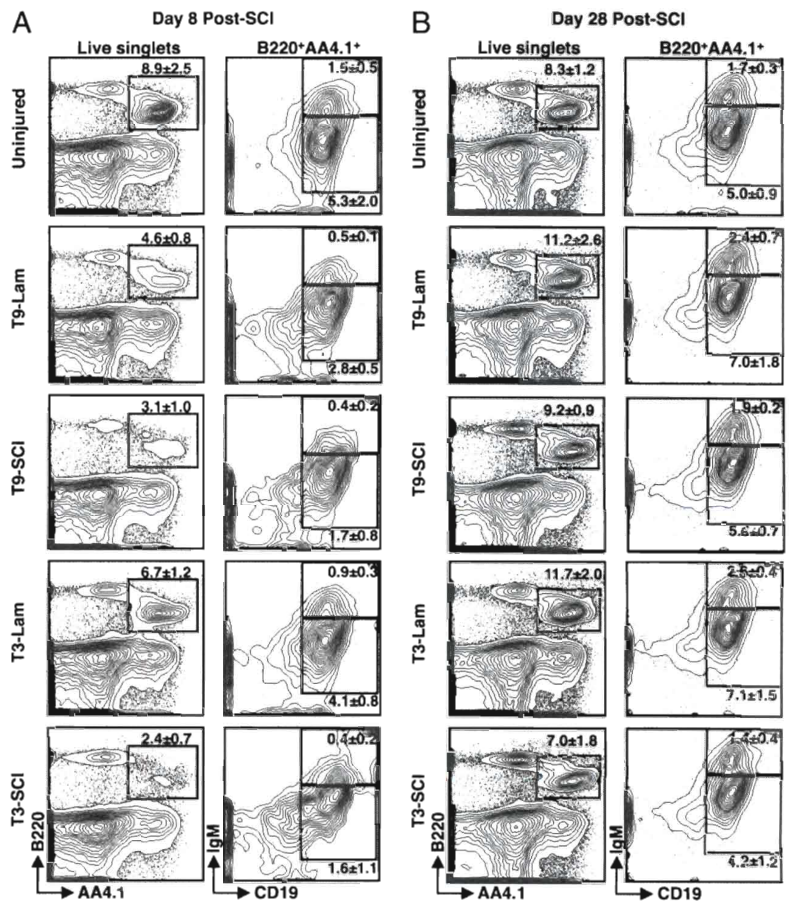

FIGURE 1. The frequency of B cell progenitors in BM is reduced following acute SCI. Representative FACS gating strategy to identify B cell subsets in the BM at day 8 (**A**) and day 28 (**B**) after T3 SCI, T9 SCI, T3 laminectory (Lam), T9 Lam or in uninjured control mice. Contour plots show the mean frequency and SD of $B220^+AA4.1^+$ developing B cells in the BM (*left panels*) and further subsets of IgM^+CD19^+ immature (IMM) and IgM^-CD19^+ pro-/pre-B cell populations (*right panels*) among live singlets. At day 8 after SCI, $n = 5$ for uninjured, $n = 8$ for T9 Lam, $n = 6$ for T9 SCI, $n = 8$ for T3 Lam, and $n = 5$ for T3 SCI. At day 28 after SCI, $n = 5$ for uninjured, $n = 7$ for T9 Lam, $n = 5$ for T9 SCI, $n = 7$ for T3 Lam, and $n = 6$ for T3 SCI.

Table I. Numbers of BM B cell subsets during acute and chronic SCI

		Total Cells	Pro-/Pre-B Cells	Immature B Cells
Uninjured[a]		37.3 ± 7.6	2.47 ± 1.23	0.76 ± 0.43
Day 8 after injury[b]	T9 Lam	38.03 ± 8.7	1.06 ± 0.29[d]	0.18 ± 0.05[d]
	T9 SCI	46.32 ± 8.88[d]	1.07 ± 0.95[d]	0.28 ± 0.37[d]
	T3 Lam	46.05 ± 5.6[d]	1.85 ± 0.35	0.43 ± 0.13
	T3 SCI	47.97 ± 9.49[d]	0.68 ± 0.83[d,e]	0.17 ± 0.17[d]
Day 28 after injury[c]	T9 Lam	34.61 ± 4.42	2.32 ± 0.6	0.84 ± 0.25
	T9 SCI	47.39 ± 9.56[d,e]	2.78 ± 0.92	0.88 ± 0.32
	T3 Lam	36.5 ± 5.47	2.54 ± 0.44	0.95 ± 0.14
	T3 SCI	52.83 ± 9.7[d,e]	2.51 ± 0.98	0.81 ± 0.23

Mean ± SD of cells ($\times 10^6$) per hind limb, gated according to representative FACS plots shown in Fig. 1. Data have been pooled across six experiments for uninjured controls, one for laminectomies, and three for SCI at each level.

[a] $n = 25$ for uninjured.

[b] $n = 8$ for T9 Lam, $n = 13$ for T9 SCI, $n = 8$ for T3 Lam, and $n = 12$ for T3 SCI.

[c] $n = 6$ for T9 Lam, $n = 13$ for T9 SCI, $n = 7$ for T3 Lam and $n = 13$ for T3 SCI.

[d] Denotes significant differences from uninjured control at $p < 0.05$.

[e] Denotes significant differences from Lam controls at $p < 0.05$.

Lam, Laminectomy.

these subsets turnover quickly and therefore deplete rapidly when B lymphopoiesis stops. Following the TR differentiation stages, B cells complete maturation and enter either the FO or MZ pools. Both of these mature, preimmune subsets lack the AA4.1 marker and are distinguished by differential IgM and CD21/35 expression (Fig. 2) (17, 24, 25). Under normal conditions, most developing

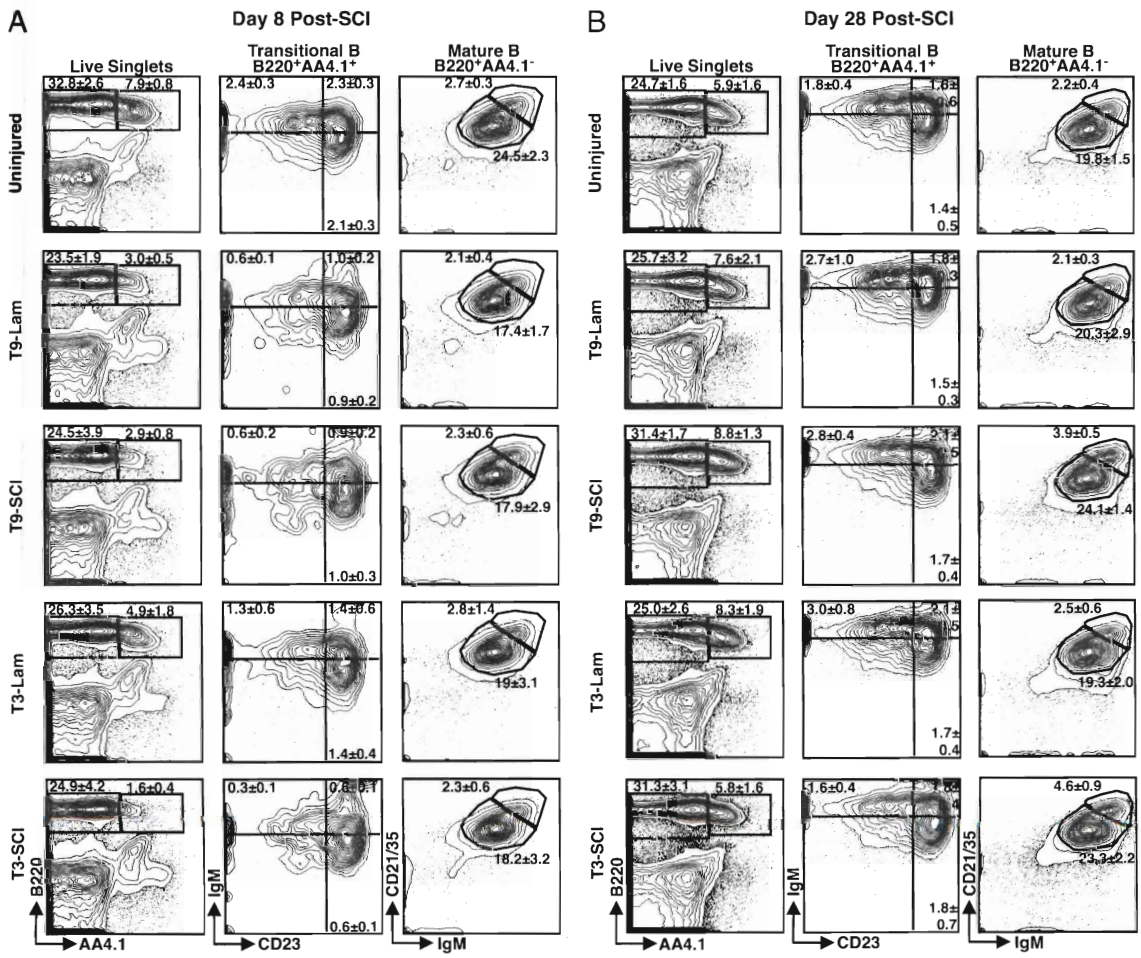

FIGURE 2. The frequency of B cell progenitors in spleen is reduced following acute SCI. Representative FACS gating strategy to identify B cell subsets in the spleen at day 8 (**A**) and day 28 (**B**) after T3 SCI, T9 SCI, T3 laminectomy (Lam), T9 Lam, or in uninjured control mice. Contour plots show the mean frequency and SD of developing B220$^+$AA4.1$^+$ and mature B220$^+$AA4.1$^-$ splenic B cells (*left panels*), and further subsets of IgM^{hi}CD23$^-$ TR1, IgM^{hi} CD23$^+$ TR2, and IgM^{lo}CD23$^+$ TR3 developing B cells (*middle panels*, gates configured clockwise) as well as IgM^{hi}CD21/35^{hi} MZ B and IgM^{lo}CD21/35^{lo} FO B cells (*right panels*) among live singlets. At day 8 after SCI, $n = 5$ for uninjured, $n = 8$ for T9 Lam, $n = 6$ for T9 SCI, $n = 8$ for T3 Lam, and $n = 5$ for T3 SCI. At day 28 after SCI, $n = 5$ for uninjured, $n = 7$ for T9 Lam, $n = 5$ for T9 SCI, $n = 7$ for T3 Lam, and $n = 6$ for T3 SCI.

Table II. Numbers of splenic B cell subsets during acute and chronic SCI

		Total Cells	TR1	TR2	TR3	FO	MZ
Uninjured[a]		131.6 ± 26.2	2.7 ± 0.6	2.5 ± 0.8	2.3 ± 0.7	28.9 ± 5.4	3.2 ± 0.5
Day 8 after injury[b]	T9 Lam	138.0 ± 9.2	0.9 ± 0.1[d]	1.3 ± 0.2[d]	1.2 ± 0.2[d]	24.1 ± 3.0	2.9 ± 0.6
	T9 SCI	104.3 ± 15.6[d,e]	0.6 ± 0.2[d]	0.9 ± 0.2[d]	1.0 ± 0.3[d]	18.3 ± 2.1[d]	2.3 ± 0.5
	T3 Lam	111.1 ± 22.1	1.5 ± 0.9[d]	1.6 ± 0.7[d]	1.6 ± 0.5	21.2 ± 6.5[d]	3.1 ± 1.9
	T3 SCI	111.3 ± 18.5	0.3 ± 0.1[d,e]	0.7 ± 0.2[d,e]	0.7 ± 0.2[d,e]	20.2 ± 3.9[d]	2.5 ± 0.5
Day 28 after injury[c]	T9 Lam	149.7 ± 32.9	3.8 ± 0.8[d]	2.6 ± 0.4	2.2 ± 0.4	30.4 ± 8.2	3.2 ± 0.7
	T9 SCI	86.8 ± 12.5[d,e]	2.5 ± 0.6[e]	2.2 ± 0.6	2.0 ± 0.7	21.1 ± 4.0[e]	3.4 ± 0.4
	T3 Lam	119 ± 22.2	3.5 ± 0.7	2.5 ± 0.5	2.0 ± 0.4	22.9 ± 4.6	2.9 ± 0.5
	T3 SCI	93.6 ± 24.1[d]	1.6 ± 0.4[d,e]	1.8 ± 0.5	1.8 ± 1.0	22.3 ± 5.9	4.3 ± 1.3[d,e]

Number ± SD of cells ($\times 10^6$) per spleen, representative FACS plots shown in Fig. 2. Numbers shown are representative of three experiments at each postinjury time point. Data shown have been pooled across two experiments for uninjured controls, one for laminectomies, and one for SCI at each level.

[a] $n = 10$ for uninjured.

[b] $n = 8$ for T9 Lam, $n = 6$ for T9 SCI, $n = 8$ for T3 Lam, and $n = 5$ for T3 SCI.

[c] $n = 7$ for T9 Lam, $n = 5$ for T9 SCI, $n = 7$ for T3 Lam, and $n = 6$ for T3 SCI.

[d] Denotes significant differences from uninjured control at $p < 0.05$.

[e] Denotes significant differences from Lam controls at $p < 0.05$.

Lam, Laminectomy.

B cells adopt the FO fate, but differentiation skews to the MZ fate under conditions of B lymphopenia or reduced BM output (25). Unlike developing B cell subsets, MZ and FO B cells turn over slowly and persist for months to years (22, 26, 27), so their numbers are modestly affected by short-term perturbations in B cell genesis.

At day 8 after SCI, the proportions of all developmental B cell subsets were profoundly reduced in both T3 and T9 SCI compared with uninjured mice (Figs. 1A, 2A). These declines reflect significant decreases in the numbers of BM pro-B, pre-B, and immature B cells, as well as all splenic TR subsets (Figs. 1A, 2A, Tables I, II). In contrast to acute injury, BM developmental subsets were fully restored during chronic injury (Fig. 1B, Table I). The splenic TR subsets also recovered but were sometimes still mildly reduced at 28 d postinjury (Fig. 2B, Table II). Rapid depletion and subsequent reconstitution of B cell progenitor pools indicates that SCI engenders transient cessation of B cell genesis that is largely re-established within 28 d after injury.

Because B cell genesis resumes within 28 d after SCI, it is unlikely that sustained dysregulation of sympathetic activity underlies blunted B cell genesis during acute injury. Instead, they likely reflect injury-related stress or inflammation, both of which can yield depression of BM B cell genesis via corticosteroid hormones or inflammatory cytokines, respectively (3, 28, 29). Consistent with this possibility, similar transient reductions in developing B cells were observed in mice receiving laminectomy injuries (Figs. 1, 2, Tables I, II), and serum corticosterone levels were elevated following SCI or laminectomy, regardless of injury location (Supplemental Table I). However, serum corticosterone returned to uninjured levels 28 d following laminectomy, yet remained elevated 28 d after T9 or T3 SCI, despite recovery of B cell genesis in all treatment groups. Accordingly, although elevated corticosterone may contribute to blocked B cell production during acute SCI, it is alone insufficient to mediate this effect and probably acts in concert with additional inhibitors of B cell genesis that are temporarily present following injury.

FO, but not MZ, B cells are reduced during SCI

We next addressed how acute and chronic SCI affect mature splenic B cell pools. For all experiments, FO B cell numbers were modestly reduced following acute or chronic SCI. These consistent reductions frequently, but not always, achieved statistical significance (Table II). In contrast, MZ B cell numbers were always either preserved or increased during both acute and chronic SCI (Table II). Variability in the degrees to which FO B cell numbers decreased and MZ B cell numbers expanded likely reflects a dynamic interplay of several factors. Foremost among these are the extent to which B lymphopoiesis is blunted and how quickly it is re-established, as these parameters will determine the degree of impact on FO B cell numbers and the associated skewing of cells into the MZ pool (25). Collectively, our findings extend prior observations demonstrating that reduced numbers of splenic B cells during acute SCI reflect a severe truncation of TR subsets, combined with a consistent but less profound depletion of FO, but not MZ, B cells (9). Moreover, we find that during chronic SCI, B lymphopoiesis has resumed, but mild reductions in TR and FO B cell numbers persist. Nonetheless, a large proportion of the mature B cell pool remains intact and, if functional, should be able to respond to antigenic challenge during chronic stages of SCI.

The TI type 2 immune response is profoundly decreased after chronic T3 injury

Despite the persistence of substantial mature B cell numbers, prior studies showed that acute SCI yields blunted primary TD Ab responses (9). Whether these defects extend to TI responses, as well as whether they continue during chronic injury, has not been determined. Accordingly, we examined NP-specific responses during chronic SCI. The NP-specific response in C57BL/6 mice is dominated by $Ig\lambda^+$ B cells, and thus responding cells can be tracked based on both NP-binding and Igλ expression (30). Additionally, numbers of NP-specific Ab-secreting cells (ASCs) and Ab concentrations can be assessed by ELISPOT and ELISA, respectively.

Using this model, we first tested whether chronic SCI affects TI type 2 responses using NP conjugated to the carbohydrate polymer Ficoll (NP-Ficoll). MZ B cells are responsible for most of the TI type 2 response and, as their numbers are not reduced following SCI, any differences in response magnitude likely reflect altered functionality (31). Mice were challenged with NP-Ficoll i.p. 28 d postinjury and responses were assessed 4.5 d later, at the peak of the response (32) (Fig. 3A). Compared to uninjured controls, mice with chronic T3 SCI, but not T3 laminectomy, had profoundly diminished NP-specific responses. This was evidenced by significantly decreased numbers of responding splenic $NP^+Ig\lambda^+$ B cells and NP-specific IgM-producing ASCs (Fig. 3B–D). In multiple experiments, serum NP-specific Ab concentrations were correspondingly reduced by 3- to 4-fold (data not shown). Although often mildly reduced compared with uninjured controls, TI type 2 responses in mice receiving T9 SCI were highly variable and less severely affected than those in mice receiving T3 SCI. Thus chronic T3 SCI severely impacts responsiveness to TI type 2 Ags despite the presence of mature B cells, including normal to elevated numbers of MZ B cells.

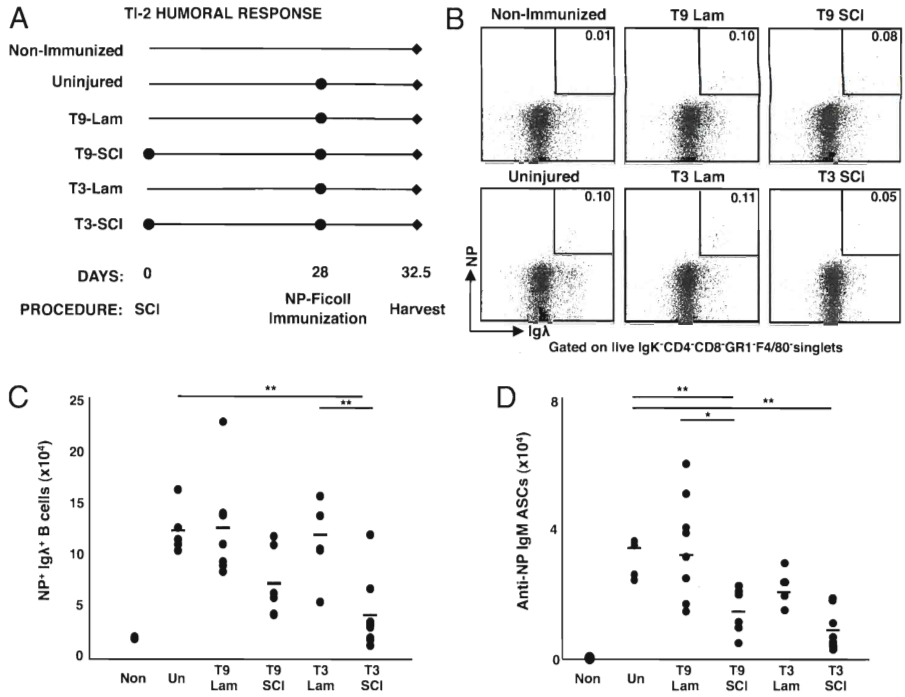

FIGURE 3. Humoral responses to TI type 2 Ags are decreased during chronic T3 SCI. (**A**) Experimental design for NP-Ficoll immunization: 28 d after SCI or postlaminectomy, mice were immunized with NP-Ficoll and the splenic tissue was analyzed at day 4.5. (**B**) Representative FACS gating strategy to identify NP-specific B cells. Dot plots show the frequency of Aqua$^-$CD4$^-$CD8$^-$F4/80$^-$Gr1$^-$Igκ^-Igλ^+NP$^+$ responding B cells among live singlets. (**C**) Total number of NP-specific B cells. (**D**) Total number of NP-specific IgM ASCs. For all data sets, n = 5 for uninjured, n = 7 for T9 laminectory (Lam), n = 6 for T9 SCI, n = 7 for T3 Lam, and n = 8 for T3 *0.01 $\leq p < 0.05$, ** $p < 0.01$.

Primary TD responses are decreased during chronic T3 SCI

To extend studies examining acute SCI, we asked whether defects in TD responses would persist during chronic SCI. Therefore, mice were challenged 28 d after SCI with NP-CGG, and responding cells in the spleen and bone marrow were evaluated (Fig. 4A). NP-specific TD responses peak between 10 and 14 d postimmunization (33), and thus we assessed the responses at day 14. Compared to uninjured and T9-injured controls, there was a significant reduction in the frequency and number of splenic NP$^+$Igλ^+ B cells in T3-injured mice (representative staining; Fig. 4B, 4C, Supplemental Fig. 2A). Furthermore, both the number and frequency of NP-specific IgG1 ASCs were significantly reduced in the BM and spleen after T3, but not T9, injury when compared with uninjured mice (Fig. 4D, Supplemental Fig. 2B). Consistent with a defect in the generation of these cells, serum levels of anti–NP-specific IgG1, but not IgM, were also significantly reduced in T3-injured mice compared with uninjured controls (Fig. 4E). In sum, chronic T3 injury severely diminishes the magnitude of primary TD responses.

Affinity maturation is intact during chronic SCI despite reductions in GC B cells and total high-affinity Ab

Effective TD responses require the formation of GCs, where B cells undergo class switch recombination, somatic hypermutation, and selection to yield the high-affinity, isotype-switched B cell clones that populate the memory and long-lived ASC compartments (34). Because the magnitude of TD responses was severely reduced during chronic SCI, we assessed whether the GC reaction was also compromised. NP-specific GC B cells can be identified cytofluorometrically by gating on activated B cells (IgD$^-$CD19$^+$) that bind NP and PNA and express high levels of Fas (representative gating strategy is shown in Fig. 5A) (35–37). At 14 d after immunization, T3-injured mice had significantly reduced numbers of NP-specific splenic GC B cells compared with uninjured mice (Fig. 5B).

Diminished GC B cell numbers could reflect either the loss of total responding cells, and therefore a loss of B cells initiating the GC fate, or an alteration to GC function resulting in the premature termination of GC B cell differentiation. Previous studies have established that whereas cells with both low and high affinity for NP bind to highly substituted NP-BSA (NP_{33}), only those with high affinity for NP bind to lowly substituted NP-BSA (NP_4) (33, 38). In this way, both total and high-affinity NP-specific Ab can be detected via ELISPOT and ELISA. Therefore, to examine whether reductions in GC B cells resulted in a loss of GC function, we assessed the relative affinity of responding ASCs and Abs from injured mice. Although the numbers and frequencies of high-affinity IgG1 ASCs in the spleen and BM were significantly reduced in T3-injured mice compared with uninjured mice, they were nonetheless detectable (Fig. 5C, Supplemental Fig. 2C). Similarly, ELISA revealed that both chronic T3 and T9 SCI mice had high-affinity NP-specific IgG1, albeit at significantly reduced levels compared with uninjured mice (Fig. 5D). The presence of high-affinity NP-specific ASCs and Abs suggests that the somatic hypermutation and selection processes required for affinity maturation are indeed intact following SCI. To confirm that the loss in total GC B cells and high-affinity NP-specific ASCs was due primarily to a paucity of responding B cells, we compared the ratio of high-affinity/total IgG1 ASCs and found that they were comparable to uninjured mice (Fig. 5E). Taken together, these observations show that the affinity maturation process per se is intact during chronic SCI, but diminished numbers of responding cells yield fewer high-affinity effectors.

Pre-existing B cell memory in the spleen is unaffected by chronic SCI

Memory B cells and long-lived plasma cells formed during primary humoral responses provide protective immunity to subsequent Ag exposures and are the mechanistic basis for most current vaccines (39). Accordingly, a potential risk facing those with SCI is loss of acquired immunity from prior natural immunizations or vaccinations. Thus, we addressed the affects of chronic SCI on established B cell memory and the ability to mount secondary responses. We immunized mice with NP-CGG, waited 54 d for the primary response to

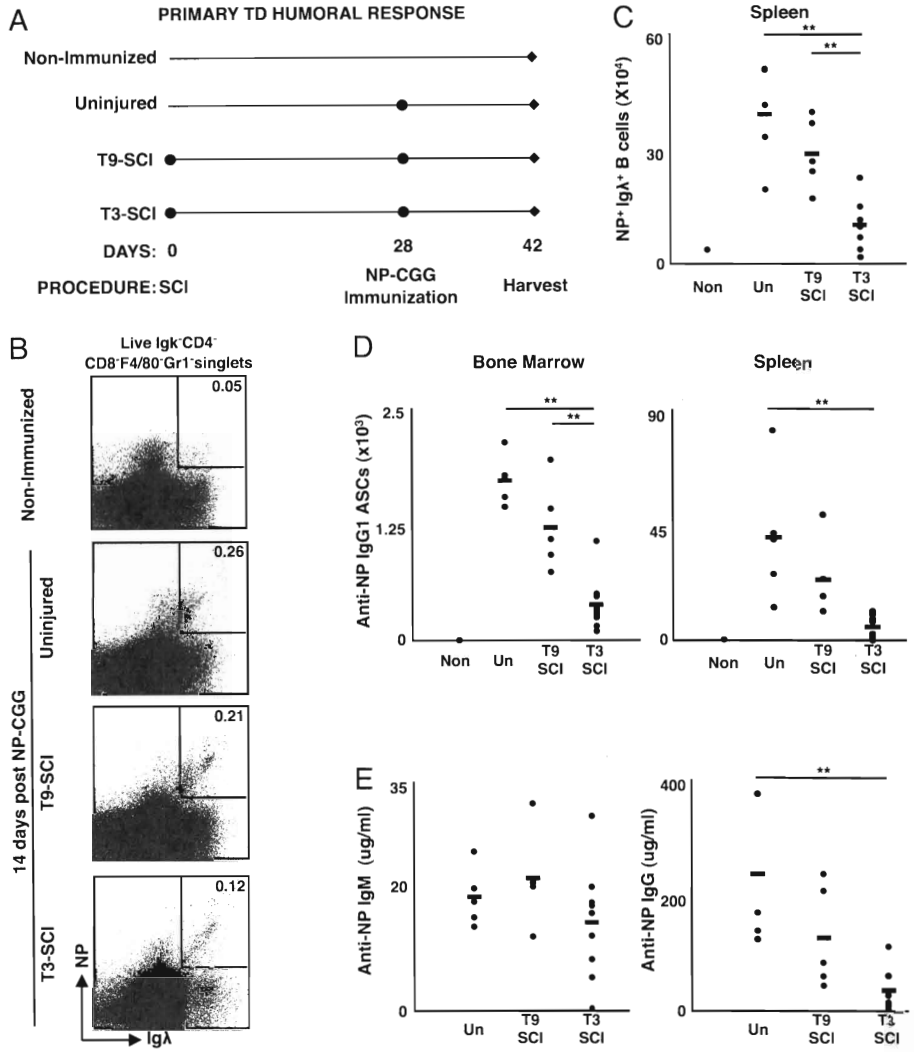

FIGURE 4. The magnitude of TD responses is reduced during chronic SCI. (**A**) Experimental design for NP-CGG immunization: 28 d after SCI, mice were immunized with NP-CGG, and at day 14 postimmunization, spleen and bone marrow from two hind limbs were analyzed. (**B**) Representative FACS gating strategy to identify NP-specific B cells. Dot plots show the frequency of $Aqua^-CD4^-CD8^-F4/80^-Gr1^-Ig\kappa^-Ig\lambda^+NP^+$ responding B cells among live singlets. (**C**) The total number of splenic NP-specific cells. (**D**) Total number of NP-specific IgG1 ASCs present in BM and spleen. (**E**) Serum levels of NP-specific IgM and IgG1 Ab above non-immunized controls. For all data sets, $n = 5$ for uninjured, $n = 5$ for T9 SCI, and $n = 9$ for T3 SCI. Data are representative of two experiments. $^{**}p < 0.01$.

wane while allowing memory pools to establish (33, 40), and then induced SCI (Fig. 6A). Twenty-eight days postinjury, one cohort of mice was analyzed without further treatment (designated as resting memory) and the second cohort was boosted with NP-CGG (designated as boosted memory). In the latter group, high-affinity ASCs were assessed 7 d postimmunization, at a time point well before new responders have undergone affinity maturation (33). Strikingly, we found no differences between the treatment groups in either the frequency or total number of high-affinity splenic ASCs (Fig. 6B) or the concentration of high-affinity Abs (Fig. 6C) in either the resting or the boosted memory responses. Thus, we conclude that despite the impaired capacity to mount primary TD responses following SCI, previously established humoral memory and the ability to respond to secondary challenge remain intact.

Discussion

These studies probe the impact of acute and chronic SCI on B lymphopoiesis, preimmune B cell homeostasis, and the capacity to mount primary or memory humoral responses. Our results reveal a transient cessation of BM B lymphopoiesis during acute T3 or T9 SCI that resolves within 4 wk injury. Consistent with this decreased BM output, splenic TR B cell numbers show a corresponding transient decline and resurgence. FO B cell numbers were consistently but mildly reduced following injury, and were most severely affected following T3 injury. In contrast, MZ B cell numbers were either unaffected or increased. Despite the presence of these mature preimmune B cells, both TI type 2 and primary TD humoral immune responses were profoundly reduced during chronic T3 injury. Finally, we find that SCI affects neither existing memory B cell numbers nor their ability to respond upon rechallenge. Collectively, these findings reveal previously unappreciated temporal and functional complexity in the impact of SCI on B cell compartments and humoral immunity.

The transient cessation of B lymphopoiesis following SCI likely reflects a combination of mechanisms. Stress hormones can both inhibit B lymphopoiesis and favor myelopoiesis, and they are elevated during acute injury (3, 9). However, B lymphopoiesis recovered within 4 wk despite continued elevation of corticosterone in chronically injured mice. These observations are consistent with those in chronic SCI patients, who have normal numbers of bone marrow lymphocytes despite elevated stress hormones (41, 42). Accordingly, transiently reduced B lymphopoiesis likely reflects additional or alternative mechanisms. Similar temporary reductions in B lymphopoiesis occur during inflammatory responses following immunization or infection (28, 29) and are associated with increases in proinflammatory cytokines such as TNF-α, IL-1b, or IFN-α/β (43–45). Inasmuch as these cytokines transiently increase at the site of spinal injury (46), it is tempting

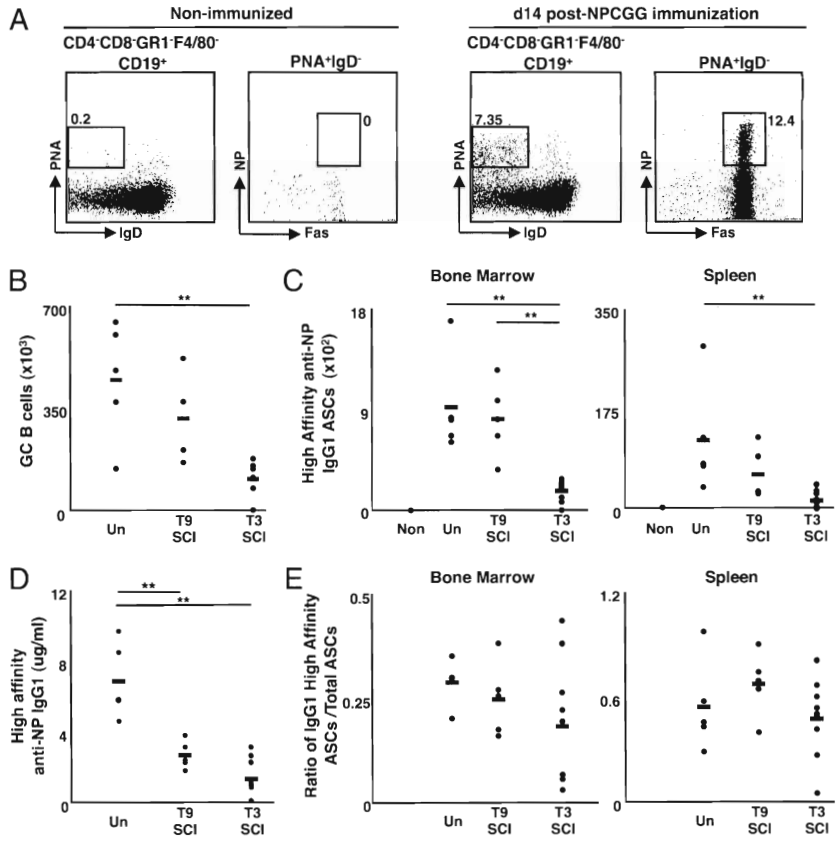

FIGURE 5. GC B cells and high-affinity ASCs are reduced in T3-injured mice after TD immunization. (**A**) Representative FACS plots for identification of NP-specific GC B cells in uninjured mice ($CD19^+CD4^-CD8^-F4/80^-Gr1^-IgD^-PNA^+NP^+Fas^+$). Numbers shown in each dot plot are percentages of each population within the parent gate. (**B**) The absolute number of GC B cells in the spleen (n = 4–5 mice/group). (**C**) Total number of high-affinity NP-specific IgG1 ASCs in BM and spleen. (**D**) Serum concentrations of NP-specific high-affinity IgG1 Ab above nonimmunized controls. (**E**) Affinity maturation within the spleen as determined by the ratio of high-affinity to total IgG1 ASCs (Fig. 4D); For (C)–(E), n = 5 for uninjured, n = 5 for T9 SCI, and n = 9 for T3 SCI. Data are representative of two experiments. $^{**}p < 0.01$.

to speculate that both inflammatory cytokines and stress hormones impact B lymphopoiesis during acute injury. B cell progenitors from T3, but not T9, SCI mice were significantly decreased when compared with laminectomy controls, and thus it remains possible that injury level impacts the extent to which B cell genesis is inhibited. However, we favor the notion that this reflects different levels of stress and inflammation, rather than a loss of sympathetic regulation, for several reasons. First, B cell genesis was significantly reduced in both T9 and T3 injured mice compared with uninjured controls, and both showed restoration after 4 wk,

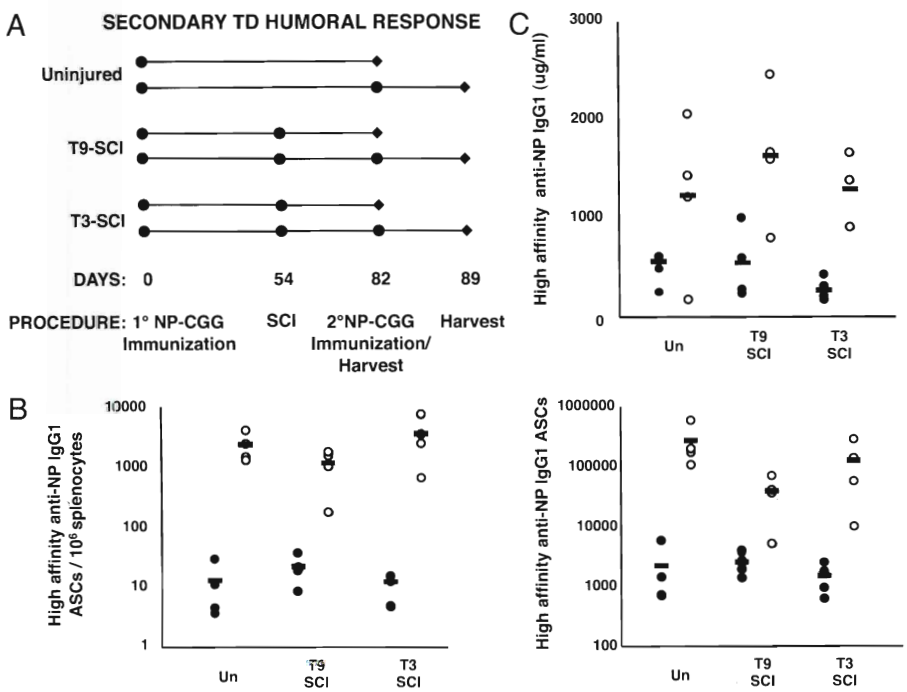

FIGURE 6. Secondary humoral responses are intact following chronic SCI. (**A**) Experimental design for assessment of secondary TD responses in injured and uninjured mice. Mice were immunized 54 d prior to SCI, and then 28 d after injury resting (●) and boosted (○) memory responses were assessed. (**B**) The frequency per million and total number of splenic high-affinity IgG1 ASCs and (**C**) high-affinity IgG1 anti-NP Ab above nonimmunized controls both before and after secondary challenge are shown (n = 4–5 mice/group). Data are representative of two experiments.

something that would not be predicted to occur if it were due to the disruption of supraspinal sympathetic regulation. Moreover, we also observed reductions in B cell genesis following laminectomy, further supporting the notion that stress and inflammation associated with injury per se, rather than supraspinal regulation, causes the blunting of B lymphopoiesis.

Although consistent with prior studies demonstrating reductions in total $CD19^+$ splenic B cells 3 d after T3 SCI (9), the detailed subsetting in this study reveals that multiple factors contribute to this overall phenomenon. Thus, reductions in the TR pools contribute substantially to initial splenic B cell losses, reflecting truncated BM output. Likewise, there is a reduction in the number of mature FO B cells following acute injury. Despite blunted B cell genesis, a large fraction of FO B cells and all MZ B cells remain, consistent with their comparatively slow turnover rates (22). Surprisingly, although BM output resumes, steady-state TR and FO B cell numbers are variably reduced during chronic injury (Table II). These continued reductions in splenic B cell populations 28 d after injury may reflect the severity of initial loss of B cell genesis and consequently differing rates of its restoration. Alternatively, reduced numbers of splenic B cell subsets are consistent with prior findings that acute T3 SCI increases the proportion of apoptotic cells found among splenic lymphocytes (9, 16). Because the ability to bind the cytokine B lymphocyte stimulator (BLyS, also termed BAFF) through BLyS receptor 3 (BR3) is essential for FO B cell survival (47–49), we assayed BR3 levels on FO B cells and found no difference between uninjured and injured mice (data not shown). The preservation of MZ B cell numbers under all conditions is consistent with the selective preservation of this subset even under B lymphopenic conditions (25). This likely reflects both a larger proportion of TR B cells assuming an MZ B fate as they mature, as well as the acquisition of MZ B cell characteristics by FO B cells driven by B lymphopenia. Taken together, these findings strongly suggest that SCI has a direct affect on peripheral B cell homeostasis.

Despite differential effects on mature, quiescent B cell pools, chronic T3 injury clearly impacts the magnitude of both TI and TD primary responses, which are dominated by MZ or FO B cells, respectively (31). The number of MZ B cells was not reduced after T3 injury, and thus simple reductions in numbers cannot explain the diminished TI responses. Whether SCI also negatively affects B1 *B* cells, which can contribute to TI immunity (50), or other cell types such as bystander T cells, which contribute to the magnitude of TI type 2 humoral responses, will be an important avenue for future exploration. Consistent with observations made during the acute phase of T3 SCI (9), the inability to mount optimal TD immune responses persisted during chronic injury, likely in part due to the reduced numbers of FO B cells observed. Alternatively, decreased FO B cell responsiveness may be due to SCI-induced impairments in T cell responses as reported (9, 10). Nevertheless, affinity maturation is unaffected by SCI, indicating that the process of somatic hypermutation and selection of high-affinity B cells within the GC reaction are largely independent of neuronal regulation. Because these key features of TD responses remain intact, strategies to induce protective humoral immunity after SCI might best be focused on increasing the numbers of initially responding B cells. The mechanism by which SCI results in such a dramatic loss of B cell function is likely multifaceted. Laminectomy did not hinder responses to TI challenge, suggesting that SNS innervation in part regulates these responses. However, administration of a β_2-adrenergic receptor blocker, butoxamine (5 mg/kg/d, administered i.p.), which inhibits signaling downstream of SNS-derived norepinephrine, did not restore B cell function during chronic SCI when administered days 0–4 after NP-Ficoll immunization (data not shown). Furthermore, SCI mice, but not laminectomy controls, had elevated levels of corticosterone during chronic injury, implicating the HPA axis. Collectively, these findings suggest that a combination of dysregulated SNS signaling and HPA axis stress hormones contributes to decreased B cell responsiveness following SCI.

Strikingly, SCI does not ablate previously established B cell memory, nor does it blunt secondary humoral responses, implying that memory B cells are unimpaired by injury. It is thus tempting to speculate that memory B cells are refractory to the immediate effects of SCI, and are independent of neuronal regulation. Regardless of underlying factors, this finding suggests that protective immunity established by prior vaccination or pathogen will remain intact following SCI. Whether protective immunity and memory can be established upon exposure to new TD Ags after SCI remains an open question. Indeed, primary TD responses are diminished during chronic SCI, but the ability to generate high-affinity B cells, and presumably the mechanisms to establish humoral memory, remain. Therefore, postinjury vaccination regimes might best focus on augmenting the frequency and expansion of initially responding cells to achieve protection. Alternatively, TI Ags will remain problematic, since prior memory for such Ags will not exist, and TI responses are persistently and severely compromised after T3 injury. Consequently, the use of conjugate vaccines, which couple TI epitopes to proteins to enable TD responses (51), might prove effective.

Acknowledgments

We thank the Reeve–Irvine Research Center and the Roman Reed Spinal Cord Research Program for facility and personnel support.

Disclosures

The authors have no conflicts of interest.

References

1. Meisel, C., J. M. Schwab, K. Prass, A. Meisel, and U. Dirnagl. 2005. Central nervous system injury-induced immune deficiency syndrome. *Nat. Rev. Neurosci.* 6: 775–786.
2. DeVivo, M. J., P. L. Kartus, S. L. Stover, R. D. Rutt, and P. R. Fine. 1989. Cause of death for patients with spinal cord injuries. *Arch. Intern. Med.* 149: 1761–1766.
3. Igarashi, S., K. L. Medina, T. Yokota, M. I. Rossi, N. Sakaguchi, P. C. Comp, and P. W. Kincade. 2005. Early lymphoid progenitors in mouse and man are highly sensitive to glucocorticoids. *Int. Immunol.* 17: 501–511.
4. Kohm, A. P., and V. M. Sanders. 1999. Suppression of antigen-specific Th2 cell-dependent IgM and IgG1 production following norepinephrine depletion *in vivo*. *J. Immunol.* 162: 5299–5308.
5. Nance, D. M., and V. M. Sanders. 2007. Autonomic innervation and regulation of the immune system (1987–2007). *Brain Behav. Immun.* 21: 736–745.
6. Podojil, J. R., and V. M. Sanders. 2003. Selective regulation of mature IgG1 transcription by CD86 and β_2-adrenergic receptor stimulation. *J. Immunol.* 170: 5143–5151.
7. Felten, S. Y., and J. Olschowka. 1987. Noradrenergic sympathetic innervation of the spleen: II. Tyrosine hydroxylase (TH)-positive nerve terminals form synapticlike contacts on lymphocytes in the splenic white pulp. *J. Neurosci. Res.* 18: 37–48.
8. Tabarowski, Z., K. Gibson-Berry, and S. Y. Felten. 1996. Noradrenergic and peptidergic innervation of the mouse femur bone marrow. *Acta Histochem.* 98: 453–457.
9. Lucin, K. M., V. M. Sanders, T. B. Jones, W. B. Malarkey, and P. G. Popovich. 2007. Impaired antibody synthesis after spinal cord injury is level dependent and is due to sympathetic nervous system dysregulation. *Exp. Neurol.* 207: 15–84.
10. Held, K. S., O. Steward, C. Blanc, and T. E. Lane. 2010. Impaired immune responses following spinal cord injury lead to reduced ability to control viral infection. *Exp. Neurol.* 226: 242–253.
11. Inman, D. M., and O. Steward. 2003. Physical size does not determine the unique histopathological response seen in the injured mouse spinal cord. *J. Neurotrauma* 20: 33–42.
12. Tuli, J. S., J. A. Smith, and D. B. Morton. 1995. Corticosterone, adrenal and spleen weight in mice after tail bleeding, and its effect on nearby animals. *Lab. Anim.* 29: 90–95.
13. Teasell, R. W., J. M. Arnold, A. Krassioukov, and G. A. Delaney. 2000. Cardiovascular consequences of loss of supraspinal control of the sympathetic nervous system after spinal cord injury. *Arch. Phys. Med. Rehabil.* 81: 506–516.

14. Cano, G., A. F. Sved, L. Rinaman, B. S. Rabin, and J. P. Card. 2001. Characterization of the central nervous system innervation of the rat spleen using viral transneuronal tracing. *J. Comp. Neurol.* 439: 1–18.
15. Inman, D., L. Guth, and O. Steward. 2002. Genetic influences on secondary degeneration and wound healing following spinal cord injury in various strains of mice. *J. Comp. Neurol.* 451: 225–235.
16. Lucin, K. M., V. M. Sanders, and P. G. Popovich. 2009. Stress hormones collaborate to induce lymphocyte apoptosis after high level spinal cord injury. *J. Neurochem.* 110: 1409–1421.
17. Allman, D., R. C. Lindsley, W. DeMuth, K. Rudd, S. A. Shinton, and R. R. Hardy. 2001. Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. *J. Immunol.* 167: 6834–6840.
18. Cancro, M. P. 2004. Peripheral B-cell maturation: the intersection of selection and homeostasis. *Immunol. Rev.* 197: 89–101.
19. Hardy, R. R., Y. S. Li, D. Allman, M. Asano, M. Gui, and K. Hayakawa. 2000. B-cell commitment, development and selection. *Immunol. Rev.* 175: 23–32.
20. Martin, F., and J. F. Kearney. 2002. Marginal-zone B cells. *Nat. Rev. Immunol.* 2: 323–335.
21. Osmond, D. G. 1986. Population dynamics of bone marrow B lymphocytes. *Immunol. Rev.* 93: 103–124.
22. Allman, D. M., S. E. Ferguson, V. M. Lentz, and M. P. Cancro. 1993. Peripheral B cell maturation, II: Heat-stable antigen^{hi} splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. *J. Immunol.* 151: 4431–4444.
23. Rolink, A. G., J. Andersson, and F. Melchers. 1998. Characterization of immature B cells by a novel monoclonal antibody, by turnover and by mitogen reactivity. *Eur. J. Immunol.* 28: 3738–3748.
24. Lindsley, R. C., M. Thomas, B. Srivastava, and D. Allman. 2007. Generation of peripheral B cells occurs via two spatially and temporally distinct pathways. *Blood* 109: 2521–2528.
25. Srivastava, B., W. J. Quinn, III, K. Hazard, J. Erikson, and D. Allman. 2005. Characterization of marginal zone B cell precursors. *J. Exp. Med.* 202: 1225–1234.
26. Sprent, J., and A. Basten. 1973. Circulating T and B lymphocytes of the mouse, II: Lifespan. *Cell. Immunol.* 7: 40–59.
27. Förster, I., and K. Rajewsky. 1990. The bulk of the peripheral B-cell pool in mice is stable and not rapidly renewed from the bone marrow. *Proc. Natl. Acad. Sci. USA* 87: 4781–4784.
28. Nagaoka, H., G. Gonzalez-Aseguinolaza, M. Tsuji, and M. C. Nussenzweig. 2000. Immunization and infection change the number of recombination activating gene (RAG)-expressing B cells in the periphery by altering immature lymphocyte production. *J. Exp. Med.* 191: 2113–2120.
29. Ueda, Y., M. Kondo, and G. Kelsoe. 2005. Inflammation and the reciprocal production of granulocytes and lymphocytes in bone marrow. *J. Exp. Med.* 201: 1771–1780.
30. Riley, R. L., and N. R. Klinman. 1985. Differences in antibody repertoires for (4-hydroxy-3-nitrophenyl)acetyl (NP) in splenic vs immature bone marrow precursor cells. *J. Immunol.* 135: 3050–3055.
31. Guinamard, R., M. Okigaki, J. Schlessinger, and J. V. Ravetch. 2000. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat. Immunol.* 1: 31–36.
32. García de Vinuesa, C., P. O'Leary, D. M. Sze, K. M. Toellner, and I. C. MacLennan. 1999. T-independent type 2 antigens induce B cell proliferation in multiple splenic sites, but exponential growth is confined to extrafollicular foci. *Eur. J. Immunol.* 29: 1314–1323.
33. Takahashi, Y., P. R. Dutta, D. M. Cerasoli, and G. Kelsoe. 1998. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl, V: Affinity maturation develops in two stages of clonal selection. *J. Exp. Med.* 187: 885–895.
34. Tarlinton, D. 1998. Germinal centers: form and function. *Curr. Opin. Immunol.* 10: 245–251.
35. Kraal, G., I. L. Weissman, and E. C. Butcher. 1982. Germinal centre B cells: antigen specificity and changes in heavy chain class expression. *Nature* 298: 377–379.
36. Lagresle, C., C. Bella, P. T. Daniel, P. H. Krammer, and T. Defrance. 1995. Regulation of germinal center B cell differentiation: role of the human APO-1/Fas (CD95) molecule. *J. Immunol.* 154: 5746–5756.
37. Rose, M. L., M. S. Birbeck, V. J. Wallis, J. A. Forrester, and A. J. Davies. 1980. Peanut lectin binding properties of germinal centres of mouse lymphoid tissue. *Nature* 284: 364–366.
38. Herzenberg, L. A., S. J. Black, T. Tokuhisa, and L. A. Herzenberg. 1980. Memory B cells at successive stages of differentiation: affinity maturation and the role of IgD receptors. *J. Exp. Med.* 151: 1071–1087.
39. Elgueta, R., V. C. de Vries, and R. J. Noelle. 2010. The immortality of humoral immunity. *Immunol. Rev.* 236: 139–150.
40. Weiss, U., and K. Rajewsky. 1990. The repertoire of somatic antibody mutants accumulating in the memory compartment after primary immunization is restricted through affinity maturation and mirrors that expressed in the secondary response. *J. Exp. Med.* 172: 1681–1689.
41. Iversen, P. O., A. Nicolaysen, N. Hjeltnes, A. Njå, and H. B. Benestad. 2004. Preserved granulocyte formation and function, as well as bone marrow innervation, in subjects with complete spinal cord injury. *Br. J. Haematol.* 126: 870–877.
42. Campagnolo, D. I., J. A. Bartlett, R. Chatterton, Jr., and S. E. Keller. 1999. Adrenal and pituitary hormone patterns after spinal cord injury. *Am. J. Phys. Med. Rehabil.* 78: 361–366.
43. Ueda, Y., K. Yang, S. J. Foster, M. Kondo, and G. Kelsoe. 2004. Inflammation controls B lymphopoiesis by regulating chemokine CXCL12 expression. *J. Exp. Med.* 199: 47–58.
44. Dorshkind, K. 1988. IL-1 inhibits B cell differentiation in long term bone marrow cultures. *J. Immunol.* 141: 531–538.
45. Lin, Q., C. Dong, and M. D. Cooper. 1998. Impairment of T and B cell development by treatment with a type I interferon. *J. Exp. Med.* 187: 79–87.
46. Pineau, I., and S. Lacroix. 2007. Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved. *J. Comp. Neurol.* 500: 267–285.
47. Harless, S. M., V. M. Lentz, A. P. Sah, B. L. Hsu, K. Clise-Dwyer, D. M. Hilbert, C. E. Hayes, and M. P. Cancro. 2001. Competition for BLyS-mediated signaling through Bcmd/BR3 regulates peripheral B lymphocyte numbers. *Curr. Biol.* 11: 1986–1989.
48. Miller, D. J., and C. E. Hayes. 1991. Phenotypic and genetic characterization of a unique B lymphocyte deficiency in strain A/WySnJ mice. *Eur. J. Immunol.* 21: 1123–1130.
49. Schneider, P., F. MacKay, V. Steiner, K. Hofmann, J. L. Bodmer, N. Holler, C. Ambrose, P. Lawton, S. Bixler, H. Acha-Orbea, et al. 1999. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J. Exp. Med.* 189: 1747–1756.
50. Martin, F., A. M. Oliver, and J. F. Kearney. 2001. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* 14: 617–629.
51. Lesinski, G. B., and M. A. Westerink. 2001. Novel vaccine strategies to T-independent antigens. *J. Microbiol. Methods* 47: 135–149.