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Stroke Induces a Rapid Adaptive Autoimmune Response to Novel Neuronal Antigens

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Abstract

Stroke affects millions of people worldwide every year. Despite this prevalence, mechanisms of long-term injury and repair within the ischemic brain are still understudied. Sterile inflammation

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occurs in the injured brain after stroke, with damaged tissue exposing central nervous system (CNS)-derived antigen that could initiate potential autoimmune responses. We used a standard immunology-based recall response assay for murine immune cells, isolated from the cervical lymph nodes and spleen after transient stroke, to determine if stroke induces autoreactivity to CNS target antigens. Our assays included novel neuronal peptides, in addition to myelin-, nuclear-, glial-, and endothelial-derived peptides. Autoimmune responses to an antigen were considered positive based on proliferation and activation over non-stimulated conditions. Stroke induced a significant increase in autoreactive CD4⁺ and CD8⁺ T cells, as well as autoreactive CD19⁺ B cells, as early as 4 days after stroke onset. Mice with large infarct volumes exhibited early T and B cell autoreactivity to NR2A, an NMDA receptor subunit, in cells isolated from lymph nodes but not spleen. Mice with small infarct volumes exhibited high autoreactivity to MAP2, a dendritic cytoskeletal protein, as well as myelin-derived peptides. This autoimmunity was maintained through 10 days post-stroke in both lymph nodes and spleen for all lymphocyte subsets. Sham surgery also induced early autoreactive B cell responses to MAP2 and myelin. Based on these observations, we hypothesize that stroke induces a secondary, complex, and dynamic autoimmune response to neuronal antigens with the potential to potentiate, or perhaps even ameliorate, long-term neuroinflammation.

Introduction

Stroke is caused by a loss of blood flow to the brain, which can result in permanent neurological damage. Worldwide, approximately 15 million individuals will suffer a stroke each year, with 800,000 in the U.S. alone (Go *et al.*, 2013). At present, there is only one pharmacological agent, tissueplasminogenactivator, approved for treating ischemic stroke in the acute 4-hour window after onset (Wardlaw *et al.*, 2012). While advances in experimental animal models have identified a substantial number of other promising neurotherapeutics, none have proved efficacious in clinical trials (Kidd, 2009). This suggests the need for a fundamental paradigm shift in stroke research to focus on long-term mechanisms of injury and repair in order to extend potential interventions beyond the acute window. To this end, both preclinical and clinical evidence shows that there is a complex and dynamic interplay between the post-stroke CNS and the immune system that potentially influences stroke pathology for weeks to months after injury onset (Chamorro *et al.*, 2012; Doyle *et al.*, 2015; Planas *et al.*, 2012b; Sardi *et al.*, 2011).

Animal stroke models reveal that circulating leukocytes diapedese into the brain and accumulate at the site of injury within hours after stroke, with various subsets of leukocytes either exacerbating or ameliorating injury (Chu *et al.*, 2014; Gelderblom *et al.*, 2009; Kipnis *et al.*, 2002). B cells, T cells, and natural killer T cells (NK-T) cells comprise the adaptive arm of the immune system, with their interactions initiated by recognition of antigen (see reviews Iadecola and Anrather, 2011; Jin *et al.*, 2010; Sardi *et al.*, 2011). In response to activation, these lymphocytes secrete cytokines, chemokines, and antibodies, in addition to directly binding target cells, to promote an active adaptive immune response. CD4 T cells (i.e., T helper cells) are activated when peptide antigens are presented on the surface of major histocompatibility class (MHC) II antigen presenting cells, such as macrophages, dendritic cells, or B cells. CD4 T cells are robustly activated after stroke, leading to an

induction of an effector population that contributes to post-stroke pathology (Chamorro *et al.*, 2012; Kleinschnitz *et al.*, 2010; Lazar-Molnar and Tebo, 2015; Yilmaz *et al.*, 2006). CD8 T cells (i.e., cytotoxic T cells) respond to antigen peptides on MHC I molecules, which are present on most cell types, and are also actively involved in post-stroke inflammation (Gelderblom *et al.*, 2009). Membrane-bound immunoglobulin allows B cells to bind antigens with great specificity and, if activated by cytokines from CD4 T cells, to secrete antibodies specific to a particular antigen. It is a common view that unlike T cells, B cells do not contribute to stroke pathology (Chamorro *et al.*, 2012; Kleinschnitz *et al.*, 2010; Yilmaz *et al.*, 2006), though they do have the potential to contribute to neuroprotection and post-stroke repair (Bodhankar *et al.*, 2013; Monson *et al.*, 2014b).

Most animal studies of CNS-antigenic specificity after stroke have focused on myelin antigens, common targets during demyelinating diseases like multiple sclerosis (Hussain *et al.*, 2014). In our study, using a common form of ischemic stroke injury, we sought to determine if there was an early adaptive autoimmune response to myelin-derived antigens (e.g., myelinbasicprotein; MBP), but also novel neuronally-derived antigens [e.g., microtubule-associated protein 2 (MAP2) and N-methyl-D-aspartate (NMDA) receptor subunit NR2A]. In total, we investigated autoreactive responses to myelin, neuronal, glial, and endothelial peptides, as well as responses to nuclear receptor subfamily 2, group F, member 2 (NR2F), a protein that is critical for neuronal and vascular development (Kim *et al.*, 2009; Lin *et al.*, 2011) and found in the adult mouse brain (Fuentelba *et al.*, 2010). We also determined if the level of braininjury influenced the breadth and scope of antigen-specific responses. Our detailed study of the adaptive immune response following stroke, and in particular the novel rapid response we found even in mice exhibiting small infarct volumes, should be foundational for future studies focusing on neuronal autoimmunity in the injured CNS. Understanding the progression of neuronally-derived autoimmune responses — and whether these responses may contribute to long-term mechanisms of recovery — will aid the development of more effective and clinically translatable immunotherapeutic strategies for the extended treatment of stroke.

Experimental Methods and Design

Transient middle cerebral artery occlusion (tMCAo)

All experiments were approved by the University of Texas Southwestern Medical Institutional Animal Care and Use Committee. Outbred Swiss Webster male mice, 6-8 weeks, were maintained on a 12-hour light cycle, and allowed food and water *ad libitum*. All experimental time points were replicated on a minimum of two separate days. Mice were initially randomized to either sham or tMCAo groups and researcher-blinded for the remainder of the study. As previously described (Monson *et al.*, 2014b), mice were anesthetized (2% isoflurane/70% NO₂/30% O₂) at 37°C and the common carotid artery (CCA) permanently ligated. Transcranial laser doppler flowmetry assessed baseline cerebral blood flow (CBF) in the middle cerebral artery (MCA) prior to insertion of an intraluminal filament through the CCA to the MCA origin. Upon successful occlusion (>80% reduction CBF), mice were placed in an incubator. After 60 minutes, mice were re-anesthetized and the suture removed. Reperfusion (>50% baseline CBF) was confirmed, and mice not

meeting the occlusion/reperfusion criteria were removed from the study (n=8 from 38 mice). An additional 4 mice died post-operatively. Sham mice were anesthetized for CCA ligation but without filament insertion (n=19). Mice were monitored during recovery from anesthesia and returned to their home cages. At 4, 8, or 10 days after tMCAo, animals were sacrificed by isoflurane overdose and spleens and cervical lymph nodes harvested for autoreactivity assays. Whole brains were dissected, placed into an acrylic brain matrix, and sectioned at 1 mm intervals. Brain slices were placed into 2% 2,3,5-triphenyl tetrazolium chloride (TTC) solution for approximately 5 minutes. TTC solution was replaced with 4% paraformaldehyde and tissues allowed to fix overnight. Infarct volumes were quantified by a blinded observer using Image J analysis software and corrected for edema on corresponding right hemispheric areas as control (Stowe *et al.*, 2011). Five mice did not undergo TTC analysis during the first experiment, as it was not part of the original experimental design.

Determination of autoreactive responses

Individual cervical lymph nodes and spleens were processed through 70 μm mesh filters and splenic cells purified using Lympholyte-M treatment, as per manufacturer's instructions (Ortega *et al.*, 2013). Antigen-specific responses were determined using the carboxyfluorescein succinimidyl ester (CFSE; Invitrogen)-based dilution assay, adapted for mice, using bulk splenocytes and cervical lymphnode cells. Cells were resuspended ($1 \times 10^6/\text{mL}$ in 0.1 M PBS) and incubated for 7 minutes (0.18 μM CFSE; 37°C). Cells were washed twice with serum-containing media, resuspended in culture media ($1 \times 10^6/\text{mL}$ in RPMI 1640, supplemented with 10% fetal calf serum, L-glutamine, penicillin, streptomycin, HEPES buffer, non-essential amino acids, sodium pyruvate, and β -mercaptoethanol), and cultured with indicated antigens at 37°C and 5% CO_2 . We determined responses to well characterized myelin peptides, such as myelin oligodendrocyte protein (MOG)₃₅₋₅₅, proteolipid protein (PLP)₁₇₈₋₁₉₁, and MBP_(Ac1-11), synthesized by the UT Southwestern Protein Chemistry core (Miller *et al.*, 2001; Stromnes and Goverman, 2006). We also generated novel CNS specific antigens NR2A (UniProt Q12879), MAP2 (P11137), and NR2F (Entrezgene proteins) with overlapping 15-mers peptides synthesized by BioSynthesis and purity qualified by RP-HPLC, with specific peptide sequences shown in **Table 1**. In order to use cells more efficiently, we used peptides pools. On day 6 of culture, cells were washed with fluorescence activated cell sorting (FACS) buffer (PBS with 1% bovine serum albumin and 0.1% Na-Azide) and stained with fluorescently tagged antibodies against TCR β -BV510 (Biolegend), CD4-v450, CD19-PE, CD8-APC, and CD25-PCY7 (all Tonbo) for 30 minutes at 4°C in the dark. Cells were washed and fixed (1% paraformaldehyde). Flow cytometric data (100,000 lymphocyte-gated events) were acquired on a BD FACS Canto flow cytometer (UT Southwestern Children's Research Institute FlowCytometry Facility) using BD FACS Diva software v8.0. A response was considered positive when the delta proliferation fraction (PF, test condition – non-stimulated condition) exceeded 1% and the stimulation index (SI, test condition/non-stimulated condition) was greater than 2 (Crawford *et al.*, 2004; Ortega *et al.*, 2013).

Statistics

All studies were performed by researchers blinded to experimental conditions. Statistical analyses between groups were performed using Graph Pad Prism 6.0. Between-group

differences in PF by day were evaluated using an unpaired two-tailed Student t test. Within group differences across days were evaluated for each group by one-way ANOVA with Tukey post-hoc analysis. All values are shown as mean \pm standard deviation (SD) and $p < 0.05$ was considered statistically significant.

Results

An early splenic CD4 T cell autoimmune response to neuronal and myelin antigen is associated with better recovery

The magnitude of stroke-induced, CNS-antigen specific autoimmunity was determined at 4, 8, and 10 days following tMCAo in immune cells isolated from the cervical lymph nodes and spleen (**Figure 1**). Four days after stroke, we observed a robust global, CNS-specific autoimmune response in CD4 T cells from both the cervical lymph nodes ($p < 0.0001$; Figure 1C) and spleen ($p < 0.0001$; Figure 1D). This early autoimmune response coincided with elevated infarct volumes ($38 \pm 36 \text{ mm}^3$; $n = 11$; $p = 0.06$) compared to sham ($10 \pm 7 \text{ mm}^3$; $n = 7$; data not graphed). It should be noted that the sham CCA ligation still resulted in areas of injury, as identified by TTC. The largest magnitude of autoreactivity for CD4 T cells isolated from the cervical lymph nodes was found 10 days post stroke (Figure 1C). When considering the between-day progression of autoreactivity by one-way ANOVA, day 10 was elevated over both day 4 ($p < 0.01$) and day 8 ($p < 0.001$) levels ($F_{(2,316)} = 8.19$; $p < 0.001$). CD4 T cells from the cervical lymph nodes were minimally activated at day 8 post-stroke. In contrast, autoreactivity peaked at 8 days after stroke in splenic CD4 T cells ($p < 0.001$) with a subsequent decline at day 10 ($p < 0.01$, day 8 vs. day 10; $F_{(2,467)} = 6.35$; $p < 0.01$).

Next, we sought to both identify the peptides eliciting the activation, and correlate the responses to the magnitude of infarct volume (**Figure 2**, Table 1) at 4 days post-stroke. The consistent autoimmune response from CD4 T cells isolated from the cervical lymph nodes included responses to myelin, NR2A ($p < 0.01$), MAP2, and NR2F peptides. Splenic CD4 T cells also responded to myelin ($p < 0.05$), MAP2 ($p < 0.01$), and NR2F, with no response to the NMDA receptor subunit NR2A. The two animals with the largest infarct volumes exhibited autoimmune responses to NR2A in the cervical lymph nodes, in the complete absence of splenic NR2A autoimmune responses. This suggests a potential activation of T cells by antigen presenting cells migrating from the injured CNS, instead of a blood-borne antigen response subsequent to blood-brain barrier breakdown that could activate splenic populations (Sardi *et al.*, 2011). The specificity of our CNS-derived antigen activation assay was confirmed by the lack of splenic NR2A, splenic, and lymphnode vWF autoimmune responses from these mice. In direct contrast, the most robust myelin (Figure 2B, red circle) and MAP2-derived (Figure 2B, orange circle) autoreactivity occurred in splenic CD4 T cells isolated from animals with the small ($2\text{-}18 \text{ mm}^3$) to moderate (45 mm^3) infarct volumes. Surprisingly, these animals, exhibiting the greatest resistance to stroke-induced injury, also showed the highest MAP2 autoreactivity in CD4 T cells from the cervical lymph nodes.

Table 1 shows peptide sequences for MAP2, NR2A, and NR2F that elicited CD4 T cell autoimmune responses. MAP2 is a very large protein, yet both our first and second pool of peptide sequences elicited immunogenic responses from CD4 T cells of the spleen and cervical lymph nodes. Several NR2A sequences also exhibited consistent immunogenicity

between compartments. Future studies are needed to verify the location of specific sequence regions on both of these proteins, confirm immunogenicity, and determine if autoreactive responses to MAP2 and NR2A occur in other CNS disease states. Take together, these data show that CD4 T cells specific to CNS antigens are quickly activated, and although brain injury is resolving over the course of 10 days post-tMCAo, there is still an active CNS-specific autoimmune-eliciting signal in the periphery. Furthermore, the majority of the significant response is focused to neuronal and myelin peptides which could be highly oligoclonal in nature, as stroke induces clonal T cell expansion (Liesz *et al.*, 2013), a possibility that needs to be addressed in future experiments.

Transient splenic CD8 T cell responses to CNS antigens precede activation in the cervical lymph nodes

We also quantified CD8 T cell responses to CNS antigen within the same cultures, and again found an early autoimmune response in both the cervical lymph nodes ($p < 0.01$; Figure 1E) and the spleen ($p < 0.001$; Figure 1F). Like the CD4 T cell population, the magnitude of autoreactivity in the cervical lymph node peaked at day 10 ($F_{(2,313)} = 16.27$; $p < 0.0001$) compared to the early elevation at day 4 ($p < 0.0001$) and the complete loss of autoreactivity at day 8 ($p < 0.0001$). CNS-directed autoreactivity of CD8 T cells isolated from the spleen was not significantly sustained beyond the early day 4 time point after stroke. There was no CD8 T cell-originating autoimmune response for the mouse with the largest infarct volume (122 mm^3 ; **Figure 3**) despite CD4 T cell activation in the cervical lymph node (Figure 2A, black circle). However, the significant response for NR2A in the cervical lymph nodes ($p < 0.05$) was solely reliant upon the mouse with the second largest infarct volume (74 mm^3 ; Figure 3A, grey circles). The only CD8 responses to NR2F and vWF were also from this mouse, with no concomitant autoreactivity in splenocytes isolated from this animal. This again suggests an NR2A-derived antigen signal that activates CD8 T cells, but only for moderately injured mice. Future studies should determine if very large infarcts somehow suppress or delay autoimmune responses in the draining lymph nodes. Similarly in the spleen, a moderately-injured mouse (36 mm^3 ; red circles) dominated the autoimmune responses to myelin and NR2F ($p < 0.05$). Unfortunately, the remaining responders to NR2F do not have corresponding infarct volume data. MAP2 autoreactivity in the spleen occurs only in mice with small and medium-sized infarct volumes, though unlike additional CD4 T cell-mediated MAP2 autoreactivity in the cervical lymph node, the mouse with the smallest infarct volume did not show a CD8 T cell response.

The first and second MAP2 peptide sequence pools that elicited an autoreactive CD4 T cell response also induced CD8 T cell activation (Table 1). Though as with CD4 T cell responses, many MAP2 peptide sequence pools that did not activate an autoimmune response in the cervical lymph nodes did trigger CD8 T cell responses in the spleen, again indicating antigen specific activation. There is a broad range of NR2A peptide responses for CD8 T cell activation also found in CD4 T cells, but not for either population isolated from the spleen. Taken together, these data show that CD8 T cells are robustly activated following stroke induction, but this activation may be dependent upon the size of CNS injury. CD8 T cell responses are lost by one-week post-stroke and this suggests a

trafficking of the effector population from the peripheral immune compartment to quite possibly the site of tissue pathology.

Stroke induces a rapid and sustained B cell response to both neuronal and myelin antigen

To our knowledge an autoimmune response following stroke has not been associated with CD19⁺ cells (Sardi *et al.*, 2011). Interestingly, we found an early day 4 activation of B cells to CNS-specific antigens in both the cervical lymph nodes ($p < 0.01$) and the spleen ($p < 0.01$), with at least one positive response from every sham and tMCAo-induced mouse, with the exception of the mouse with the largest infarct volume (Figure 1G-H, **Figure 4**). Unlike either T cell population, however, the magnitude of response at day 4 in the cervical lymph nodes was equivalent to the magnitude of response at day 10, suggesting an early and robust B cell activation by CNS antigen that is maintained during the first weeks after stroke. Day 10 autoreactivity remained elevated ($p < 0.05$) in the spleen, which did not occur with either of the T cell subsets. B cells from the cervical lymph nodes exhibited a similar biphasic autoreactivity, with lower levels on day 8 compared to day 4 ($p < 0.05$) and day 10 ($p < 0.05$; $F_{(2,318)} = 4.02$; $p < 0.05$).

Sham surgery also activated B cell autoimmune responses. In fact, elevations on day 8 in splenic B cells ($F_{(2,271)} = 5.24$; $p < 0.01$) preceded elevations in B cell autoreactivity on day 10 in the cervical lymph nodes ($F_{(2,174)} = 17.23$; $p < 0.0001$). This level of response, across days and in both the sham and stroke populations, may reflect a sustained B cell autoimmune activation after even very mild CNS injury, as may occur after CCA ligation and anesthesia exposure. However, CD8 T cell autoreactivity to myelin peptides has been found in healthy adults in the absence of autoimmune disease (Crawford *et al.*, 2004). Therefore, the generalized B cell autoimmune response could also reflect a previously undiscovered basal fluctuation in neuronal autoimmunity independent of CNS injury.

While almost every mouse exhibited B cell-mediated autoreactivity at 4 days after tMCAo or sham surgery (Figure 4), only MAP2 autoreactivity in the cervical lymph nodes showed significance ($p < 0.05$). As with T cells, B cell autoreactivity to myelin, NR2A, and NR2F in the cervical lymph nodes was predominated by animals with moderate to large infarct volumes. The mouse with the smallest infarct volume, however, had the highest PF for MAP2 in the cervical lymph node, which seems counterintuitive to CNS antigen exposure being secondary to ischemic tissue damage. Several sham mice also showed responses to myelin, NR2A, and NR2F, a surprising response that again seems independent of injury and warrants further investigation. The heterogeneous response of B cells to CNS antigen was recapitulated in the splenic B cell populations and included trends for both post-stroke myelin ($p = 0.06$) and NR2F autoreactivity ($p = 0.06$). As with T cells, NR2A autoreactivity is predominantly restricted to the cervical lymph nodes, though the second peptide sequence did elicit one weak response in splenic B cells (Table 1). Splenic B cells show the broadest range of MAP2 peptide responses from any of the lymphocyte populations, though future studies should attempt to determine which of these peptides is truly immunogenic. Taken together, these data show that B cells activated following stroke exhibit a global, complex, and sustained autoimmune response to CNS antigen that may indicate a very active role for T and B cells in stroke damage and/or repair.

Discussion

Understanding the role of post-stroke inflammation, including autoimmunity, is an evolving high priority for NIH research (Grotta *et al.*, 2008). Early CNS-directed autoimmunity was studied predominantly in animal models of multiple sclerosis (Hong *et al.*, 2009; York *et al.*, 2010). Now, however, CNS-directed autoimmunity is increasingly observed in other neurodegenerative diseases, including stroke (Planas *et al.*, 2012a), traumatic brain injury (Schwartz and Moalem, 2001; Weissman *et al.*, 2011), and Alzheimer's disease (Monson *et al.*, 2014a; Sardi *et al.*, 2011). The role of autoreactive adaptive immunity is not only now applicable to many CNS diseases, but is also undergoing a paradigm shift from the concept that all autoimmunity results in detrimental effects. While CD8 and CD4 T cells can target neurons to impede electrical signal, induce excitotoxicity, and promote cell death (Bien *et al.*, 2002; Laguna Goya *et al.*, 2011; Maehlen *et al.*, 1988), MBP-specific autoreactive T cells also function during health to maintain basal neurogenesis and spatial learning (Radjavi *et al.*, 2014). In fact, the adoptive transfer of MBP-specific T cells into neonatal mice does not induce autoimmune disease during adulthood (Kawakami *et al.*, 2005). The mice in our study that exhibited minimal CNS injury, despite an identical duration of stroke, demonstrated high autoreactivity to myelin and MAP2 in splenic lymphocytes. While this could be the result of a sequestration of dangerous autoreactive B and T cells in the spleen, which could reduce brain injury, high MAP2 autoreactivity also is found in the draining lymph nodes. Perhaps, instead, autoreactivity can also be used to home beneficial cell populations, including regulatory T cells and B cells (Bodhankar *et al.*, 2013; Brea *et al.*, 2014; Liesz *et al.*, 2009), to neurons at risk of injury or death, though to what mechanistic end remains to be elucidated. Either way, the adoptive transfer of CD4 T cells can improve neuronal plasticity after CNS trauma (Ishii *et al.*, 2012), which suggests some immune populations may be necessary for post-stroke neuronal anatomical and functional changes that contribute to functional recovery.

After stroke, lymphocytes encounter CNS-derived antigens and acute autoimmune responses against the brain develop (see reviews Becker, 2009; Iadecola and Anrather, 2011; Sardi *et al.*, 2011). One of the few studies of post-stroke neuronal autoimmunity found that MHC II receptors colocalized with brain-derived antigens in the cervical lymph nodes and palatine tonsils of stroke patients (Planas *et al.*, 2012a). Specifically, colocalization with myelin-derived epitopes (e.g., MBP) appeared to be detrimental at 90 days after stroke, as interactions between MBP and lymphocytes correlated with larger infarct volumes. In the same study, MHC II expression of MAP2 and NR2A both correlated with smaller infarct volumes and better long-term recovery at 90 days. Our study, however, revealed that larger infarct volumes 4 days after tMCAo corresponded with increased T and B cell autoreactivity in the cervical lymph node to NR2A, and to a lesser extent, myelin. Autoimmunity to the NMDA receptor is involved in encephalitis, with clinical presentations including memory deficits, seizures and psychosis (Lazar-Molnar and Tebo, 2015). Although this is believed to be mediated by NMDA autoantibodies from B cells, it is possible that CD4 T cell responses to NR2A, which is concomitant with B cell activation in our data, may predispose a patient to this disease. Multiple strokes in patients increase serum levels of NR2A protein (Weissman *et al.*, 2011), which could increase the risk of antigen exposure and potentially

detrimental NR2A-specific autoreactivity. As cognitive decline is common to stroke survivors and mediated by B cells in animal models of stroke (Doyle *et al.*, 2015), future studies should determine whether an NMDA-specific autoimmune response limits functional recovery following stroke.

What is more in line with the correlation of neuronal autoreactivity to better stroke recovery in the clinical data (Planas *et al.*, 2012a) is the high autoreactive response to MAP2 for both T and B cells from the spleens of mice with low to moderate CNS injury. MAP2 is a cytoskeletal protein found in dendrites that is crucial to both dendritic arborization and pruning during post-stroke neuronal plasticity that occurs in both the ischemic and the uninjured hemispheres during behavioral recovery (Hsu and Jones, 2006). Specifically, the upregulation of MAP2 associates with reduced infarct volumes and better functional recovery (Zhang *et al.*, 2012). In stroke patients, also with better functional recovery, CD68⁺ macrophages isolated from palatine tonsils and cervical lymph nodes colocalized with MAP2 (Planas *et al.*, 2012a), which indicates an innate immune response to MAP2 that may precede a MAP2-directed autoimmune CD4 T cell response. While the time frame between our data (4 days) and the clinical data (90 days) is greatly different, the correlation between high autoreactivity to MAP2 and less severe CNS injury suggests that autoimmune activation of MAP2 may either preserve neurons at-risk of injury in the ischemic brain, or promote recovery mechanisms within the injured cortex.

Unfortunately, the data regarding myelin autoreactivity on stroke outcome are ambiguous. Both MBP antigen presentation prior to stroke (Becker *et al.*, 1997) and a sustained regulatory T cell response to MBP (Kala *et al.*, 2011) were associated with improved long-term recovery in mice. But the determination of whether myelin autoimmune responses are detrimental or beneficial after stroke in patients is confounded by post-stroke co-morbidities with infections. A clinical study of 114 patients showed that at 90 days after stroke onset, the severity of functional recovery correlated with enhanced CD4 T cell responses to MBP, with autoreactivity exacerbated by infection and fever within the first 15 days after stroke (Becker *et al.*, 2011). The authors thus concluded that autoimmune responses to myelin were associated with worse outcome. A Letter to the Editor in response to this assumption pointed out that the autoimmune responses to MBP, PLP, and even GFAP, at either 3 or 7 days after stroke onset had no correlation with long-term functional outcome at 90 days (Urrea *et al.*, 2012). Only the 90-day immune response correlated with the 90-day severity of stroke. These data highlight the complex interplay between autoantigen presentation, injury, and recovery in the ischemic cortex and begs the question: is the higher autoimmune response to myelin contributing to the loss of functional recovery, or is the inability to recover lost function driving a myelin-derived — and potentially neuronal-derived — autoimmune response in an effort to promote plasticity during continued recovery?

Several future studies should address limitations in our data. First, we observed a profound reduction in autoreactivity at day 8 in the cervical lymph nodes, which at least visually rebounded by day 10. The day 8 group had an n=5 for tMCAo, though this experiment was duplicated on two separate days. Also, global immunoreactivity from T cell splenocytes did not reach significance compared to shams at days 8 and 10 post stroke. Therefore, it will be necessary to add replicates in order to confirm the day 8 loss of autoreactivity, though this

did not simultaneously occur in the spleen. If a loss of autoreactivity at day 8 is confirmed, these data may be explained by either a loss of active brain injury, a mobilization of autoreactive immune cells to other tissues, or post-stroke immunosuppression, possibilities which will be investigated in future studies. As also mentioned above, long-term autoreactive CD4 T cell responses were associated with poor outcome in stroke patients (Becker *et al.*, 2011). CNS-directed post-stroke autoreactivity was investigated in a 2010 study with several genetic lines of mice previously used in mouse models of multiple sclerosis (Kleinschnitz *et al.*, 2010). Restriction of autoreactivity to a non-CNS peptide in both CD8 and CD4 T cells, and restriction of autoreactivity to only MOG₃₅₋₅₅, did not contribute to T cell-mediated growth of infarct volumes. However, injury was evaluated only at 24 hours after stroke onset, within a timeframe that precedes activation of the adaptive immune system in the injured CNS (Kamel and Iadecola, 2012).

Future studies should first adoptively transfer myelin, or more importantly NR2A or MAP2-stimulated T and B cells, at least 1 day after stroke to determine the long-term contribution of neuronal autoreactivity to post-stroke neuronal plasticity and functional recovery. Therapeutic strategies could eventually be aimed at suppressing unfavorable immune activation through immune modulating drugs, or augmenting favorable immune responses through cell-based immunotherapy or vaccinations, to accelerate neurological recovery in stroke patients. Secondly, future studies should also address the possibility that the memory aspect of an autoimmune response may play a significant role in secondary stroke severity or complications, particularly with regard to a long-term circulation of CNS-directed lymphocytes. Finally, NR2F is an orphan nuclear receptor that mediates brain development (Lin *et al.*, 2011) and contributes to post-stroke angiogenesis (Li *et al.*, 2013). Originally intended to be a neuronal protein control for NR2A and MAP2 identified in the clinical study (Planas *et al.*, 2012b), future studies should investigate the specific ramifications of an autoimmune response to a neuronal nuclear protein, especially as autoreactivity is thus far confined to mice with larger infarct volumes.

Conclusions

Although the immune system does not play a role in the initiation of stroke, there is a growing perception that stroke-induced immune activation may potentiate either neuropathology or neurorepair. Elucidating novel neuronal surface antigens that are targets for immune cells offers the first unique insights into potential cellular and systemic consequences of autoimmunity during post-stroke neuronal plasticity. There is a dire need to further investigate if the autoreactive immune responses contribute to secondary brain injury, if they predispose an individual to other neurological diseases, or if there is a role for autoreactivity in successful neurorepair. By observing similar types of adaptive immune responses in mice that are mirrored in patient studies, we now have a specific and sensitive immune-based assay that can be used to elucidate the mechanisms of neuronal autoreactivity following ischemic brain injury.

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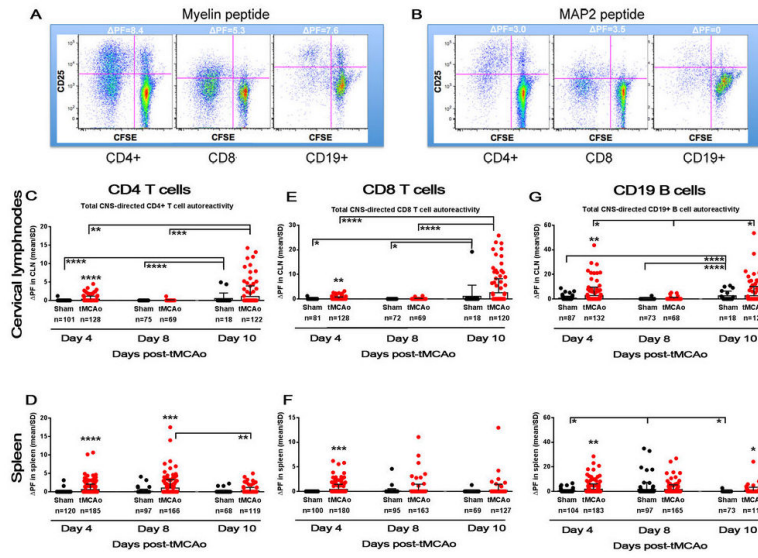
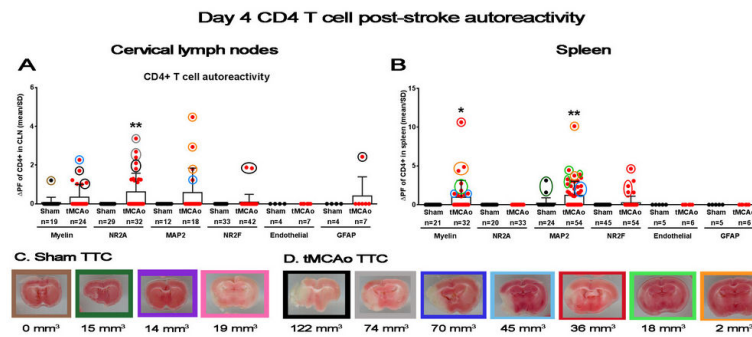


Figure 1. CNS-derived antigen induces autoimmune responses in both B and T cells by four days after stroke. (A-B) Autoreactivity is measured by the change in proliferation fraction (PF) of peptide over non-antigen conditions. Dot plots of individual immune cells following myelin (MOG, left) and MAP2 (right) exposure are shown. CFSE gradient along the x axis (dilution of CFSE intensity from right to left designates proliferation) and CD25 expression on the y axis (increase in CD25 expression from bottom to top designates activation) create the PF. (C-H) The timeframe for autoreactivity (PF; y axis) in both sham populations (black circles) and tMCAo populations (red circles) at 4, 8, and 10 days after tMCAo (x axis) are shown for (C-D) CD4 T cells, (E-F) CD8 T cells, and (G-H) CD19 B cells. Responses from lymphocytes isolated from the cervical lymph nodes (CLN) are shown in the top row, and splenic lymphocyte responses shown in the bottom row of graphs. Values for the number of wells within each group with a measurable response are shown below in parenthesis. Within-day significance is shown as asterisks over each tMCAo group (student's t-test). Between-day significance is shown by bars (one-way ANOVA). Values are mean \pm standard deviation (SD). * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

**Figure 2.**

Early splenic CD4 T cell autoimmune response to neuronal and myelin antigen is associated with smaller infarcts. **(A-B)** Graphs depict autoreactivity (%PF; y axis) for individual peptides in both sham populations (black circles) and tMCAo populations (red circles) at 4 days after tMCAo (x axis). **(A)** Left graph shows responses from CD4 T cells isolated from the cervical lymph nodes (CLN), and **(B)** right graph shows splenic CD4 T cell responses. Values for the number of wells within each group with a measurable response are shown below in parenthesis. **(C-D)** Representative TTC-stained section from each animal in both **(C)** sham and **(D)** tMCAo-treated groups. Areas of mitochondrial dysfunction (i.e., infarct volumes) appear white, while healthy tissue stains red. The total infarct volume for each animal is shown below the corresponding pictures (mm³). The border around each infarct is color-coded with the circles around individual data points in the graphs shown in (A, B). Within-day significance is shown as asterisks over each tMCAo group (student's t-test). Values are mean \pm standard deviation (SD). * $p < 0.05$, ** $p < 0.01$.

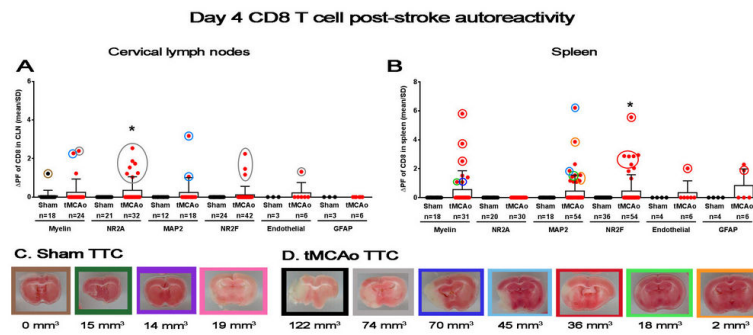


Figure 3.

Transient splenic CD8 T cell responses to CNS antigens precede activation in the cervical lymph nodes. **(A-B)** Graphs depict autoreactivity (PF; y axis) for individual peptides in both sham populations (black circles) and tMCAo populations (red circles) at 4 days after tMCAo (x axis). **(A)** Left graph shows responses from CD8 T cells isolated from the cervical lymph nodes (CLN), and **(B)** right graph shows splenic CD8 T cell responses. Values for the number of wells within each group with a measurable response are shown below in parenthesis. **(C-D)** Representative TTC-stained section from each animal in both **(C)** sham and **(D)** tMCAo-treated groups. Areas of mitochondrial dysfunction (i.e., infarct volumes) appear white, while healthy tissue stains red. The total infarct volume for each animal is shown below the corresponding pictures (mm^3). The border around each infarct is color-coded with the circles around individual data points in the graphs shown in (A, B). Within-day significance is shown as asterisks over each tMCAo group (student's t-test). Values are mean \pm standard deviation (SD). * $p < 0.05$.

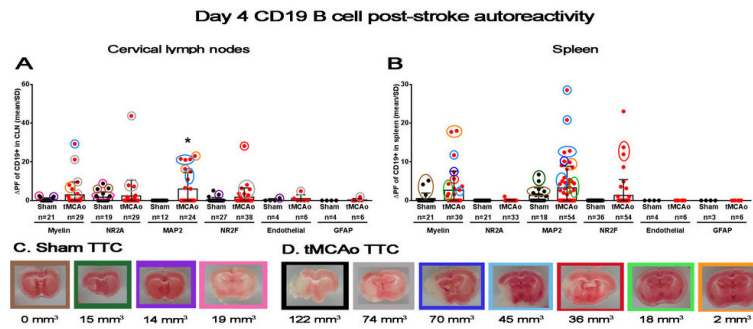


Figure 4. Stroke induces a rapid and sustained B cell response to both neuronal and myelin antigen. (A-B) Graphs depict autoreactivity (PF; y axis) for individual peptides in both sham populations (black circles) and tMCAo populations (red circles) at 4 days after tMCAo (x axis). (A) Left graph shows responses from B cells isolated from the cervical lymph nodes (CLN), and (B) right graph shows splenic B cell responses. Values for the number of wells within each group with a measurable response are shown below in parenthesis. (C-D) Representative TTC-stained section from each animal in both (C) sham and (D) tMCAo-treated groups. Areas of mitochondrial dysfunction (i.e., infarct volumes) appear white, while healthy tissue stains red. The total infarct volume for each animal is shown below the corresponding pictures (mm³). The border around each infarct is color-coded with the circles around individual data points in the graphs shown in (A, B). Within-day significance is shown as asterisks over each tMCAo group (student's t-test). Values are mean \pm standard deviation (SD). * p<0.05.

Table 1

Peptides Used in CFSE Proliferation Study.

Name	Peptide Pool ID	Peptide ID & Sequences	CD4 CLN	CD4 SPL	CDS CLN	CDS SPL	CD19 CLN	CD19 SPL
MAP	MAP-1	MADERKDEAKAPHWT TEASAHSHPEIKDQ	APHWDTSAPLTEASA EIKDQGGAGEGLVRS	+	+	+	+	+
	MAP-2	GLVRSANGFPYREDE EHGSQGTYSNTKENG	YREDEEGAFGEHGSQ TKENGINELTSADR	+	+	+	+	+
	MAP-3	TSADRETAEEVLKGE QHDKQTAALPLAAEE	VLKGEQEKEAQHKDQ LAAEETANLPPSPPP	-	-	-	+	+
	MAP-4	PSPPPSPASEQTVTV GESALAPSVFKQAKD	QTVTVEEAAGGESAL KQAKDKVSDGVTKSP	+	-	+	+	+
	MAP-5	VTKSPEKRSSLPRPS RRGVSGDRDENSFSL	LPRPSSILPPRRGVS NSFSLNSSISSARR	-	+	-	+	-
	MAP-6	SSARRTTRSEPIRRA STPTTPGSTAITPGT	PIRRAGKSGTSTPTT ITPGTPPSYSSRTPG	-	-	-	+	+
	MAP-7	SRTPGTPGTPSYPR TPKSAILVPSEKKVA	SYPRTPHTPGTPKSA EKKVAIIRTPPKSPA	-	+	-	+	-
	MAP-8	PKSPATPKQLRLINQ KNVKSIGSTDNIKY	RLINQPLPDLKNVKS DNIKYQPKGGQVQIV	-	+	-	-	-
	MAP-9	QVQIVTKKIDLSHVT LKNIRHRPGGGRVKI	LSHVTSKCGSLKNIR GRVKIESVKLDFKEK	-	-	-	-	-
	MAP-10	DFKEKAQAKVGSND GGGNVKIDSQKLNFR	GSLDNAHHVPGGGNV KLNFRHAKARVDHG	-	-	-	+	-
	MAP-11	RVDHGAEITQSPGR PRRLSNVSSSGSINL	QSPGRSSVASPRRLS GSINLLESPQLATLA	-	+	-	+	-
	MAP-12	LATLAEDVTAALAKQGL		-	+	-	-	-
NR2F	NR2F-1	RMPPTQPGQFALT		-	-	-	-	+
	NR2F-2	QFALTWGDPLNCKSY		-	+	-	+	+
	NR2F-3	NCHSYLSGYISLLLR		+	-	+	-	+
	NR2F-4	SLLLRAEPTYPSRF		-	-	+	+	-
	NR2F-5	PTSRFGSQCMQPNNI		-	+	+	+	-
	NR2F-6	QPNNIMGIEWICELA		-	+	-	+	-
	NR2F-7	ICELAARMLFSAVEW		-	+	-	+	+
	NR2F-8	SAVEWARNIPFPDL		+	-	-	-	+
	NR2F-9	ARHIPFPDLQITD		-	-	-	+	-
NMDA receptor subtype NR2A	NR2A-1	NRTDPKSLITHVCDL CDLMSGARIHGLVFG	-	+	-	-	-	+
	NR2A-2	VFGDDTDQEAVAQML QMLDFTSSQTFIPIL	+	-	+	-	-	+
	NR2A-3	PIIGHGGASMIMAD MADKDPTSTFFQFGA	+	-	-	-	+	-
	NR2A-4	GASIQQATVMLKIM KIMQDYDWHVFLVT	+	-	+	-	+	-
	NR2A-5	LVTTFPGYRDFISF ISFIKTTVDNSFVGW	+	-	+	-	+	-
	NR2A-6	VGWDMQNVITLDTSF TSFEDAQTQVQLKKI	+	-	+	-	+	-

Name	Peptide Pool ID	Peptide ID & Sequences	CD4 CLN	CD4 SPL	CDS CLN	CDS SPL	CD19 CLN	CD19 SPL
	NR2A-7	KKIHSSVILLYCSKD SKDEAVLILSEARSL	+	-	+	-	+	-
	NR2A-8	RSLGLTGYDFFWIVP IVPSLVSGNTELIPK	+	-	+	-	+	-
	NR2A-9	IPKEFPSGLISVSVD SYDDWDYSLEARVRD	+	-	+	-	-	-
	NR2A-10	VRDGLGILTTAASSM SSMLEKFSYIPEAKA	-	-	+	-	+	-
	NR2A-11	AKASCYQGTE	-	-	-	-	+	-
Myelin oligodendrocyte glycoprotein	MOG35-55	MEVGVYRSPFSRWHLRNGK	+	+	+	+	+	+
Proteolipid lipoprotein	PLP178-191	NTKTTCSIAFPSK	+	+	-	+	+	+
Myelin basic protein	MBP Ac(I-11)	ASQKRPSQRSK	+	+	+	+	+	+
von Willebrand factor	vWF	Not disclosed by vendor	-	+	+	+	+	+
Cilia fibrillaiy acidic protein	GFAP	GEVIKESKQEHKDVM	+	+	-	+	+	+

Note: + indicates a positive response, - indicates a negative lesponse.