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Sensitization and Tolerization to Brain Antigens in Stroke

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Abstract

Despite encounter of novel brain antigens by the systemic immune system following stroke, autoimmune responses to these antigens do not seem to occur. A systemic inflammatory insult at the time of stroke, however, provokes changes increase the likelihood of developing detrimental autoimmunity. These findings may help to explain why infections in the post-stroke period are associated with worse outcome. In addition, data suggest that the immune response can be manipulated in an antigen specific fashion to improve stroke outcome. Together these data argue that the nature of the post-ischemic immune response influences neurological recovery from stroke.

Keywords

autoimmune; T_H1; T_H3/T_{REG}; MBP; mucosal tolerance

Introduction

An antigen non-specific inflammatory response occurs within the brain and peripheral circulation following stroke. This inflammatory response is characterized by activation of microglia and the influx of neutrophils and monocytes into the brain, as well as by the elaboration of leukocyte adhesion molecules and inflammatory cytokines that can be detected within the brain parenchyma and the systemic circulation (Kriz, 2006). In addition, ischemic compromise of the blood-brain barrier (BBB) allows for the infiltration of lymphocytes into brain and for injured neurons and glial cells to “leak” antigens into the peripheral circulation (Schroeter et al., 1994, Jander et al., 1995, Cunningham et al., 1996, Abraha et al., 1997, Missler et al., 1997, Bertsch et al., 2001, Becker, 2004, Foerch et al., 2004). Both of these situations allow lymphocytes, the key cells of the adaptive immune system, to encounter antigens that are normally sequestered from it by the BBB; this encounter leads to the possibility of an (auto) immune response developing to those antigens. In fact, antibodies to brain antigens (myelin basic protein, neurofilaments and the NR2A/2B subtype of the N-methyl-D-aspartate receptor) are documented in persons after stroke (Wang et al., 1992, Bornstein et al., 2001, Dambinova et al., 2003). Based on these observations, we undertook a series of experiments to assess whether a cellular immune response occurs to brain antigens following stroke, and if so, the consequences of that response. Furthermore, we explored the therapeutic utility of modulating the post-ischemic immune response capitalizing on the fact that brain antigens are exposed to the systemic immune system following stroke.

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CNS Autoimmune Responses in Experimental Stroke

For all of the experiments described in this manuscript, Lewis rats were subjected to 3 hours of middle cerebral artery occlusion (MCAO) followed by varying periods of reperfusion; the cellular immune response to myelin basic protein (MBP), a prototypical central nervous system (CNS) antigen, was analyzed by performing ELISPOT assays on mononuclear cells extracted from the spleen and the ischemic hemisphere of the brain. The number of cells secreting interferon (IFN)-γ in response to stimulation with MBP (relative to the number of unstimulated cells secreting IFN-γ) was used as an indicator of a T_H1 response; the number of cells secreting transforming growth factor (TGF)-β1 in response to stimulation with MBP (relative to the number of unstimulated cells secreting TGF-β1) was used as an indicator of the T_H3/T_{REG} response. The ratio of the relative increase in the number of MBP-specific IFN-γ secreting cells to that of the relative increase in the number of MBP-specific TGF-β1 secreting cells was used as a measure of the T_H1 response to MBP. Figure 1 depicts the T_H1 response to MBP among brain lymphocytes and splenocytes in individual animals. We considered animals to be "sensitized" to MBP if the ratio of the relative increase in the number of MBP specific IFN-γ secreting cells to the relative increase in MBP specific TGF-β1 secreting cells was at least 1.48. This value was chosen to dichotomize those with and without a $T_H1(+)$ response to MBP based on a previous study in our laboratory where animals were injected with MBP in complete Freund's adjuvant, which is a typical method of inducing experimental autoimmune encephalomyelitis (EAE). In Figure 2, it can be seen that 1.48 represents the lower quartile of values for the ratio of the IFN:TGF response in animals "sensitized" to MBP and effectively excludes naïve/control animals. Based on these data, we considered animals to have a $T_H1(+)$ response to MBP if the ratio of the IFN:TGF response was ≥ 1.48; conversely, a T_H3/T_{REG} was considered to be induced if this ratio was ≤ 0.68 (*ie*. $1.00 \div 1.48$). Based on the data presented in Figure 1, it does not appear that animals develop a cellular T_H1 type immune response to following stroke; in fact, the predominant cellular immune response to MBP, at least in the first weeks after stroke, is that of a T_H3/T_{REG} response (Becker et al., 2005).

That we were unable to demonstrate a $T_H1(+)$ response to MBP after stroke may be related to a number of different factors. Firstly, lymphocyte activation/initiation of an adaptive immune response requires that antigen be presented to T cells in the context of the major histocompatibility class (MHC) II molecule and receive an additional costimulatory signal (June et al., 1994, Croft and Dubey, 1997). Within the CNS, however, there are a relative paucity of antigen presenting cells (APCs) and the expression of costimulatory molecules is limited (De Simone et al., 1995, Lovett-Racke et al., 1998, Perry, 1998, Suter et al., 2003). Secondly, the type of lymphocyte effector response that occurs upon antigen encounter depends upon the "milieu" of the local environment at the site of antigen encounter (expression of adhesion molecules, cytokines, *etc*.), and the "immunologic" milieu of the CNS tends to inhibit the development of an immune response (Hailer et al., 1998, Chang et al., 2001, Suter et al., 2003). Finally, cerebral ischemia induces a systemic immunodepression that inhibits the function of effector T cells (Prass et al., 2003 and Offner et al., this issue); this immunodepression would negatively impact the ability to initiate an adaptive immune response in both the CNS and in the periphery. From a teleological perspective, all of these mechanisms could be viewed as essential to preventing a detrimental autoimmune response to damaged brain tissue (Dirnagl et al., 2007).

While the microenvironment of the brain does not support the development of an immune response under usual circumstances, the expression of adhesion and costimulatory molecules, as well as the cytokines necessary for lymphocyte activation, can be induced. For instance, lipopolysaccharide (LPS), which contains a molecular motif known as a pathogen associated molecular pattern (PAMP), is a potent stimulator of the innate (antigen non-specific) immune response. When administered systemically, LPS provokes a number of changes within the brain

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and in the periphery that could support the induction of an antigen specific (adaptive) immune response (Satoh et al., 1995, de Vries et al., 1996, Henninger et al., 1997, Menendez Iglesias et al., 1997, Maciejewski-Lenoir et al., 1999, Bohatschek et al., 2001, Fraticelli et al., 2001, Kloss et al., 2001). The actions of LPS are mediated primarily through Toll-like receptor (TLR)-4 (Janeway and Medzhitov, 2002); other PAMPs activate innate immunity through other TLRs (Dalpke and Heeg, 2002). Given that infection is common after stroke, with at least 20% of hospitalized ischemic stroke patients developing a pneumonia or urinary tract infection, the possibility that systemic inflammation promotes the development of a CNS (auto)immune response needs to be considered (Davenport et al., 1996, Georgilis et al., 1999, Grau et al., 1999, Langhorne et al., 2000, Hilker et al., 2003, Aslanyan et al., 2004, Kwan and Hand, 2007). While activation of TLRs appears to be detrimental in stroke, modulation of TLR signaling can be used to induce endogenous neuroprotection (Stenzel-Poore, et al., this issue).

In order to address the effect of infection/systemic inflammation on the cellular immune response to MBP in our stroke model (3 hours MCAO), we injected a subset of animals with LPS 3 hours after the onset of ischemia (at the time of reperfusion). Given that LPS is a component of the Gram negative bacterial cell wall, and Gram negative bacteria commonly cause infection following stroke, this experimental paradigm is clinically relevant (Puri et al., 2002, Hilker et al., 2003). In these studies, we found that animals treated with LPS had increased microglial expression of the costimulatory molecule B7.1 (Becker et al., 2005). Members of the B7 family are among the most important costimulatory molecules and deliver their signal to lymphocytes through CD28 (Liang and Sha, 2002). Concomitant with the increased expression of B7.1 seen in the brains of our LPS treated animals, LPS treatment was associated with infiltration of more $CD4^+$ lymphocytes into the CNS early after ischemia and more inflammation in the brain (characterized by increased numbers of $CD8^+$ cells) one month after stroke (Becker et al., 2005). As might be predicted by this change in histology, LPS treated animals also had a greater likelihood of developing a $T_H1(+)$ response to MBP, and this response was associated with worse neurological outcome up to one month after MCAO (Becker et al., 2005). These results suggest that the post-ischemic immune response influences outcome from stroke and that the effect on outcome is durable.

Antigen Specific Modulation of the Immune Response Improves Outcome

In a previous study we showed that induction of “mucosal tolerance” to MBP (characterized as a T_H3/T_{REG} response to the antigen) could improve outcome in an animal model of stroke (Becker et al., 1997, Becker et al., 2003). In these experiments, regulatory T cells to MBP were induced by mucosal administration of MBP prior to MCAO. If an animal is “tolerized” to a given CNS antigen prior to stroke, re-encounter with that antigen after stroke leads to secretion of cytokines (TGF-β1 or IL-10) that modulate the local immune response (Chen et al., 2003, Frenkel et al., 2005). While this regulatory response is induced in an antigen specific manner, the secreted cytokines modulate the immune response in an antigen non-specific fashion, a phenomenon referred to as bystander suppression (Miller et al., 1991). Induction of a T_H3/T_{REG} response to a given antigen may therefore be of therapeutic benefit by modulating the immune response wherever that antigen is present, even if that antigen is not pathologic. By inducing a T_H3/T_{REG} response to MBP to modulate the immune response following stroke, we demonstrated that infarct size could be reduced at 24 hours and 96 hours after MCAO (Becker et al., 1997, Becker et al., 2003). This “neuroprotective” benefit was associated with decreased inflammation in the brain and could be transferred to naïve animals through MBP tolerized lymphocytes (Becker et al., 2003). Other investigators have shown that induction of mucosal tolerance to additional CNS (myelin oligodendrocyte glycoprotein) and vascular (E-selectin) antigens similarly reduces infarct volume and improves stroke outcome (Chen et al., 2003, Frenkel et al., 2003). The benefit of mucosal tolerance in these animal studies is evident within 24 hours of stroke onset, a time too early for the induction of an adaptive immune response.

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Following antigenic challenge, it generally takes weeks before a cellular immune response to the antigen can be detected (Filion et al., 1988, Rimmelzwaan et al., 2000, Mayer et al., 2002). Given the kinetics of the adaptive immune response, the early benefit of mucosal tolerance seen in these studies must occur independent of any affect on adaptive immunity; the "neuroprotection" associated with mucosal tolerance in these studies is thus more likely related to modulation of the innate immune response (through the bystander effect). That modulation of this early post-ischemic inflammatory response can improve outcome is suggested by multiple lines of evidence, although this experimental benefit has not yet translated into clinical success (Muir et al., 2007).

Systemic Inflammation is Associated with Worse Stroke Outcome

In our animal model, a $T_H1(+)$ response to MBP induced by systemic inflammation (*ie*. administration of LPS) is associated with worse outcome up to one month after MCAO (Becker et al., 2005). Given that neurological outcome is worse in patients who develop an infection following stroke, the possibility that the infection contributes to this worsened outcome (and is not just a marker for stroke severity) must be entertained (Davenport et al., 1996, Georgilis et al., 1999, Grau et al., 1999, Langhorne et al., 2000, Hilker et al., 2003, Aslanyan et al., 2004, Kwan and Hand, 2007). Clinical data to support the detrimental affects of inflammation following stroke are provided by a trial that investigated the therapeutic benefit of a *murine* monoclonal antibody to intercellular adhesion molecule (ICAM)-1 in acute ischemic stroke; in this study, patients who received the investigational antibody experienced increased morbidity and mortality compared to patients who received placebo (Investigators, 2001). Antibody treated patients were found to have an increased incidence of infection as well as aseptic inflammatory processes, including meningitis. In retrospect, these results may have been anticipated since the experimental antibody actually induces an inflammatory response and the formation of human anti-murine antibodies (Vuorte et al., 1999, Furuya et al., 2001). The premise of this trial was that inflammation contributes to cerebral ischemic injury (and that limiting the inflammatory response would improve outcome); ironically, the trial offered resounding support for this premise (see Teeling et al. and McColl et al., this issue). It could be argued that it is the activation of the innate immune system by infection that is responsible for the worse outcome seen in infected stroke patients, and that the cellular immune response to MBP seen in our model is merely an epiphenomenon of this activation (Urra *et. al.* in this issue). The fact that the T_H1 response to MBP is more predictive of outcome from stroke than whether an animal has received an inflammatory challenge with LPS in our experimental model, and that the degree of the T_H1 response correlates with performance on behavioral tasks at 1 month after MCAO, suggest that the development of an adaptive immune to MBP is pathologic (Becker et al., 2005, Gee et al., 2008)

The Nature of CNS Autoimmune Response Influences Outcome

Based on our experimental data and the fact that infection (and the attendant inflammatory response that occurs in response to infection) is so common following stroke, the circumstances that favor the development of an immune response to previously sequestered brain antigens exist in many stroke patients. Preventing infection could potentially limit the chances of developing an autoimmune response, but strategies to prevent infection following stroke have not yet been demonstrated to be effective (Chamorro et al., 2005 and Klehmet et. al. this issue). Immunomodulatory strategies could also be considered to limit the chances of developing a detrimental post-ischemic CNS autoimmune response. We thus performed another series of experiments in which we attempted to prevent the $T_H1(+)$ response to MBP using the paradigm of mucosal tolerance. Animals were "tolerized" to MBP or ovalbumin (OVA), an irrelevant antigen, prior to MCAO and administration of LPS. Pre-ischemic treatment with mucosal MBP resulted in fewer animals developing a $T_H1(+)$ response to MBP and was associated with

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improved outcome, as assessed by a number of measures, including performance on the rotarod (Figure 3a) (Gee et al., 2008). Animals with a $T_H1(+)$ response to MBP had a shorter latency to fall from the rotarod (worse performance) (Figure 3b), while those that developed a T_H3/T_{REG} response to MBP tended to perform better than those without such a response (Figure 3c). These data suggest that the nature of the immune response to brain antigens following stroke influences outcome and imply that a strategy of immunodulation may be viable for treating patients with stroke.

That the cellular immune response to MBP following stroke is more than an epiphenomenon is further demonstrated by the fact that the degree of the T_H1 response to MBP correlates to the neurological outcome at one month (using Pearson's correlation); the more robust the T_H1 response, the quicker the animals were to fall from the rotarod (P=0.020). Additionally, animals with a $T_H1(+)$ response to MBP in spleen had higher titers of anti-MBP antibodies than those without (P=0.001), and the antibody titer was correlated to the degree of the cellular immune response to MBP (P=0.001).

Other experimental studies also suggest that CNS autoreactive T cells contribute to CNS injury. For instance, transgenic animals in which greater than 95% of $CD4^+$ T cells express receptors specific for MBP recover less following spinal cord injury than wild type controls, and animals immunized to MBP prior to stroke have increased stroke related mortality (Becker et al., 1997, Jones et al., 2002). Furthermore, T lymphocytes obtained from animals shortly after spinal cord injury can precipitate histopathological changes similar to experimental allergic encephalomyelitis (EAE) when injected into naïve animals (Popovich et al., 1996). Conversely, focal cerebral injury enhances the inflammation associated with EAE, and when animals with EAE are subjected to focal cerebral injury, adoptive transfer of lymphocytes from these animals into naïve animals results in more significant inflammation of the brain than does adoptive transfer of lymphocytes from animals with EAE but no cerebral injury (Phillips et al., 1995, Lake et al., 1999).

Pathological Effects of CNS Autoimmunity

Our data thus show that induction of mucosal tolerance to a brain antigen (MBP) can modulate the post-ischemic inflammatory response to improve outcome; it also limits the chance of developing a detrimental $T_H1(+)$ response to brain antigens in animals subjected to a systemic inflammatory response following stroke. The mechanisms by which a $T_H1(+)$ immune response to MBP worsens outcome from stroke, however, are not completely clear. Potential contributing factors include the fact that activated lymphocytes secrete a number of cytokines that are either directly or indirectly neurotoxic; effector lymphocytes are also able to kill "target cells" through direct lysis or by inducing apoptosis (Hanisch et al., 1996, Lee et al., 1996, Barone et al., 1997, Hanisch et al., 1997, Hu et al., 1997, Rothwell et al., 1997, Shresta et al., 1998, Downen et al., 1999). Specifically, cytotoxic T lymphocytes (CTLs) release perforin, which integrates into the target cell membrane leading to osmotic lysis of the target cell (Tschopp et al., 1986). Once perforin is integrated into the target cell membrane, granzyme (a serine protease) can be "injected" into the target cell to trigger apoptosis (Nakajima and Henkart, 1994, Kajino et al., 1998). *In vitro*, NK cells are able to lyse neurons in a perforin dependent fashion, and pathological data suggest that $CD8^+$ lymphocytes may kill neurons through a granzyme dependent pathway (Backstrom et al., 2000, Bien et al., 2002). Lymphocytes also induce apoptosis in target cells through Fas ligand (FasL) and release of tumor necrosis factor (TNF); neurons express cognate receptors for both and are thus susceptible to lymphocyte mediated injury (Botchkina et al., 1997, Kajino et al., 1998, Raoul et al., 2000). In experimental models of stroke, apoptotic neurons appear in close approximation to invading leukocytes, suggesting that inflammatory cells may contribute to ischemic neuronal death (Braun et al., 1996). In Figure 4, perforin positive cells (FITC positive) are seen in close

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contact to neurons within the infarcted tissue following MCAO. One month after stroke in our experimental model, animals treated with LPS evidenced more brain atrophy and had more apoptotic neurons than animals that did not receive an inflammatory stimulus (Becker et al., 2005).

While there is clear evidence that the immune response contributes to post-ischemic neuropathology, as outlined above, the effects of the immune response appear to depend on the model used. As is discussed by Schwarz et. al. and Kerchensteiner et. al. in this issue, the CNS immune response can also promote recovery and repair.

Conclusions

Our data demonstrate that an inflammatory insult (*ie*. LPS) after stroke onset is associated with worse outcome. LPS initiates the innate immune response through stimulation of TLR4 and sets the stage for the adaptive immune response by creating an environment that promotes lymphocyte sensitization to antigens; if one of these antigens is a brain antigen, a CNS autoimmune response may occur. The long-term consequences of this autoimmune response are unclear, but it appears that it is associated with worse outcome for at least one month following experimental stroke. Whether a similar cellular immune response to brain antigens occurs in persons with stroke is unknown, as are the potential consequences of such a response. In persons with vascular dementia, however, there is evidence of a humoral response to brain antigens (glial fibrillary acidic protein [GFAP], S100, tubulin and neurofilament), suggesting that CNS autoimmunity may not be benign (Mecocci et al., 1995, Terryberry et al., 1998). Given that induction of T_{REG} cells to brain antigens (*ie*. MBP) can prevent the $T_H1(+)$ response to these antigens (and is associated with better outcome from stroke independent of this effect), antigen specific immunomodulation may be a viable therapeutic intervention for stroke.

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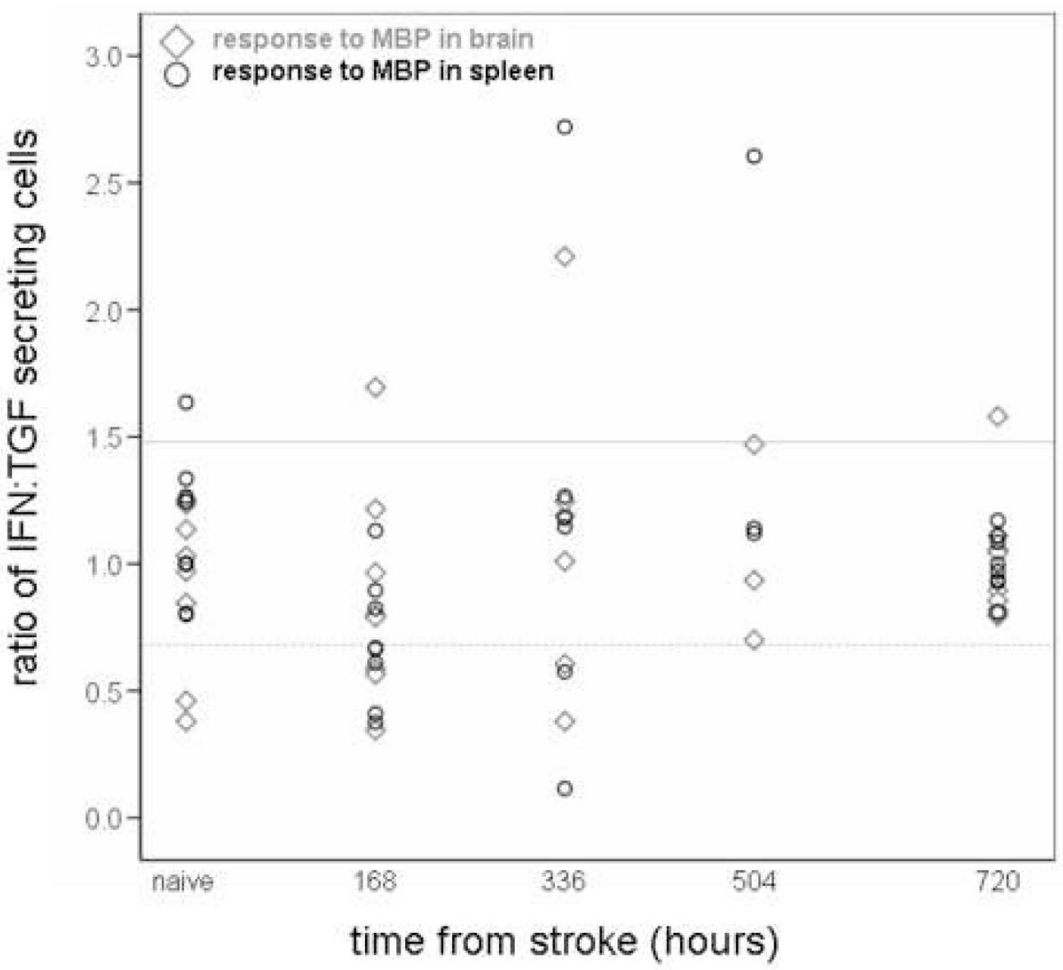

Figure 1. Immune response to MBP in brain and spleen following MCAO

Individual animal data are presented. For each animal, the response of brain lymphocytes to MBP is depicted by open black circles; the response of splenocytes to MBP is depicted by open gray triangles. The ratio of the number of MBP-specific IFN-γ secreting cells to the number MBP-specific TGF-β1 secreting cells is depicted on the Y-axis; the time from MCAO is depicted on the X-axis. A ratio ≥ 1.48 is considered indicative of a $T_H1(+)$ response (solid gray line); a ratio ≤ 0.68 is considered indicative of a T_H3/T_{REG} response (dotted gray line). There is no demonstrable immune response to MBP following MCAO.

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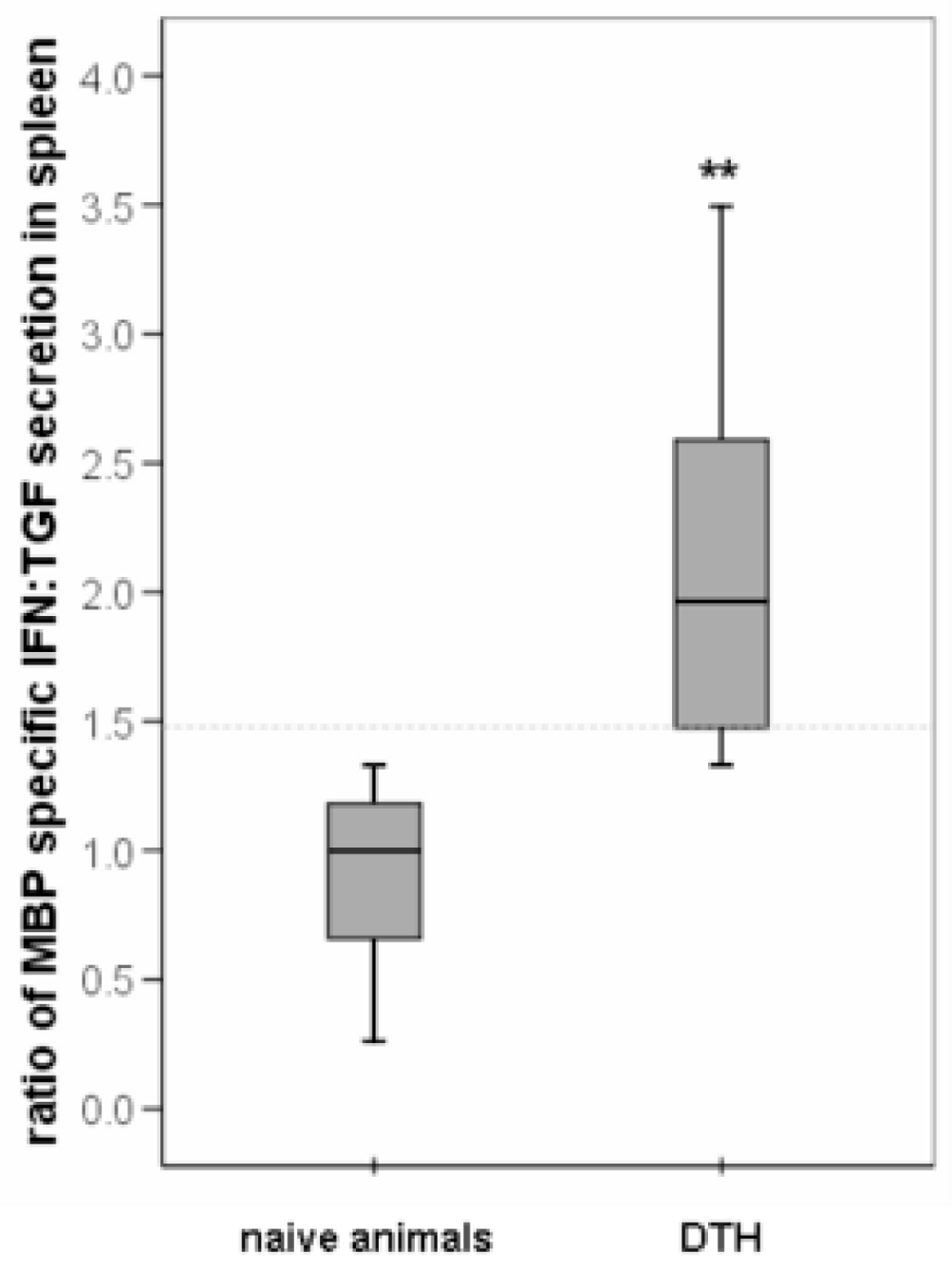

Figure 2. The immune response to MBP in spleen among naïve animals and animals "sensitized" to MBP

The ratio of the number of MBP-specific IFN-γ secreting cells to the number MBP-specific TGF-β1 secreting cells in spleen is depicted on the Y-axis. The box plots display the median and interquartile ranges; the lower interquartile range of MBP sensitized animals (1.48) effectively excludes naïve animals. *P<0.01 using the t-test.

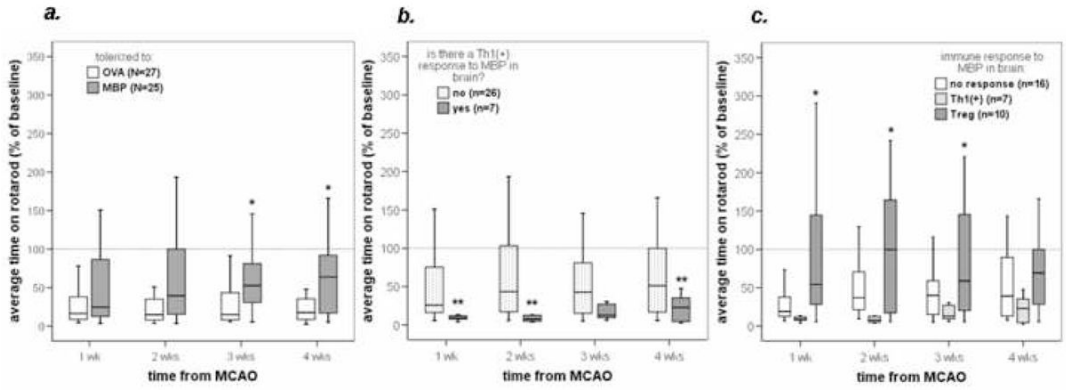

Figure 3. Rotarod performance following MCAO based on tolerization status and immune response to MBP

Animals tolerized to MBP prior to MCAO performed better than those tolerized to OVA (***a***). Animals that developed a $T_H1(+)$ response to MBP fell more quickly from the rotarod than those that did not (***b***). Animals with a T_{REG} response to MBP performed better than those with either a $T_H1(+)$ response or no response (***c***). The box plots display the median and interquartile ranges. *$P \leq 0.05$, ** $P \leq 0.01$ using the *t*-test or ANOVA, as appropriate.

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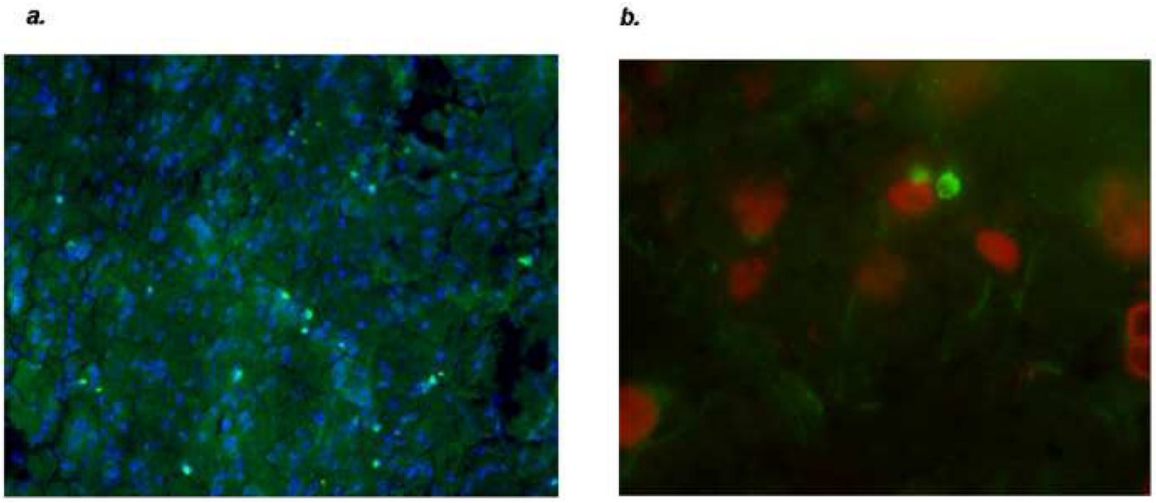

Figure 4.
There are multiple perforin positive cells (stained with FITC) within the infarcted tissue of this LPS treated animals sacrificed one month after MCAO (***a***). These perforin positive cells are seen in close juxtaposition to DAPI positive cells (40x). The DAPI positive cells were shown to be neurons using a Texas-red conjugated antibody for neuron specific enolase (100x) (***b***).