Published in final edited form as:

J Neuroimmunol. 2014 September 15; 274(0): 132–140. doi:10.1016/j.jneuroim.2014.07.009.

Pro-inflammatory T-Lymphocytes rapidly infiltrate into the brain and contribute to neuronal injury following cardiac arrest and cardiopulmonary resuscitation

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Abstract

Although inflammatory mechanisms have been linked to neuronal injury following global cerebral ischemia, the presence of infiltrating peripheral immune cells remains understudied. We performed flow cytometry of single cell suspensions obtained from the brains of mice at varying time points after global cerebral ischemia induced by cardiac arrest and cardiopulmonary resuscitation (CA/CPR) to characterize the influx in lymphocytes into the injured brain. We observed that CA/CPR caused a large influx of lymphocytes within 3 hours of resuscitation that was maintained for the 3 day duration of our experiments. Using cell staining flow cytometry we observed that the large majority of infiltrating lymphocytes were CD4⁺ T cells. Intracellular stains revealed a large proportion of pro-inflammatory T cells expressing either TNF α or INF γ . Importantly, the lack of functional T cells in TCR α knockout mice reduced neuronal injury following CA/CPR, implicating pro-inflammatory T cells in the progression of ischemic neuronal injury. Finally, we made the remarkable observation that the novel CD4⁺CD40⁺ (Th40) population of pro-inflammatory T cells that are strongly associated with autoimmunity are present in large numbers in the injured brain. These data indicate that studies investigating the neuro-immune response after global cerebral ischemia should consider the role of infiltrating T cells in orchestrating the acute and sustained immune response.

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Keywords

T cells; Cardiac arrest; global cerebral ischemia; Th40

1. Introduction

Every year in the United States more than half million people suffer from unexpected sudden cardiac arrest (CA) requiring cardiopulmonary resuscitation (CPR) (Roger et al., 2012). Recent advances in resuscitation have improved survival rates; however, no drug treatment is currently available for the often debilitating long-term neurological outcome. Up to 60% of CA survivors develop moderate to severe neurological deficit (Roine et al., 1993). Cardiac arrest directly causes global cerebral ischemia, which in turn triggers selective, delayed, neuronal cell death in vulnerable neuronal populations such as the hippocampal CA1 region (Kirino, 1982, Pulsinelli et al., 1982, Petito et al., 1987). Extensive research has focused on the mechanisms of ischemia-induced neuronal cell death, including excitotoxicity, oxidative stress and apoptosis. Unfortunately, these endeavors have not led to translatable neuroprotective findings in humans.

Recent research indicates that neuroinflammation mediated by the influx of peripheral immune cells contributes to ongoing injury in experimental stroke (Iadecola and Anrather, 2011). However, there is little evidence for the presence of peripheral immune cells in the central nervous system following CA/CPR. It has been demonstrated that global cerebral ischemia stimulates microglial activation and a pro-inflammatory state within the brain (Wagner et al., 2002, Langdon et al., 2008, Waid et al., 2008, Norman et al., 2011, Satoh et al., 2011). While resident microglia are clearly early mediators of neuroinflammation and likely effectors of injury, the maintenance of a sustained inflammatory state consistent with delayed neuronal injury requires the action of other immune cells, particularly T lymphocytes. T lymphocytes have been identified as critical mediators of inflammation, serving as the orchestrators of a sustained immune reaction by regulating the function of various other immune cells. It is well known that there are two classes of T lymphocytes: CD4⁺ (or T-helper cells; Th) and CD8⁺ (or cytotoxic T cells; T_C) T cells. The Th cell subset comprises Th1, Th2 and Th17 and regulatory, Treg cells (Brait et al., 2012). Recent studies identified a novel subset of pro-inflammatory T cells which express the CD40 receptor and were thus termed Th40 cells. Th40 cells exhibit features of both Th1 and Th17 cells, producing both IFN γ and IL-17A, which contribute to tissue damage (Vaitaitis and Wagner, 2008, 2012). Th40 cells play a central role in autoimmune diseases, such as type 1 diabetes (Wagner et al., 2002, Waid et al., 2008, Vaitaitis et al., 2010, Vaitaitis and Wagner, 2010, Carter et al., 2012).

Here, we took advantage of our novel mouse model of CA/CPR to assess the role of infiltrating lymphocytes in ischemic brain injury. The current study observed that CA/CPR-induced cerebral ischemia stimulates a rapid infiltration of activated T lymphocytes into the brain, and nearly 80% of all infiltrating T cells have a Th40 phenotype. This implies that inflammation is a very important neuronal injury mechanism in cardiac arrest-induced global cerebral ischemia. Indeed, we observed that mice lacking functional T cells are protected from hippocampal CA1 neuronal cell death following global cerebral ischemia.

Therefore, understanding the role of inflammation in determining outcome following CA/CPR could lead to new insights into therapeutic interventions.

2. Materials and Methods

Experimental animals

All experimental protocols were approved by the Institutional Animal Care and Use Committee and conformed to the National Institutes of Health guidelines for the care and use of animals in research. All experiments were performed in a blinded, randomized manner. Male C57Bl/6 mice (Charles River), male TCR α KO mice (B6.129S2-Tcr α^{tm1Mom}) and their corresponding wild type control mice C57Bl/6J (Jackson Laboratory) weighing 20–25g were used.

Cardiac arrest and cardiopulmonary resuscitation model

Mice were subjected to CA/CPR as previously described (Kofler et al., 2004, Allen et al., 2011). Anesthesia was induced with 3% isoflurane and maintained with 1.5–2% isoflurane in oxygen enriched air via face mask. Temperature probes were placed into the left ear canal and rectum. The rectal temperature was controlled at near 37 °C during surgery. For drug administration, a PE-10 catheter was inserted into the right internal jugular vein and flushed with heparinized 0.9% saline solution. Animals were endotracheally intubated using a 22G intravenous catheter, connected to a mouse ventilator (Minivent, Hugo Sachs Elektronik, March-Hugstetten, Germany). The electrocardiogram was monitored throughout the experimental procedures. Cardiac arrest was induced by injection of 50 μ l 0.5M KCl via the jugular catheter, and confirmed by the appearance of asystole on the electrocardiography monitor and no spontaneous breathing. The endotracheal tube was disconnected from the ventilator and anesthesia was stopped. During cardiac arrest, the pericranial (tympanic) temperature was maintained at 37.5 ± 0.2 °C and the body temperature was allowed to fall during the arrest to 35 °C. CPR was begun 6 min after induction of cardiac arrest in Charles River mice or 8 min after induction of cardiac arrest in Jackson Laboratory mice (hippocampal injury was too small with 6 min cardiac arrest in Jackson Laboratory mice, data not shown, by slow injection of 0.5 ml of epinephrine (16 μ g epinephrine/ml 0.9% saline), chest compressions at a rate of approximately 300 min $^{-1}$, and ventilation with 100% oxygen. As soon as restoration of spontaneous circulation (ROSC) was achieved, defined as electrocardiographic activity with visible cardiac contractions, cardiac massage was stopped. If ROSC could not be achieved within 2 min of CPR, resuscitation was stopped and the animal was excluded from the study. Mechanical ventilation was stopped and the endotracheal tube was removed when spontaneous breathing reached a rate of 60 breathes/min. Temperature probes and catheters were removed, and the skin wounds were closed. The animal was then placed into its home cage for recovery.

Health Assessment Score

Mice were weighed daily, and a health assessment score was performed on each mouse daily after CA/CPR according to a graded scoring system. The scoring system ranges from 0 to 2, 0 to 3, or 0 to 5 depending on the behavior assessed, with 0 indicating no deficit and the upper limit indicating the most impaired. The behaviors assessed included consciousness (0–

3), interaction (0–2), ability to grab a wire top (0–2), motor function (0–5), and activity (0–2). Scores in each category were summated to generate an overall health assessment score (Allen et al., 2011, Kosaka et al., 2012).

Hematoxylin & Eosin staining

Three days after CA/ CPR, animals were anesthetized with 3% isoflurane and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were removed, post-fixed with paraformaldehyde and embedded in paraffin. Coronal sections 6 μ m thick were serially cut and stained with hematoxylin and eosin (H&E). The hippocampal CA1 region was analyzed, three levels (100 μ m apart), beginning from –1.5 mm bregma. Nonviable neurons were determined by the presence of hypereosinophilic cytoplasm and pyknotic nuclei. The percentage of nonviable neurons was calculated for each brain region (average of 3 levels per region). The investigator was blinded to treatment before analyzing neuronal damage.

Mononuclear cell isolation

Mononuclear cells were isolated at various time points from the brain, spleen, and peripheral draining lymph nodes of WT C57/Bl6, CA/CPR treated, or sham CA/CPR treated mice perfused with 40–50 ml of cold saline. The brains were homogenized in Hanks Balanced Salt Solution (Sigma-Aldrich), and mononuclear cells were isolated using 37/70% discontinuous Percoll gradients (Sigma-Aldrich). The spleens and lymph nodes were homogenized in Red Blood Cell Lysing Buffer (Sigma-Aldrich) or in PBS with 2mM EDTA and 5% BSA (Running Buffer), respectively. Mononuclear cells were isolated using Lympholyte-M gradients (CedarLane). Total cell numbers were determined using a Countess Automated Cell Counter (Invitrogen), and viability was assessed by trypan blue exclusion.

Cell Staining and Flow Cytometry

Eight-color flow cytometry was conducted using total mononuclear cell preparations and was run on the MACSQuant Analyzer (Miltenyi, Auburn, CA). Antibodies purchased from eBioscience are as follows: anti-CD11b (M1/70); anti-CD18 (M18/2); anti-Ly-6G (RB6-8C5); anti-CD22 (2D6); anti-CD69 (H1.2F3); anti-MHC II (M5/114.15.2); anti-TCR β (H57-597); anti-CD44 (IM7); anti-CD14 (Sa2-8); anti-CD45 (30-F11); anti-CD62L (MEL-14); anti-CD209 (5H10); anti-CD20 (2H7); anti-IFN γ (XMG1.2); anti-TNF- α (MP6-XT22); anti-FoxP3 (FJK-16s); and anti-IL-6 (MP5-20F3). Anti-CD8a-VioBlue was purchased from Miltenyi Biotech. Antibodies made in-house include: anti-CD4 (GK1.5); anti-CD40 (1C10); anti-CD25 (PC61.5.3); anti-CD5 (53-7.313); and anti-CD3 (145-2C11). Single cell suspensions were incubated on ice with appropriate antibodies for 30 min. Cells were washed with running buffer 3 times fixed in 1% paraformaldehyde/PBS, and then suspended in running buffer for flow cytometry. Results were analyzed with FlowJo software.

Statistical analysis

Data were reported as mean $\pm$ SEM. Immune cell staining and flow cytometry data were analyzed using 1-Way ANOVA with Student-Newman Keuls post-hoc test. Histological damage and health assessment scores were compared using t test. Survival comparisons were made using a χ^2 test. A p value < 0.05 was considered statistically significant.

3. Results

Immediate asystolic cardiac arrest was observed following injection of KCl in all mice. Body weight, total ischemic time and epinephrine dose per body weight were not different between experimental groups (Table 1). Health assessment was performed daily during the 3-day survival. No difference was observed between groups in health assessment score and survival rate (Table 1). In order to characterize cell infiltrates into the brain following global cerebral ischemia induced by cardiac arrest and cardiopulmonary resuscitation (CA/CPR), brains of mice were collected 3–72 hours after resuscitation. To determine levels of infiltrating lymphocytes, single cell homogenates of one hemisphere of each brain was analyzed. Forward scatter (FSC) and side scatter (SSC) was used to confirm the cellular identity of our preparation. Cells isolated from brains of injured mice showed a FSC/SSC pattern consistent with previously described lymphocyte patterns (Fig. 1A). Importantly, the same FSC/SSC pattern was observed in cells isolated from lymph nodes (Fig. 1B). Lymphocytes were below the level of detection in brains collected from control animals (data not shown). In contrast, a large number of lymphocytes were observed at each time point analyzed after CA/CPR, with an interesting biphasic pattern emerging. Elevated levels of lymphocytes were observed acutely (3 hr), reduced significantly at 24 hours after CA/CPR ($n=3$, $p<0.05$) and increased again in the following two days ($n=3$, $p<0.05$; Fig. 1A). In contrast, lymph nodes of post injury mice had FSC/SSC profiles identical to sham treated mice at all time-points analyzed (Fig. 1B).

Cells extracted from the brains of injured animals exhibited two distinct FSC populations, further characterized as FSC^{lo} and FSC^{hi} (Fig. 2A). The FSC^{lo} population had mostly CD4^{hi} cells (Fig. 2B), associated with a naïve, un-activated phenotype (Wagner et al., 1996). The FSC^{hi} population had predominantly CD4^{lo} cells (Fig. 2C), associated with a more active phenotype (Vaitaitis and Wagner, 2010). Previously we showed that CD4^{lo} cells actually have as much CD4 protein as CD4^{hi} cells, but the CD4 molecule has been internalized (Vaitaitis and Wagner, 2010). At 3 hours post injury there were a relatively small number of forward scatter low (FSC^{lo}) cells and a much greater population of FSC^{hi} cells ($n=3$, Fig. 2D, E). At 24 hours post injury the FSC^{lo} population increased relative to levels at 3 hours ($n=3$, $p<0.05$; Fig. 2D) while the FSC^{hi} population significantly decreased ($n=3$, $p<0.05$; Fig. 2D). At 48 hours post injury the reverse occurred, FSC^{lo} cells reduced to levels seen at 3 hours and FSC^{hi} cells increased ($n=3$, $p<0.05$; Fig. 2d, E). At 72 hours post injury there was significant increase in both populations ($n=3$, $p<0.05$; Fig. 2D, E). These data demonstrate a dynamic process occurring in the brain over time after global ischemic injury.

We delved further into the phenotypes of the cells in the brains of mice post global ischemia/reperfusion. Total cellular infiltrates were high at 3 hours, and remained high throughout the duration of the experiments, 72 hours ($n=6$ per timepoint; Fig. 3A). Of those

infiltrating cells, the majority were CD4⁺ T cells (Fig. 3B). No significant differences were seen over the 72 hour time period. We found that 8% of the total cell infiltrates at 3 hours were CD8⁺ cytotoxic T cells (Fig. 3C). That percentage increased slightly, but not significantly at 48 hours, holding through 72 hours. B cells accounted for a minor population of total infiltrates (< 4%), at 3 and 24 hours (Fig. 3D). Interestingly levels of B cells spiked at 72 hours post injury. We further characterized the CD4⁺ T cell population relative to TCR expression levels, which is a classic marker of activation status (Akkaraju et al., 1997, D'Souza et al., 2003). The influx of T cells at 3 hours post injury demonstrated relatively high cell surface expression of TCR (Fig. 4). The level of cell surface TCR expression was significantly reduced at 24, 48 and 72 hours post injury (Fig. 4, $p < 0.05$ by ANOVA), indicating the presence of activated T cells. At 7 days post injury, the cell surface TCR expression levels had recovered to the levels observed at 3 hours after injury (Fig. 4), suggesting a return to baseline activation status at this more chronic timepoint.

Given the role of CD4⁺ T cells in driving inflammation, we further examined the role of CD4⁺ T cells in ischemic brain injury. We hypothesized that infiltrating T cells contribute to ongoing injury and ischemic neuronal cell damage. Therefore, we analyzed neuronal injury in TCR α KO mice that have a population of T cells that are incapable of being activated and contributing to neuro-inflammation. Cardiac arrest and CPR was performed in TCR α KO mice (and corresponding wild-type (WT) control mice) and hippocampal CA1 neuronal cell death was measured 3 days after resuscitation using standard histological methods, H&E staining (Allen et al., 2011, Kosaka et al., 2012). Figure 5 illustrates our observation that ischemic damage to hippocampal CA1 neurons was significantly less in TCR α KO mice ($4.5 \pm 11.5\%$, $n=9$) compared to WT control mice ($37.3 \pm 0.7\%$, $n=7$, $p < 0.05$). These data support the hypothesis that T cells contribute to neuronal injury following global cerebral ischemia and further implicate pro-inflammatory T cells in the injury process.

In previous work we described a subset of CD4⁺ T cells that express the CD40 biomarker and termed these cells Th40. They produce pro-inflammatory cytokines and uniquely express both IFN γ (a Th1 defining cytokine) and IL-17 (a Th17 defining cytokine) (Vaitaitis et al., 2010, Vaitaitis and Wagner, 2010). Th40 cells are highly pro-inflammatory (Wagner et al., 2002, Vaitaitis et al., 2003, Waid et al., 2004, Waid et al., 2007, Siebert et al., 2008, Vaitaitis and Wagner, 2008, Waid et al., 2008, Vaitaitis et al., 2010, Vaitaitis and Wagner, 2010, Carter et al., 2012, Vaitaitis and Wagner, 2012). At 3 hours post injury a large proportion (45%) of T cells in the brain were CD40⁺, demonstrating a major influx of Th40 cells early after injury. At 48 hours the relative abundance of CD40⁺ T cells reduced to approximately 20% ($n=6$, $p < 0.05$) and increased again at 72 hours to 50% ($n=6$, $p < 0.05$; Figure 3E).

We examined T cells in brains during CA/CPR injury for cytokine production. When total T cells were examined for Th1, pro-inflammatory cytokines TNF α and IFN γ , a very different pattern emerged over the 72 hour time frame. TNF α expressing T cells showed a biphasic pattern with low levels (15%) at 3 hours that increased at 24 hours ($n=5$, $p < 0.05$). At 48 hours post injury a very low level of TNF α ⁺ cells were detected ($n=6$, $p < 0.05$; Fig. 6A). At 72 hours post injury the highest level of TNF α ⁺ cells was seen ($n=5$, $p < 0.05$). Relative to IFN γ at 3 hours moderate numbers (30%) of T cells were IFN γ ⁺ with a modest decrease at

24 hours (Fig. 6B), that reversed with the observation of a substantial increase in IFN γ ⁺ cells was seen at 72 hours ($n=5$, $p<0.05$; Fig. 5B). At early time points the majority of total CD4⁺ T cells were producing IFN γ and at 72 hours the majority of CD4⁺ T cells were producing TNF α .

Th40 cells are a subset of total CD4⁺ cells that have high pathogenic potential (Wagner et al., 2002, Vaitaitis et al., 2003, Waid et al., 2004, Waid et al., 2007, Siebert et al., 2008, Vaitaitis and Wagner, 2008, Waid et al., 2008, Vaitaitis et al., 2010, Vaitaitis and Wagner, 2010, Carter et al., 2012, Vaitaitis and Wagner, 2012). Th40 cells drive inflammatory cascades, given the systemic nature of the ischemia/reperfusion in this model. We examined cytokine production in the Th40 subset. At the earlier time points, through 48 hours, Th40 cells accounted for very little of the overall TNF α production (Fig. 6C). At 72 hours however, a moderate portion (33%) of Th40 cells were TNF α ⁺. A substantial portion of Th40 cells (60%) were IFN γ ⁺ at the 3 hour time point (Fig. 6D). That level receded slightly at 24 hours and significantly at 48 hours (Fig. 5D). At 72 hours almost 60% of Th40 cells were once again IFN γ ⁺.

We examined the effects of CA/CPR on peripheral lymphocytes. Spleens from injured animals were compared to spleens from untreated animals and analyzed. Spleens from animals at each time point showed a classic CD4/CD8 profile (Supplemental Figure 1). As expected in untreated animals, only a small percentage of splenic cells had an activated, CD69^{hi}, phenotype (Supplemental Figure 1B). At the 3 hour time point a small portion of CD8⁺ and CD4⁺ cells demonstrated an activated phenotype, 17.6% and 18.6% exhibiting CD69^{hi} (Supplemental Figure 1B). At 24 hour and 72 hours there was a significant increase in activation status (Supplemental Figure 1B), however the peak was observed to occur at 48 hours (Supplemental Figure 1B).

4. DISCUSSION

The brain has been thought to be a relatively immune-privileged organ which contains resident immune competent cells that regulate local immune reactions. However, following injuries that damage the blood-brain barrier (BBB), such as experienced after ischemic stroke, extensive infiltration of peripheral immune cells is observed (Iadecola and Anrather, 2011). In contrast, the inflammatory state following global cerebral ischemia has long been considered to be mediated predominantly by resident microglia and possibly astrocytes. Here, we made the unexpected observation that lymphocytes rapidly infiltrate into the brain parenchyma following global cerebral ischemia induced by cardiac arrest and cardiopulmonary resuscitation (CA/CPR). We observed that the vast majority of infiltrating lymphocytes are pro-inflammatory T cells. Furthermore, we showed that hippocampal CA1 neuronal cell death is significantly reduced in TCR α KO mice compared to their WT controls, implicating infiltration of pro-inflammatory T cells in CA/CPR-induced injury. Thus, regulation of T cell infiltration and the consequent immune response following cardiac arrest may represent a new therapeutic approach.

The timing of peripheral immune cell infiltration has been characterized following experimental stroke, with neutrophils appearing earliest (within a few hours) followed by the

appearance of lymphocytes 12–24 hours after stroke (Park et al., 1999, Stevens et al., 2002, Yilmaz et al., 2006, Yilmaz and Granger, 2010). In contrast, little is known about immune cell infiltration following global cerebral ischemia. We made the surprising observation that significant numbers of lymphocytes are present in the brain following CA/CPR-induced global cerebral ischemia within 3 hours of resuscitation and remain at high levels up to 72 hours. CD4⁺ T lymphocytes were detected within the brain 3 hours after insult, and accounted for the majority of the total infiltrating cells. The T cells examined after 3 hr, 24hr and 48 hr were consistently CD4^{lo}, suggesting an early and relatively persistent activation state. Similarly, when we examined cell surface expression of TCR, levels at 3 hrs were higher than at 24 and 48 hrs. Given that these cells were isolated from perfused brains, this would suggest that during extravasation CD4 levels decrease but cell surface TCR levels do not until the cells are localized in the tissue. In addition TNF α producing cells, indicating T cell effector status, were low at 3 hr and significantly higher at 24 and 72 hrs. Therefore, while global cerebral ischemia and focal cerebral ischemia both stimulate the appearance of T cells within the injured brain, the timing of their appearance and activation status is significantly different. It remains unclear what causes this interesting difference, but it is likely due to the different cell injury mechanisms between global and focal cerebral ischemia. The damage in focal cerebral ischemic area (infarction occurs within 24 hours) develops more intensely and rapidly than global cerebral ischemia (ischemic vulnerable area, especially hippocampal CA1 region injury occurs after 48–72 hours). Regardless, the appearance of large numbers of lymphocytes within the brain following CA/CPR opens important new research areas. T cell activation leads to dynamic changes in phenotype.

Most importantly, what role do the infiltrating lymphocytes play in the progression of injury? Our data showed that hippocampal CA1 neuronal cell damage of TCR α KO mice significantly decreased compared to WT mice following CA/CPR. There are two types of T-cell antigen receptor (TCR), $\alpha\beta$ ⁺ and $\gamma\delta$ ⁺ TCR. As T cells develop they express one or the other type. TCR α KO mice express the beta TCR chain, but do not express the alpha chain (Kaufmann, 1996). The cells are stunted with a pre-TCR that is not functional. Because the TCR delta gene lies within the TCR alpha gene, $\gamma\delta$ ⁺ T cells are not expressed in TCR α KO mice either. While T cells do develop in these mice, they do not have antigen recognition capability (MacDonald and Wilson, 1998). Hence, our data indicated that the activation of infiltrated T cells in the brain following CA/CPR may be strongly associated with neuronal cell damage. We hypothesize that activated T cells are directly responsible for neuronal injury, via the release of pro-inflammatory cytokines (TNF α and IFN γ). However, it remains possible that T cells enhance neuronal injury via stimulation of microglia. We stained post-ischemic brains from both WT and TCR α KO mice with the activated microglia marker Mac-2 and observed that regardless of genotype, Mac-2 staining directly correlated with magnitude of neuronal injury (data not shown). Unfortunately, this does not resolve the fundamental issue because microglia are known to be stimulated by injured neurons, therefore, it is equally possible that lack of T cells reduces microglial activation by loss of T cell-microglial signaling or indirectly by protecting neurons and thus resulting in decreased microglial stimuli from injured neurons. The complex signaling between the adaptive and innate immune system following CA/CPR remains an important topic and the focus of ongoing follow-up studies. Importantly, the protection observed in the TCR α KO mice is

consistent with the previous findings in experimental stroke models, with studies using T cell deficient mice consistently showed a smaller infarct volume and better functional outcome compared to the WT animals (Yilmaz et al., 2006, Hurn et al., 2007, Liesz et al., 2011). Further studies demonstrated that different T cell subtypes play different roles following stroke. Mice deficient in CD4⁺ and/or CD8⁺ T cell had smaller infarct volumes following experimental stroke than WT mice (Yilmaz et al., 2006, Liesz et al., 2011). However, depletion of regulatory T cells exhibited either exacerbation of brain damage or no effect after stroke (Liesz et al., 2009). Furthermore, $\gamma\delta$ T cell-deficient mice had similar injury with WT mice (Kleinschnitz et al., 2010). Similarly, the role of B lymphocytes in brain ischemia is controversial (Hurn et al., 2007, Ren et al., 2011). Therefore, further work is needed to begin to unravel the role of the various lymphocytes attracted to the injured brain following cardiac arrest.

CD40 is a transmembrane receptor expressed on a variety of cells, such as antigen-presenting cells (APC), interacts with CD40 ligand which is primarily expressed on active T cells and serve as a second signal for APC activation (Chatzigeorgiou et al., 2009). Recently, CD40 has been observed to be expressed on CD4⁺ T cells, representing a new T cell subset. It has been shown that Th40 cells are associated with autoimmune disease (Wagner et al., 2002, Waid et al., 2007, Waid et al., 2008, Vaitaitis et al., 2010). We made a notable observation that a majority of the CD4⁺ cells infiltrating the brain after CA/CPR are Th40 cells. The activated CD4⁺ T lymphocytes play an important role in controlling effector mechanisms of inflammation by producing a variety of cytokines (Brait et al., 2012). Activated T cells are a major source of interferon γ (IFN γ) (Zwacka et al., 1997, Li et al., 2001). Extensive studies showed that IFN γ promotes inflammatory reactions, activates microglial and exacerbates ischemic brain damage (Li et al., 2001, Yilmaz et al., 2006, Liesz et al., 2011). Tumor necrosis factor- α (TNF α) is produced primarily by macrophages, but can be also produced by CD4⁺ T lymphocytes and other cell types (Gahring et al., 1996). TNF α has an important role in mediating inflammatory pathways and cell death signaling (Wajant et al., 2003, Chio et al., 2013, Han, 2013). We observed that after ischemic insult, about 30% of the CD4⁺ T lymphocytes produce IFN γ , while about 60% of the Th40 T lymphocytes are IFN γ positive. Similarly, we observed reduced surface expression of T cell receptor β in T cells obtained from the injured brain, consistent with the presence of activated T cells.

It is understood that phenotypic Th1 cells produce pro-inflammatory cytokines, e.g. IFN γ , TNF α and IL-2, while Th2 cells produce IL-4, IL-10 and TGF β , cytokines that are associated with anti-inflammatory responses. A relatively new classification of helper T cells includes Th17 cells that produce IL-17 and IL-22 that appear to be predominantly pro-inflammatory (Liesz et al., 2011). Extensive evidence demonstrates that each of the various pro-inflammatory cytokines can contribute to injury via various cell death pathways, thus the balance between pro- and anti-inflammatory T cells is likely to influence overall injury. T cell activation results in other phenotypic changes including cell surface upregulation or down regulation of molecules. For example, antigen mediated TCR signals induce increased expression of CD154 and CD25 (Liesz et al., 2009) while causing decreased expression of CD4 and CD3 (Vaitaitis and Wagner, 2008). An interesting observation in the current study is that CD4 total protein levels in CD4^{lo} cells was as much as in CD4^{hi} cells, suggesting that

T cell activation causes cell surface down modulation of the CD4 molecule (Vaitaitis and Wagner, 2008). CD3 and CD4 expression are coordinated in lipid rafts (Hurn et al., 2007), and our data further demonstrate that CD4 cells isolated from brains post injury have activation phenotypes. Over the 72 hr period a biphasic response is seen with higher numbers of activated cells at 3 and 72 hrs. This observation is confirmed by our data using intracellular stains to measure the pro-inflammatory cytokines IFN γ , TNF α . We observed that approximately 30–50% of infiltrating T cells are pro-inflammatory, exhibiting a Th40 phenotype. In other studies Th40 cells proved to be highly pathogenic. In a type 1 diabetes disease model for example, Th40 cells directly lead to islet inflammation and destruction (Wagner et al., 2002, Vaitaitis et al., 2003). It is interesting that an autoimmune associated, destructive T cell type infiltrates the brain post traumatic injury.

Conclusions

In summary, our data provides new insight into the immune response after CA/CPR-induced ischemic brain damage. Importantly, we have demonstrated rapid infiltration of lymphocytes, predominantly pro-inflammatory CD4⁺ T cells. In addition, we made the remarkable observation that the novel Th40 sub-population of T cells are present in large numbers in the injured brain. Furthermore, we showed that a deficiency in T cells confers neuronal protection in global cerebral ischemia. Therefore, our data indicates that strategies to reduce infiltration of the highly pro-inflammatory T cells may represent a new therapeutic approach to reduce brain injury following cardiac arrest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Project funded by NIH grants NS046072 & NS080851 and a Walter S. and Lucienne Driskill Foundation grant

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Highlights

- Cardiac arrest & CPR causes rapid lymphocyte infiltration into brain
- Majority of infiltrating T cells are CD4+ and pro-inflammatory
- Remarkable numbers of Cd4+CD4+ T cells in ischemic brain
- T cell receptor deficient mice have reduced neuronal injury following CA/CPR

Figure 1.

Lymphocytes in the brain during CA/CPR. Mice that had undergone CA/CPR procedure were analyzed for lymphocyte infiltrations in brain. (A) Brains from 3 hr., 24 hr., 48 hr. and 72 hr. post injury were excised and one hemisphere was made in to a single cell preparation and analyzed by flow cytometry for forward (FSC) versus side scatter (SSC). Each dot plot represents 3 mice at each time point. (B) Peripheral lymph nodes including brachial, axillary, and inguinal were collected, of CA/CPR mice at each time point were made in to single cell suspensions and analyzed by flow cytometry.

Figure 2.

Characterization of FSC cells from brains of CA/CPR induced mice. (A) A representative dot plot was examined for CD4 expression (histograms). CD4 levels in FSC^{lo} and FSC^{hi} populations were compared. (B, C). The level of FSC^{lo} and (D) FSC^{hi} (E) cells from injured mice at each time point. Statistics were ANOVA done using the Graph Pad Prism program.

Figure 3.

Phenotypes of lymphocytes infiltrating brains during CA/CPR. (A) We determined the total number of infiltrating cells in brains of CA/CPR injured mice at 3 hr., 48 hr., and 72 hr. Brains were excised and one hemisphere put in to a tissue digester to make single cell suspensions. Total cells were passed over percoll gradients to isolate lymphocytes. Cells were stained and characterized as (B) CD4+ cells; (C) CD8+ cells; (D) B cells; (E) Th40 cells [CD4+CD40+ population after the exclusion of microglial cells].

Figure 4.

T cells isolated from brains of CA/CPR demonstrate a more activated phenotype after 24 hours of injury. Analysis of T Cell Receptor β (TCR β) expression in CD4⁺ T cells show a transient decrease in TCR expression, indicating a population of highly activated T cells in the injured brain. Lymphocytes were isolated directly from brains then stained for CD4 and TCR β . Mean fluorescent intensity, which indicates cell molecular expression levels, were determined. * $P < 0.05$ compared to TCR levels at the 3 hr timepoint. ** $P > 0.05$, 3 hr compared to 7 day timepoints.

Figure 5.

T-cell deficiency protects hippocampal CA1 neurons from CA/CPR-induced cerebral ischemia. Representative photomicrographs of hippocampal CA1 neurons from WT control mice (A) and TCR α KO mice (B) following 8 min cardiac arrest and stained with H&E 3 days later. C) Quantification of ischemic neurons in CA1 region of hippocampus 3 days after CA/CPR, indicating significantly less injured neurons in TCR α KO mice. * $P < 0.05$.

Figure 6.

Cytokine production from lymphocytes in brains of CA/CPR mice. Lymphocytes were isolated from CA/CPR induced mice at 3 hr., 24 hr. and 72 hr. post injury. Cells were stained intracellularly for the Th1, pro-inflammatory cytokines TNF α and IFN γ . (A) Cytokines in total CD4+ T cells; (B) Cytokines in the Th40 subset of CD4+ cells.

Table 1

Body weight, cardiac arrest parameters, survival rate, and general health assessment between WT mice and TCR α KO mice.

	WT	TCR α KO
N	9	7
Body weight (BW, g)	23.4 $\pm$ 0.7	24.1 $\pm$ 0.5
Total ischemia time (sec)	589 $\pm$ 5	596 $\pm$ 10
Epinephrine (μ g/g BW)	0.37 $\pm$ 0.02	0.35 $\pm$ 0.04
Surviving animals (%)	45(9/20)	70(7/10)
Health Assessment Score		
POD 1	4.8 $\pm$ 0.5	3.4 $\pm$ 0.6
POD 2	4.2 $\pm$ 1.0	3.1 $\pm$ 1.0
POD 3	4.0 $\pm$ 1.0	2.4 $\pm$ 1.2

KO, knockout mouse; WT, wild type mice; POD, post-operative day.