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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 of lymphocytes into the injured brain. We observed that CA/CPR caused a large influx of lymphocytes within 3 h 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 TNFα or INFγ. Importantly, the lack of functional T cells in TCRα 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.

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 debilitative 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 IFNγ and IL-17A, which contribute to tissue damage (Vaitaitis and Wagner, 2008,

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2012). Th40 cells play a central role in autoimmune diseases, such as type 1 diabetes (Wagner et al., 2002; Waid et al., 2008; Vaitaitis and Wagner, 2010; Vaitaitis et al., 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

2.1. 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 TCRα KO mice (B6.129S2-Tcra^{tm1Mom}) and their corresponding wild type control mice C57Bl/6J (Jackson Laboratory) weighing 20–25 g were used.

2.2. 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 22 G 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 μl 0.5 M 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 started 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 μg 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.

2.3. 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).

2.4. 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 μm thick were serially cut and stained with hematoxylin and eosin (H&E). The hippocampal CA1 region was analyzed, three levels (100 μm 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.

2.5. 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 2 mM 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.

2.6. 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$_{\beta}$ (H57-597); anti-CD44 (IM7); anti-CD14 (Sa2-8); anti-CD45 (30-F11); anti-CD62L (MEL-14); anti-CD209 (5H10); anti-CD20 (2H7); anti-IFNγ (XMG1.2); anti-TNF-α (MP6-XT22); anti-FoxP3 (FJK-16 s); 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.

2.7. Statistical analysis

Data were reported as mean ± 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