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Full-length Article

Expansion of brain T cells in homeostatic conditions in lymphopenic *Rag2*$^{-/-}$ mice

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ABSTRACT

The concept of the brain as an immune privileged organ is rapidly evolving in light of new findings outlining the sophisticated relationship between the central nervous and the immune systems. The role of T cells in brain development and function, as well as modulation of behavior has been demonstrated by an increasing number of studies. Moreover, recent studies have redefined the existence of a brain lymphatic system and the presence of T cells in specific brain structures, such as the meninges and choroid plexus. Nevertheless, much information is needed to further the understanding of brain T cells and their relationship with the central nervous system under non-inflammatory conditions. In the present study we employed the *Rag2*$^{-/-}$ mouse model of lymphocyte deficiency and reconstitution by adoptive transfer to study the temporal and anatomical expansion of T cells in the brain under homeostatic conditions. Lymphopenic *Rag2*$^{-/-}$ mice were reconstituted with 10 million lymphoid cells and studied at one, two and four weeks after transfer. Moreover, lymphoid cells and purified CD4^{+} and CD8^{+} T cells from transgenic GFP expressing mice were used to define the neuroanatomical localization of transferred cells. T cell numbers were very low in the brain of reconstituted mice up to one week after transfer and significantly increased by 2 weeks, reaching wild type values at 4 weeks after transfer. CD4^{+} T cells were the most abundant lymphocyte subtype found in the brain followed by CD8^{+} T cells and lastly B cells. Furthermore, proliferation studies showed that CD4^{+} T cells expand more rapidly than CD8^{+} T cells. Lymphoid cells localize abundantly in meningeal structures, choroid plexus, and circumventricular organs. Lymphocytes were also found in vascular and perivascular spaces and in the brain parenchyma across several regions of the brain, in particular in structures rich in white matter content. These results provide proof of concept that the brain meningeal system, as well as vascular and perivascular spaces, are homing sites of lymphocytes and suggest the possibility of a brain specific T cell subtype.

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1. Introduction

An increasing number of studies indicate that T cells may perform diverse functions in the brain in addition to their classical role of immune surveillance and pathogen clearance. T cells have been implicated in complex brain processes including memory (Brynskikh et al., 2008; Derecki et al., 2010; Kipnis et al., 2004, 2012; Ziv et al., 2006), emotional behavior (Brachman et al., 2015; Kim et al., 2012; Lewitus et al., 2008), stress responsiveness (Clark et al., 2014b), as well as neurogenesis (Wolf et al., 2009; Ziv et al., 2006) and brain development and brain sexual differentiation (Rilett et al., 2015). The existence of a brain lymphatic system, recently re-defined by two independent studies (Aspelund et al., 2015; Louveau et al., 2015), provides the anatomical basis to support the concept that cellular interaction of T cells with CNS elements are likely part of natural physiological processes of the brain. This in turn provides additional support for a role of lymphocytes and T cells in neurobehavioral function. All these studies show that CD3^{+} T cells are present in specific structures of the brain constituting a brain lymphatic system anatomically related to the meninges and the choroid plexus, where they may influence brain homeostasis through the production of cytokines such as interleukin-4 (IL-4) (Derecki et al., 2010; Kipnis et al., 2012; Radjavi et al., 2014).

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Much information on T cell function in the brain has been gained by comparing immunocompetent wild type rodents with lymphocyte deficient animals. The effects on neurobehavioral functions of several lymphocyte subsets has been studied by adoptive transfer of functional cells into lymphocyte deficient animals, a process referred to as reconstitution by adoptive transfer (Riddell et al., 1994). The beneficial effects of lymphocytes and $CD4^+$ T cells on hippocampal dependent memory processes (Radjavi et al., 2014), neurogenesis (Wolf et al., 2009; Ziv et al., 2006) and emotional behavior (Brachman et al., 2015; Rattazzi et al., 2013) has been reported by several of these types of studies. Moreover, detrimental effects on emotionality have also been shown for inflammatory Th17 cells (Beurel et al., 2013) while some discrepancies exist about the specific roles of regulatory T cells in behavior (Cohen et al., 2006; Kim et al., 2012). These studies have employed different models of lymphocyte deficiency, some of which are the result of deleting genes that are expressed in the CNS. For example, the Rag1 gene, which is also expressed in the CNS, has been proposed to modulate the expression of behavior (McGowan et al., 2011; Rattazzi et al., 2013).

Lymphocyte deficient *Rag2*$^{-/-}$ mice are widely employed to study T cell differentiation and function (Dasgupta et al., 2011; Dorsey et al., 2013; Spanopoulou, 1996). Functional T and B cell deficiency is produced by deletion of the recombination activation gene 2 (RAG2) necessary for the V[D]J re-arrangement process of the T and B cell receptor (Shinkai et al., 1992). There is increasing interest in the use of this model to study the role of T cells on brain function and behavior (Brachman et al., 2015; Clark et al., 2014a,b; McGowan et al., 2011; Rattazzi et al., 2013) due to the restricted expression of the *Rag2*$^{-/-}$ gene in peripheral immune cells (Chun et al., 1991; Clark et al., 2014b). These mice are good acceptors of functional lymphocytes. T cells in particular were shown to proliferate and expand in peripheral tissues and organs (Min et al., 2004). This process, initially called homeostatic expansion in a lymphopenic setting (Goldrath et al., 2000; Murali-Krishna and Ahmed, 2000), has been shown to involve two distinct proliferative responses of T cells. A rapid proliferative response that is independent of interleukin-7 (IL-7) and a slower response dependent on IL-7 (Min et al., 2004, 2005; Min and Paul, 2005; Troy and Shen, 2003). The first response has been referred to as endogenous proliferation and the second as homeostatic proliferation (Min and Paul, 2005). To our knowledge, there is no information on the profiles of brain T cell expansion and anatomical localization in the model of adoptive transfer in immune deficient mice. Thus, the objective of the present studies was to provide a temporal and anatomical characterization of lymphocytes, and in particular $CD4^+$ and $CD8^+$ T cells, in the brain during endogenous and homeostatic expansion in lymphopenic *Rag2*$^{-/-}$ mice. The results of the present studies provide proof of concept that T cells home and expand into the brain under homeostatic conditions and localize mostly in the brain lymphatic system. They also reveal a significant degree of interaction with vascular and perivascular cells across the entire brain during this process.

2. Materials and methods

2.1. Animals and tissue processing

Six to eight week old C57Bl/6 wild type mice were obtained from Taconic Farms (Germantown, NY) and used as donors of lymphocytes (n = 22 females) or for control reference group (n = 8, males and 8 females) in flow cytometry experiments. Six week old transgenic C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ male mice (n = 22) were obtained from Jackson laboratories (Farmington, CT. Stock #006567) and used as donors of lymphocytes for fluorescent microscopy analyses. Six to eight week old *Rag2*$^{-/-}$ mice (n = 22 females and 22 males) were obtained from Taconic Farms (Germantown, NY) and used as recipients of lymphocytes or purified T cells. After arrival, all mice were housed in microisolator cages under strict sanitary conditions and allowed to acclimate for one week before any procedures. Mice were euthanized by CO_2 inhalation followed by cervical dislocation (donors) or were anesthetized with isoflurane followed by perfusion with 20 ml PBS (Fisher Scientific, Waltham MA) and 0.01% heparin to remove blood from the brain. Brains were immediately dissected by splitting the skull along the sagittal suture and across the interparietal bone. As the brain was removed the dura mater, whose major connections to the skull were severed, was also collected. The underside of the skullcap and base of the skull were inspected to insure that the meninges were fully removed. The tissue was directly placed in DMEM (Corning Cellgro, Tewksbury, MA) supplemented with 5% foetal bovine serum (FBS) (Gemini Bioproducts, Sacramento CA) and freshly processed for mononuclear cell (MNC) preparation. Spleens and lymph nodes were collected during the dissection procedure and also processed for lymphocyte preparation. Groups of mice reconstituted with lymphocytes from transgenic mice were additionally perfused with 30 ml ice cold 4% paraformaldehyde in phosphate buffered saline. The brains were extracted with dissections intended to preserve as much of the meninges attached to the brain. For confocal microscopy, the brains were post-fixed 24 h in 4% PFA at 4 °C and transferred to a 30% sucrose solution until they sunk and then processed for sectioning. For two-photon microscopy the brains were post fixed one to two days in 4% PFA at 4 °C and then transferred to PBS for one day. They were then lightly embedded in 10% porcine gelatin in PBS, chilled and then fixed in 4% PFA for an additional day and finally transferred to PBS. All procedures were carried out under approved IACUC protocols and institutional guidelines at the University of Maryland, School of Medicine.

2.2. Preparation of peripheral lymphoid and T cells for adoptive transfer

All experiments involving adoptive transfer of lymphoid cells were carried out on a 1:1 donor to recipient basis according to sex. Experiments of adoptive transfer of purified $CD4^+$ and $CD8^+$ T cells were carried out on a 2:1 donor to recipient basis. Lymph nodes (LNs: cervical, brachial, inguinal and mesenteric) were processed from each mouse in 5 ml DMEM and centrifuged at 400*g* (1500 rpm) for 5 min. Red blood cells were removed by using ACK lysis buffer (Quality Biological INC, Gaithersburg, Maryland). The pellet was resuspended with 5 ml PBS and passed through a 40 μm cell strainer to make a single cell suspension. Dead cells were stained with Trypan Blue (Corning Cellgro) and live cells counted using a hemocytometer. For specific $CD4^+$ and $CD8^+$ reconstitution, T cells were further isolated by negative selection using the EasySep T cell isolation kit specific for $CD4^+$ or $CD8^+$ T cells (Stem Cell Technologies, Vancouver, BC, Canada) following manufacturer's guidelines for manual separation. *Rag2*$^{-/-}$ mice were reconstituted with 10 million cells in a total volume of 200 μl *via* tail vein injections and used for flow cytometry analysis at one, two and four weeks after reconstitution and at two and four weeks after for fluorescent microscopy studies.

2.3. Preparation of mononuclear cells from whole brains

Whole brains including the meninges were collected and finely sectioned and passed through a cell strainer (70 μm) (Fisher Scientific) to make a single cell suspension. The cells were precipitated by centrifugation and washed twice in 5% FBS in DMEM (Corning Cellgro). After centrifugation, cells were resuspended in 10 ml 30% Percoll (Sigma Aldrich) in PBS and gently layered on top of