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Role of dihydrotestosterone in post-stroke peripheral immunosuppression after cerebral ischemia

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Abstract

Stroke is a sexually dimorphic disease with male gender considered a disadvantage in terms of risk and disease outcome. In intact males, stroke induces peripheral immunosuppression, characterized by decreased splenocyte numbers and proliferation and altered percentages of viable T, B and CD11b⁺ cells. To investigate whether the potent androgen and known immunomodulator, dihydrotestosterone (DHT), exacerbates post-stroke immunosuppression in castrated male mice after focal stroke, we evaluated the effect of middle cerebral artery occlusion (MCAO) on peripheral and central nervous system (CNS) immune responses in castrated mice with or without controlled levels of DHT. MCAO reduced spleen cell numbers in both groups, but altered T cell and B cell percentages in remaining splenocytes and concomitantly increased the percentage of CD11b⁺ blood cells solely in DHT-replaced animals at 24 h. Furthermore, DHT-replacement reduced splenocyte proliferation which was accompanied by an increased percentage of immunosuppressive regulatory T cells relative to castrates 96 h post-MCAO. In brain, the percentages of immune cell populations in the ischemic hemisphere relative to the non-ischemic hemisphere were similar between castrated and DHT-replaced mice after MCAO. These data suggest DHT modulates peripheral immunosuppression after MCAO but with relatively little effect on early immune response of the recovering CNS.

Keywords

Ischemia; Stroke; Hormone; dihydrotestosterone; testosterone; Immunosuppression; regulatory T lymphocyte; neuroprotection

INTRODUCTION

Male sex is considered a risk factor for stroke. Stroke occurs more frequently in men than women (Giroud et al., 1991) and outcomes after experimental stroke are worse in male

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animals relative to females (Herson et al., 2009). Although most studies have focused on brain outcome, it is becoming increasingly clear that stroke induces profound effects on the peripheral immune system (Dirnagl et al., 2007; Meisel et al., 2005). For example, male mice demonstrate initial activation (6–24 h) (Offner et al., 2006a) followed by suppression (96 h) of the peripheral immune system after MCAO, as evidenced by profound reduction of spleen size, splenocyte numbers and proliferative response to mitogens (Offner et al., 2006b; Vendrame et al., 2006). Of the remaining viable splenocytes, T cell abundance is curiously increased, including immunosuppressive regulatory T cells (Treg), while B lymphocytes are decreased (Liesz et al., 2009a; Offner et al., 2006b). In blood, CD11b⁺ cells are greatly increased (Offner et al., 2006b). These abnormalities are important, because post-stroke immunosuppression in humans and animals results in high frequency of pneumonia and septicemia (Chamorro et al., 2007; Dirnagl et al., 2007; Liesz et al., 2009a; Meisel et al., 2005). Furthermore, the importance of understanding contributors to peripheral immune suppression after stroke is highlighted by the fact that fatal infection is the most common cause of death in humans who have survived the initial stroke (Chamorro et al., 2007; Dirnagl et al., 2007; Meisel et al., 2005).

Peripheral immunosuppression is the proximate mediator of increased risk for pneumonia and septicemia post-stroke. Mice develop spontaneous pneumonia and septicemia within days after focal cerebral ischemia in parallel with the downregulation of the innate and adaptive immunity (Dirnagl et al., 2007). Animals that suffer from severe leukocyte depression show bacterial growth in blood cultures and in lung homogenates (Liesz et al., 2009a). Likewise, Prass et al. (Prass et al., 2003) observed decreased monocyte and T cell activation in animals that developed spontaneous bacterial infections after stroke. T cells are crucial in combating bacterial infection and adoptive transfer of T cells from wild-type mice but not IFN γ deficient mice inhibits stroke-induced bacterial infections demonstrating a link between cell-mediated immune response and increased susceptibility to bacterial infection.

Sex hormones modulate immune function during normal and disease-related processes (Liu et al., 2009). Testosterone, the primary circulating male hormone, and its more potent metabolite dihydrotestosterone (DHT), have well-established immunosuppressive effects in the periphery following disease and injury (Olsen and Kovacs, 1996; Palaszynski et al., 2004). Despite androgens' well-recognized immunosuppressive potential, the importance of the intracellular androgen DHT to post-ischemic immune function has not been well-examined. We hypothesized that DHT heightens post-stroke immunosuppression and contributes in this manner to adverse outcomes in the male.

MATERIALS AND METHODS

Animals

All experiments were conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals. Age-matched sexually mature 8–10 weeks of age, male mice C57BL/6J; Charles River Laboratories, body weight 20–25 g were used in all experiments.

Castration and DHT replacement

Castration was performed in male mice 1 week before transient focal cerebral ischemia. At the time of castration, DHT was replaced via 30 day release subcutaneous pellets containing 0.5 mg DHT (Innovative Research of America, Toledo, OH), as previously described (Uchida et al., 2009). Because testosterone may be metabolized into either estrogen, which improves immune outcome after stroke (Zhang et al. 2010) or DHT, we used DHT to eliminate the confounding immunoprotective effects of estradiol. We chose a sustained

subcutaneous dose of the DHT based on our previous demonstration that the implants yield physiologically relevant plasma steroid levels and based on the dose's ability to alter brain ischemic damage at 24 h post-MCAO (Uchida et al., 2009). Castrated mice did not receive placebo pellets.

Middle cerebral artery occlusion (MCAO) in mice

Reversible focal cerebral ischemia was induced by MCAO via the intraluminal suture technique under isoflurane anesthesia for 90 min as previously described (Offner et al. 2006a). Body and head temperatures were controlled at $36.5 \pm 1.0^\circ\text{C}$, with a warming blanket and heat lamps during surgery and ischemia. A small laser-Doppler probe was affixed to the skull to monitor cortical perfusion and verify vascular occlusion and reperfusion. Occlusion was confirmed by a decrease in laser-Doppler signal to less than 25% of baseline. A silicone-coated 6-0 nylon monofilament was inserted into the right internal carotid artery via the external carotid artery until a drop in laser-Doppler signal was observed. After securing the filament in place, the surgical site was closed, and the animal was awakened. At 90 min of occlusion, the mouse was re-anesthetized, the laser-Doppler probe re-positioned over same site on the skull, and the occluding filament withdrawn to allow for reperfusion. Mice were then allowed to recover for 96 h. For the sham MCAO animal group, the same procedure was conducted except the common, external, and internal carotid arteries were exposed but were otherwise left undisturbed.

Hormone Assays

Serum levels for total and free testosterone and DHT were measured by radioimmunoassay (Diagnostic Products, Los Angeles, CA, USA). The lower limits of detection for total and free testosterone and for DHT were 0.025ng/mL, 0.56 pg/mL, and 0.025ng/mL, respectively. While calculating mean hormone levels per treatment group, a value of zero was assigned to hormone values less than the lower limits of detection.

Infarct Volume Assessment

Infarction volume was measured by standard TTC histology as in previous studies (Offner et al., 2006b; Offner et al., 2006a). Infarct size was measured at 96 h after MCAO in 2-mm thick coronal brain sections (five total) using 2,3,5-triphenyltetrazolium chloride staining and digital image analysis. Sections were incubated in 1.2% 2,3,5-triphenyltetrazolium chloride in saline for 30 min at 37°C , and then fixed in 10% formalin for 24 h. Slices were photographed, and analyzed for infarction size with SigmaScan Pro 5.0 software (Systat Software Inc., San Jose, CA, USA).

Isolation of leukocytes from spleen, thymus, blood and brain

Spleen and thymus from individual sham and MCAO mice were removed and a single-cell suspension is prepared by passing the tissue through a 100- μm nylon mesh (BD Falcon, Bedford, MA). The cells were washed using RPMI 1640 and the red cells lysed using 1x red cell lysis buffer (eBioscience, Inc., San Diego, CA) and incubated for 3 min. The cells were then washed twice with RPMI 1640, counted, and resuspended in stimulation medium (RPMI, containing 10% FBS, 1% sodium pyruvate, 1% L-glutamine, .4% βME . Cardiac blood was collected and placed in heparinized tubes (Fisher Scientific, Pittsburgh, PA). Cells were pelleted and 1x red cell lysis buffer was added to the cell pellet and incubated for 7 min. Cells were washed twice with RPMI, counted and resuspended in RPMI 1640. The brain was divided into the ischemic (right) and nonischemic (left) hemisphere, dissociated mechanically through a 150-mesh screen, resuspended in 80% percoll overlaid with 40% percoll and centrifuged for 30 min at 1600 RPM. The cells were then washed twice with RPMI 1640, counted, and resuspended in stimulation medium. Individual brain hemispheres

were pooled according to similar spleen cell counts for flow cytometry. However, there were no differences in cell populations in hemispheres between low spleen (less than 10 million) and higher (greater than 10 million cells/spleen) so the data for hemispheres within each group were combined.

Splenic Proliferation assay

A total of 400 000 viable cells/well were plated without regard to the overall differences in the total number of recovered cells between the treatment groups in triplicate on standard 96-well flat-bottom tissue-culture plates at 37°C at 7% CO₂ with and without plate-bound anti-CD3 (5 µg/ml) and anti-CD28 (1 µg/ml) (BD Falcon, Bedford, MA) antibodies. Cells were incubated for 24 h and 0.5 µCi of [³H] was added 6–8 h prior to harvesting. Cells were harvested onto glass fiber filters and [³H] uptake was determined by liquid scintillation. Cells were plated in triplicate.

Analysis of cell populations by fluorescence-activated cell sorting (FACS)

All antibodies were purchased from (BD Biosciences, San Jose, CA) or (eBioscience, Inc., San Diego, CA) as published (Offner et al., 2006b; Offner et al., 2006a). Four-color (FITC, PE, APC and PerCP) fluorescence flow cytometry analyses were performed to determine the phenotypes of splenocytes, thymocytes and blood leukocytes, as previously published (Offner et al., 2006a). One million cells were washed with staining medium (PBS containing 0.1% NaN₃ and 1% bovine serum albumin (Sigma, Illinois) and incubated with the combinations of the following monoclonal antibodies: CD4 (GK1.5), CD8 (53–6.7), CD11b (MAC-1), CD45 (Ly-5) and CD11c (HL-3), CD19 (1D3), CD25 (PC61) for 10 mins at 4°C. 1 ml of staining buffer was added to wash the cells. Propidium iodide was added to identify dead cells. The FoxP3 staining kit was used according to the manufacturer's protocol (eBioscience) and as previously described (Zhang et al., 2010).

Statistical analysis

Differences between infarct volumes and percentages of cellular subtypes for FACS analysis were analyzed with a Student's t-test. Splenocyte counts, thymocyte counts or stimulation index for spleen cell proliferation assays were analyzed by one-way analysis of variance (ANOVA) followed by a Neuman-Kuels *post hoc* test. The criterion for statistical significance was $p < 0.05$. All values are reported as mean $\pm$ SD, with the exception of hormone levels which are reported as mean $\pm$ SEM. Fisher's exact test and X² tests were used for 24 and 96 h survival analysis, respectively. Differences were considered statistically significant with $P < 0.05$. Statistical analyses were carried out using SigmaStat Statistical Software (Version 3.0, SPSS, Chicago, IL).

RESULTS

Plasma hormone levels

As expected plasma DHT levels were lower in castrated (0.19 ± 0.09 pg/mL) ($n = 12$) versus in DHT-replaced mice (0.97 ± 0.32 pg/mL) ($n = 18$) at 96 h post-reperfusion. The plasma DHT levels from DHT-replaced animals in our study were similar to endogenous levels observed in intact naïve male mice (Uchida et al., 2009). Plasma total testosterone levels were low and similar between groups; 0.21 ± 0.013 ng/mL in castrated mice versus 0.15 ± 0.07 in DHT-replaced mice. Free testosterone levels were 0.0 ± 0.0 ng/mL in both groups.

Effect of DHT on peripheral immune cells prior to MCAO

We first compared the total number of splenocytes, responsiveness to T cell receptor (TCR) activation with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) and composition of

splenocyte populations between castrated (CAST) and DHT-replaced (CAST+DHT) mice prior to experimental stroke. The total number of spleen cells was lower in DHT-replaced as compared to castrated animals (Fig. 1A). However, there was neither a difference in proliferation in response to TCR stimulation (Fig. 1B) nor percentage of leukocyte populations (Fig. 1C and Fig. 1D) between the two groups. In particular, the immunosuppressive regulatory T cell ($CD4^+CD25^+FoxP3^+$) population was similar between castrated and DHT-replaced animals, suggesting DHT-replacement did not suppress T cell function prior to injury. A hormone effect on cell number was also observed in another immune organ, the thymus. Thymocyte numbers were similarly reduced in DHT-replaced animals; values were 131.5 ± 27.2 vs 60.6 ± 7.0 million cells in castrated and DHT-replaced animals, respectively ($p=0.046$). No differences in blood cell counts (CAST 1.8 ± 0.62 and CAST+DHT 2.1 ± 0.41 million cells per ml) or percentages of leukocyte populations within blood were observed between castrated and DHT-replaced animals (data not shown).

Effect of DHT on spleen cell numbers and thymic cell numbers after MCAO

The overall mortality was lower, although not statistically significant, in castrated versus DHT-replaced animals; 3.8% in castrated versus 9.5% in DHT-replaced at 24 h and 23.1% in castrated versus 33.8% in DHT-replaced animals at 96 h. Of the remaining animals, experimental stroke reduced the total mononuclear cells per spleen in both CAST and DHT-replaced mice euthanized at 24 h (Fig. 2A) post-MCAO relative to corresponding sham groups. By 96 h, an even greater reduction occurred in both castrated and DHT-replaced animals relative to corresponding sham groups (Fig. 2B). Although DHT-replacement decreased spleen cell counts prior to injury, no difference in splenocyte numbers were observed between castrates and DHT-replaced animals after 24 h and 96 h MCAO, suggesting stroke-induced splenocyte loss was not exacerbated by the presence of DHT. Although MCAO had no effect on thymocyte numbers at 24 h post-MCAO; (140.9 ± 44.3 vs 173.5 ± 70.8 for Sham vs MCAO in castrates, 48.4 ± 29.4 vs 38.9 ± 42 for Sham vs MCAO in DHT-replaced animals), at 96 h, thymocyte numbers were drastically reduced in both CAST and DHT-replaced animals; 264.2 ± 74.8 vs 5.2 ± 4.2 for Sham vs MCAO in castrates and 107.0 ± 44.3 vs 1.3 ± 1.7 million cells in Sham vs MCAO in DHT-replaced animals. Like the spleen, MCAO not DHT-replacement, was the major contributor in reducing thymocytes after experimental stroke.

MCAO induced changes in cell populations from spleen and blood from castrated and DHT-replaced animals

As shown in Fig. 2, MCAO reduced spleen cell numbers in both castrated and DHT-replaced males. To determine whether DHT altered the composition of the residual peripheral leukocyte populations toward an immunosuppressive phenotype, spleen and blood cells were isolated and evaluated for percentages of T ($CD4^+$, $CD8^+$), B ($CD19^+$), and myeloid ($CD11b^+$) cells at 24 and 96 h after MCAO. DHT-replaced animals had significantly higher percentages of $CD4^+$ and $CD8^+$ T cells in post-stroke spleen at 24 h than castrates and an increase in the percentage of $CD8^+$ cells relative to DHT-replaced shams. MCAO induced a decrease in B cell percentage solely in the DHT-replaced group, which was also lower than the castrated group after MCAO. There were no differences in the splenic $CD11b^+$ populations among the 24 h post-MCAO groups (Fig. 3).

In blood, both castrated and DHT-replaced animals had approximately 50% reduction in leukocyte numbers compared to respective shams (data not shown). The percentage of $CD8^+$ T and B cells decreased in the DHT-replaced animals compared to corresponding shams (Fig. 4). Blood leukocytes from DHT-replaced animals exhibited a large increase in the percentage of $CD11b^+$ myeloid cells after MCAO compared to DHT-shams and even a larger increase relative to the castrated animal cohort. This was remarkably different from

post-stroke castrates which showed the opposite changes in blood when compared to DHT-replaced animals after 24 h MCAO. In castrates, the percentage of myeloid cells decreased after stroke with a complimentary increase in CD4⁺, CD8⁺ and CD19⁺ percentages that is not characteristic of immunosuppression after stroke.

96 h after MCAO, DHT-replaced animals attained significantly increased percentages of CD4⁺ and CD8⁺ T cells in post-stroke spleen compared to respective shams. Castrated animals also exhibited an increased CD4⁺ T cell percentage after stroke (Fig. 5). MCAO induced a decrease in the B cell percentage solely in the castrated animals at 96 h post-MCAO. The decreased B cell percentage in viable splenocytes occurred earlier in DHT-replaced (24 h) than in castrated animals (96 h) but was ameliorated by 96 h. In blood, there were no differences in CD4⁺, CD8⁺, CD19⁺ and CD11b⁺ cell percentages among the groups although the percentage of CD11b⁺ myeloid cells in both castrated and DHT-replaced animals was substantially increased relative to respective shams (Fig. 6).

Effect of DHT-replacement on regulatory T cells after MCAO

In both castrated and DHT-replaced males, MCAO significantly increased the percentage of CD4⁺ splenic T cells relative to respective sham animals at 96 h post-MCAO. Therefore, we further assessed whether DHT-replaced animals would have a higher percentage of immunosuppressive CD4⁺CD25⁺ FoxP3⁺ Treg cells within the CD4⁺ cell population than their castrated counterparts in spleen at this time (Fig 7). Of the remaining CD4⁺ cells, the Treg population was enriched in the DHT-replaced animals 96 h after MCAO compared to both respective shams and castrates. In contrast, the percentage FoxP3⁺ cells was not increased in the CAST group after MCAO suggesting the increased percentage of Tregs after MCAO is DHT dependent.

Effect of DHT-replacement on splenocyte proliferation after MCAO

To determine whether DHT reduced immune function of remaining splenocytes, we evaluated T cell proliferation responses induced by anti-CD3 and anti-CD28 mAbs using equal numbers of viable cells per culture. As shown in Fig 8., proliferation responses were detectable in sham-treated animals and significantly and robustly increased in splenocytes from castrated males after MCAO. In contrast, proliferation responses were strongly reduced in the recovered viable splenic leukocyte population from the DHT-replaced animals relative to respective shams.

MCAO-induced changes in cell populations in brain from castrated and DHT-replaced animals

To determine whether these changes in the peripheral immune system translated to changes in activated or infiltrating immune cell populations present in the brain, leukocytes were isolated from non-ischemic and ischemic brain and evaluated for percentages of T (CD4⁺, CD8⁺) and B (CD19⁺) cells, and activated microglia/macrophages (CD45hi/CD11bhi) at 96 h after MCAO. This timepoint is relevant as the infarct is largely mature and peripheral immunosuppression is present. DHT-replaced mice had significantly smaller infarct volumes than did castrated male mice after 96 h MCAO (45.2±8.6 in CAST+DHT versus 61.0±10.4 in CAST) in the total hemisphere as determined by student's t test (Fig. 9). This protection was apparent in the cortex (56.8±9.7 in CAST+DHT versus 65.3±11.6 in CAST) and the striatum (85.7±8.8 in CAST+DHT versus 113.7±15.0 in CAST) (by student's t test). Since DHT-treatment exacerbated multiple attributes of immunosuppression, we expected reduced immunocyte infiltration into the brain of DHT-treated animals relative to castrates after stroke. However, we found no differences in overall total brain mononuclear cell number for castrated vs DHT-replaced animals in the ischemic (203 333±224 400 vs 219 200±214 320) or non-ischemic (54 000±40 988 vs 96 464±38 799) hemispheres. We

identified specific cell populations in hemispheres by flow cytometry. MCAO significantly increased the percentage of CD45hi/CD11bhi double positive cells that represent activated macrophages and microglia in the ischemic hemisphere of both the castrated and DHT-replaced mice relative to corresponding non-ischemic sides. With the exception of the CD8⁺ cell population, there were no differences between castrated and DHT-replaced and animals after MCAO. The overall percentage of CD8⁺ cells was lower in the DHT-replaced relative to the CAST cohort after MCAO but was similar to the DHT-replaced non-ischemic side (Fig 10).

DISCUSSION

These data provide the first evidence that post-ischemic peripheral immunosuppression is exacerbated by the potent androgen DHT. While MCAO reduced spleen cell density in all animals, DHT further altered surviving cell subsets. Blood-borne CD11b⁺ cells were also more strongly elevated in DHT-replaced animals during the early post-stroke period. DHT reduced splenocyte proliferation in response to TCR stimulation and increased splenic CD4⁺CD25⁺FoxP3⁺ Tregs by 96 h post-MCAO. This was in striking contrast to castrated animals in which robust response to TCR stimulation occurred and no increase in splenic Tregs was observed. In contrast to its peripheral actions, DHT availability did not alter the immune cell migration into the injured brain. These data, taken together, suggest DHT modulates peripheral immunosuppression after MCAO but with relatively little effect on early immune response of the recovering CNS.

Previous work has defined characteristic changes in peripheral immune cell populations after brain injury in intact male rodents. Focal cerebral ischemia induces spleen and thymic degeneration, in part through apoptotic death mechanisms (Brait et al., 2010; Liesz et al., 2009a; Offner et al., 2006b; Prass et al., 2003), and in part due to cellular transmigration from spleen to brain with deleterious consequences (Ajmo, Jr. et al., 2008; Yilmaz et al., 2006). Splenic atrophy occurs through direct adrenergic receptor signaling and can be prevented by β adrenergic antagonists, suggesting a catecholamine initiated origin (Ajmo et al., 2009; Dirnagl et al., 2007). Decreased spleen cell numbers have been linked to increased susceptibility to infection, highlighting the previously unrecognized importance of the spleen to post-stroke recovery (Liesz et al., 2009a). The composition of surviving splenocytes, e.g. the percentage of immunosuppressive Tregs relative to B lymphocytes may also be important (Gee et al., 2009; Offner et al., 2006b). In addition, splenocyte proliferation in response to stimuli is markedly reduced after MCAO (Becker et al., 1997; Offner et al., 2006b). Our current results expand on these observations to suggest that DHT may be responsible for some of these previously reported changes in the male.

It is well characterized that sex steroids influence immune functions; for recent review see (Fish, 2008). Females have greater susceptibility to autoimmune diseases, and more robust cell-mediated and humoral responses against infection and sepsis, than do males. Similarly, we recently showed that estradiol (E2) enhances immune function after experimental stroke in ovariectomized females. After MCAO, E2-replacement improves splenocyte numbers, increases proliferation in response to stimulation with anti-CD3 and anti-CD28 antibodies and prevents the increased CD4⁺CD25⁺FoxP3⁺ Treg cell percentage as compared to E2 deficient animals (Zhang et al., 2010). The decreased mRNA expression for cytokines, chemokines and chemokine receptors observed in E2 deficient animals is normalized by E2 replacement after MCAO.

In contrast, androgens have been categorized as immunosuppressive. For example, testosterone and DHT reduce autoimmune disease severity in experimental multiple sclerosis (Bebo, Jr. et al., 1999; Palaszynski et al., 2004). Androgens suppress cytokine

production and splenocyte proliferation in some reports (Angele et al., 1998; Wichmann et al., 1996), e.g. increased anti-inflammatory IL-10 production by CD4⁺ T cells (Liva and Voskuhl, 2001)). Splenocyte proliferation and IL-2 release from stimulated splenocytes are decreased after trauma and hemorrhage shock, effects that can be prevented by castration (Wichmann et al., 1997) and reversed by DHT replacement (Angele et al., 1998).

Of the few reports comparing the effects of testosterone and DHT on immune cell subsets, the data suggest similar sensitivity of T and B lymphocytes to testosterone and DHT. Selective loss of CD4⁺CD8⁺ double positive thymocytes is observed in both testosterone- and DHT-replaced castrated animals (Olsen et al., 1991; Olsen et al., 2001). Both testosterone and DHT increase IL-10 secretion in T cell lines (Bebo, Jr. et al., 1999) and testosterone or DHT-replacement reduces the percentage of B220⁺ B cells in bone marrow (Viselli et al., 1997). Virtually nothing is known, to the best of our knowledge, comparing the effects of testosterone and DHT on Tregs or the CD11b⁺ cell population.

Our data also suggest DHT has similar effects as testosterone on the post-stroke immunosuppression. In the current study, we hypothesized that DHT might be the key androgen that controls post-ischemic immune function in the male. While we did not directly compare castrates or DHT-replaced animals to gonadally intact males, the data suggest that DHT induces similar, albeit not identical, characteristics of immunosuppression in blood and spleen to those we previously characterized in intact males. In intact males, previous reports indicate immunosuppression is accompanied by increased CD11b⁺ cells in blood 96 h after MCAO (Offner et al., 2006b). We also observed that MCAO increased the percentage of CD11b⁺ cells in remaining blood borne leukocytes in DHT-replaced mice although this increase occurred earlier at 24 h post-MCAO. In spleen, T cell percentages were increased in the current study at 96 h (CD4⁺, CD8⁺ and CD4⁺CD25⁺FoxP3⁺) consistent with our previous findings in intact males showing increased T cell percentages (CD4⁺, CD8⁺ and CD4⁺CD25⁺FoxP3⁺) 96 h post-MCAO. Likewise T cell function was reduced in DHT-replaced animals 96 h after MCAO, consistent with our previous report indicating decreased splenocyte proliferation in response to T cell receptor stimulation with either concavalin A or anti-CD3/CD28 antibodies in intact males, 96 h post-MCAO (Offner et al., 2006b). In the current study, we observed the B cell percentage (CD19⁺) was decreased at 24 h in DHT-replaced animals. This decrease in CD19⁺ B cells occurred earlier than the decrease in B cell percentage at 96 h observed in intact male mice and was no longer apparent by 96 h.

In contrast to the actions of DHT, circulating blood CD11b⁺ cells decreased after 24 h MCAO and no changes were observed in the distribution of spleen cell subpopulations in castrated male mice. Although MCAO reduced splenocyte numbers and altered viable spleen cell subsets at 96 h post-MCAO in castrates, the Treg subpopulation was not increased and proliferation of splenocytes was robust after TCR stimulation suggesting remaining splenocytes from animals lacking androgen maintain proper immune function after MCAO but those from DHT-replaced animals are immunocompromised.

The difference in proliferation, an indicator of immune function, between DHT-replaced and castrated animals observed after MCAO may be related to differing percentages of CD4⁺CD25⁺FoxP3⁺ Tregs in spleen. Tregs limit inflammation and suppress autoimmune disease by maintaining self tolerance. Tregs inhibit CD4⁺ and CD8⁺ T cells, B cells and CD11b⁺ monocytes (Maloy and Powrie, 2001). However, after an immune system attack, with net loss of cells, overabundance of Tregs in the periphery could result in further suppression of the immune system after stroke. Tregs are increased in spleen and blood in mice and humans (Offner et al., 2006b; Yan et al., 2009), respectively and others have reported that Tregs are involved in stroke outcome (Ishibashi et al., 2009; Liesz et al.,

2009b; Offner et al., 2006b). The concept that Tregs reduce proliferative responses in the spleen in DHT-replaced animals is in agreement with our previous report in estradiol-replaced females demonstrating that the percentage of splenic Tregs is not increased after MCAO and proliferation is robust in response to mitogens (Zhang et al., 2010).

The steady state levels of circulating DHT may account for the earlier changes in cellular subtypes observed in DHT-replaced animals compared to castrates and to those previously reported for intact males (Offner et al., 2006b). Testosterone, the primary circulating hormone in intact males, can be aromatized intracellularly to either estrogen or DHT. Adding to the complexity, testosterone levels in intact males are suppressed by experimental stroke at 24 h (Uchida et al., 2009). Therefore, the confounding effects of both estradiol which improves immune function, as well as the drop in testosterone levels which occurs after experimental stroke, were avoided using a sustained subcutaneous dose of the DHT that cannot be further metabolized. However, we recognize that this is simultaneously also a limitation of using DHT to model androgen replacement. Such a sustained dose of DHT does not mimic the diurnal fluctuations of male androgens (Lucas and Eleftheriou, 1980). In addition, both testosterone and DHT act via the androgen receptor, but DHT is the more pharmacologically potent of the two.

One clinically relevant prevailing supposition is that post-ischemic immunosuppression provides endogenous CNS protection from secondary injury by reducing influx of peripheral immunocytes thus reducing brain inflammation and damage (Dirnagl et al., 2007; Meisel et al., 2005; Prass et al., 2003). As a consequence, the host is left with a compromised defense against infection. We used a low dose of DHT previously shown to reduce infarct size at 24 h in our model (Uchida et al., 2009). As an extension of our previous observations, DHT-replacement reduced histological damage as assessed at 96 h reperfusion in the current study. Because DHT promoted immunosuppression after experimental stroke in the periphery, it was surprising that the number of infiltrating cells, distribution of cellular phenotypes, with the exception of the CD8⁺ T lymphocyte population, and activated macrophage/microglia (CD45hi/CD11bhi) were similar between castrates and hormone replaced animals after MCAO. One possible explanation may be that DHT alters the function of the infiltrating cells, thereby reducing their ability to promote damage. Alternatively, androgens directly protect neurons after ischemic insult as has been observed in several reports both *in vitro* and *in vivo* (Ahlbom et al., 2001; Hammond et al., 2001; Nguyen et al., 2010; Pike et al., 2008). DHT also promotes the survival of non-neuronal cell types, which contribute to supporting neuronal survival after ischemia. Gatson et al. demonstrated that DHT protects astrocytes from cell death after ischemic and metabolic insult (Gatson and Singh, 2007) and activates protective signaling pathways in a glial cell line (Gatson et al., 2006). Androgens influence a multitude of genes after ischemia including those involved in inflammation, apoptosis, and maintenance of the extracellular matrix, dysregulation of the blood brain barrier, metabolism and ionic imbalance (Cheng et al., 2007). Recently, Cheng et al. showed DHT enhances post-ischemic SIK1 expression, a novel neuroprotective kinase, in brain (Cheng et al., 2010). DHT may also suppress deleterious inflammatory responses directly in brain rather than reducing infiltrating peripheral immunocytes. In activated microglia, DHT reduces damaging nitrate and TNF α production (Brown et al., 2007). These data taken together, suggest DHT may reduce infarct volume independently of effects on immune cell trafficking or by enhanced peripheral immunosuppression. We did observe a lower percentage of CD8⁺ cells in ischemic hemisphere of the DHT-replaced mice relative to castrates. However, the reduction of CD8 in the ischemic DHT group may not be specific. A lower percentage of CD8⁺ T cells was also observed in the non-ischemic hemisphere of DHT-replaced animals. DHT may have reduced the percentage of CD8 positive cells independent of ischemia because higher

variance in the castrate non-ischemic hemisphere may result in a false negative comparison relative to the non-ischemic side in DHT-replaced animals.

The effects of male sex steroids on regulating the immune system are in the early stages of investigation. We demonstrate a first avenue by which male androgen DHT suppresses immune responses following stroke. Although statistically males show a higher incidence of stroke, stroke-related deaths are divided nearly evenly between aged males and females when hormone levels decline in both genders (Di et al., 2003). This data suggests the lack of androgen is beneficial for post-stroke survival in the aged. One reason may be that lack of androgen improves immune function and therefore reduces susceptibility to post-stroke infections in the aged male, but future studies will be required to determine whether this phenomenon occurs. We used young male animals to compare with the initial studies characterizing post-stroke immunosuppression in intact males. However, it is important to note that 25% of all strokes occur in a non-elderly population (Volpato et al., 2004). The current findings are important for understanding hormonal modulation of immune system function for improving the survival and outcome of a male stroke survivor who is immunologically disadvantaged relative to females. Therapeutic agents that block the harmful effects of circulating male androgen may be beneficial to the recovering peripheral immune system.

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FIGURE 1. Effect of castration and DHT-replacement prior to stroke

Spleen cell counts (A) proliferative response to TCR activation (B) representative flow cytometry plots (C) and percentages of different cell types (D) from animals castrated (CAST) or castrated with DHT replacement (CAST+DHT) 1 week prior to harvesting. Proliferation was evaluated 24 h after stimulation with anti-CD3 and anti-CD28 mAbs. Spleen cells were isolated and flow cytometry was used to evaluate percentages of viable CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, and CD4⁺CD25⁺ Foxp3⁺ regulatory T cells. The presence of regulatory T cells was determined by gating on the CD4⁺ population and the percentage of CD25⁺FoxP3⁺ double positives represents events within the CD4⁺ population. n=4 for each group. * significant difference compared to CAST group.

FIGURE 2.

Effect of castration (CAST) and DHT-replacement (CAST+DHT) on spleen cell counts at 24 h (A) and 96 h (B) post-MCAO as compared with corresponding sham cohorts. 24 h; CAST Sham n=5, CAST MCAO n=11, CAST+DHT Sham n=6, CAST+DHT MCAO n=8. 96 h; CAST Sham n=6, CAST MCAO n=11, CAST+DHT Sham n=6, CAST+DHT MCAO n=18. * indicates significant difference compared to corresponding sham-treated mice; & significant difference compared to CAST Sham.

FIGURE 3. Percentage of different cell types 24 h after MCAO in spleen
Spleen cells isolated from sham-treated and MCAO-treated mice that were castrated (CAST) or castrated with DHT replacement (CAST+DHT) were evaluated for expression of CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, and CD11b⁺ myeloid cells by flow cytometry. Representative flow cytometry plots (A) from combined data (B). CAST Sham n=5, CAST MCAO n=6, CAST+DHT Sham n=6, CAST+DHT MCAO n=10. * significant difference in stroke mice vs sham-treated mice. & significant difference in CAST+DHT mice vs CAST mice after MCAO.

FIGURE 4. Percentage of different cell types 24h after MCAO in blood

Blood mononuclear cells isolated from sham-treated and MCAO-treated mice that were castrated (CAST) or castrated with DHT replacement (CAST+DHT) were evaluated for expression of CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, and CD11b⁺ myeloid cells myeloid cells by flow cytometry. Representative flow cytometry plots (A) from combined data (B). CAST Sham n=5, CAST MCAO n=5, CAST+DHT Sham n=6, CAST+DHT MCAO n=5. * significant difference in stroke mice vs sham-treated mice. & significant difference in CAST +DHT mice vs CAST mice after MCAO.

FIGURE 5. Percentage of different cell types in spleen 96 h after MCAO

Spleen cells isolated from sham-treated and MCAO-treated mice that were castrated (CAST) or castrated with DHT replacement (CAST+DHT) were evaluated for expression of CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, and CD11b⁺ myeloid cells by flow cytometry.

Representative flow cytometry plots (A) from combined data (B). CAST Sham n=9, CAST MCAO n=8, CAST+DHT Sham n=9, CAST+DHT MCAO n=10. * significant difference in stroke mice vs sham-treated mice. & significant difference in CAST+DHT mice vs CAST mice after MCAO. # significant difference CAST+DHT sham-treated vs CAST sham-treated mice.

FIGURE 6. Percentage of different cell types in blood 96 h after MCAO

Blood mononuclear cells isolated from sham-treated and MCAO-treated mice that were castrated (CAST) or castrated with DHT replacement (CAST+DHT) were evaluated for expression of CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, and CD11b⁺ myeloid cells by flow cytometry. Representative flow cytometry plots (A) from combined data (B). CAST Sham n=5, CAST MCAO n=5, CAST+DHT Sham n=6, CAST+DHT MCAO n=9. # significant difference CAST+DHT sham-treated vs CAST sham-treated mice.

FIGURE 7. Percentage of Tregs in remaining splenocytes 96 h after MCAO

Splenocytes were obtained from sham-treated and MCAO-treated animals that were castrated (CAST) or castrated with DHT replacement (CAST+DHT) and the percentage of Tregs was evaluated by flow cytometry. The presence of regulatory T cells was determined by gating on the CD4⁺ population and the percentage of CD25⁺FoxP3⁺ double positives represents events within the CD4⁺ population. Representative plots (A) from combined data (B). CAST Sham n=5, CAST MCAO n=6, CAST+DHT Sham n=6, CAST+DHT MCAO n=10. * significant difference compared to respective sham group. & significant difference compared to CAST MCAO.

FIGURE 8. Proliferative response of splenocytes to TCR activation 96 h after MCAO
Splenocytes were obtained from sham- and MCAO-treated mice in castrated (CAST) or DHT-replaced (CAST+DHT) animals 96 h after MCAO. Proliferation was evaluated 24 h after stimulation with anti-CD3 and anti-CD28 mABs. Data are a representative of at least three separate experiments. Data are presented as mean $\pm$ SD of six CAST Sham, three CAST MCAO, four DHT Sham and three DHT MCAO-treated mice. * significant difference compared to respective sham group. & significant difference compared to CAST MCAO.

FIGURE 9. Effect of DHT on infarct size and percentages of immune cell populations in the brain 96 h after MCAO

Male C57BL/6 mice were castrated (CAST) and replaced with a subcutaneous DHT pellet (0.5 mg) (CAST+DHT) one week prior to ischemia. Infarction volume was assessed by TTC staining in the cortex (CTX), striatum (STR) and total hemisphere (HMSPH) at 96 h following reperfusion. & significant difference compared to CAST MCAO.

FIGURE 10. Effect of DHT on percentages of immune cell populations in the brain 96 h after MCAO

Male C57BL/6 mice were castrated (CAST) and replaced with a subcutaneous DHT pellet (0.5 mg) (CAST+DHT) one week prior to ischemia. Leukocytes were isolated from brain from non-ischemic hemispheres or ischemic hemispheres 96 h after MCAO and flow cytometry was used to evaluate the T cell (CD4, CD8), B cell (CD19) and activated macrophages/microglial populations (CD45hi/CD11bhi) in the ischemic and non ischemic hemispheres. Gating strategy for different cell populations of brain leukocytes from a representative experiment (A). Average percentages of CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, and CD45hi/CD11bhi activated microglia/macrophages from pooled non-ischemic or ischemic hemispheres processed and indicate the percentages of total gated live leukocytes (B). CAST n=4, CAST+DHT n=5. *Significant difference compared to respective sham group. & significant difference compared to CAST MCAO.