To the editor:

CD8-predominant T-cell CNS infiltration accompanies GVHD in primates and is improved with immunoprophylaxis

A recently published article in *Blood* showed that, in mice, the central nervous system (CNS) is a target of graft-versus-host-disease (GVHD).[1] However, diagnosing CNS GVHD in the clinic is challenging,[2-4] and until now, evidence in a large-animal model has been lacking. Here we demonstrate that the brain is a target of GVHD in a rhesus macaque nonhuman primate (NHP) transplant
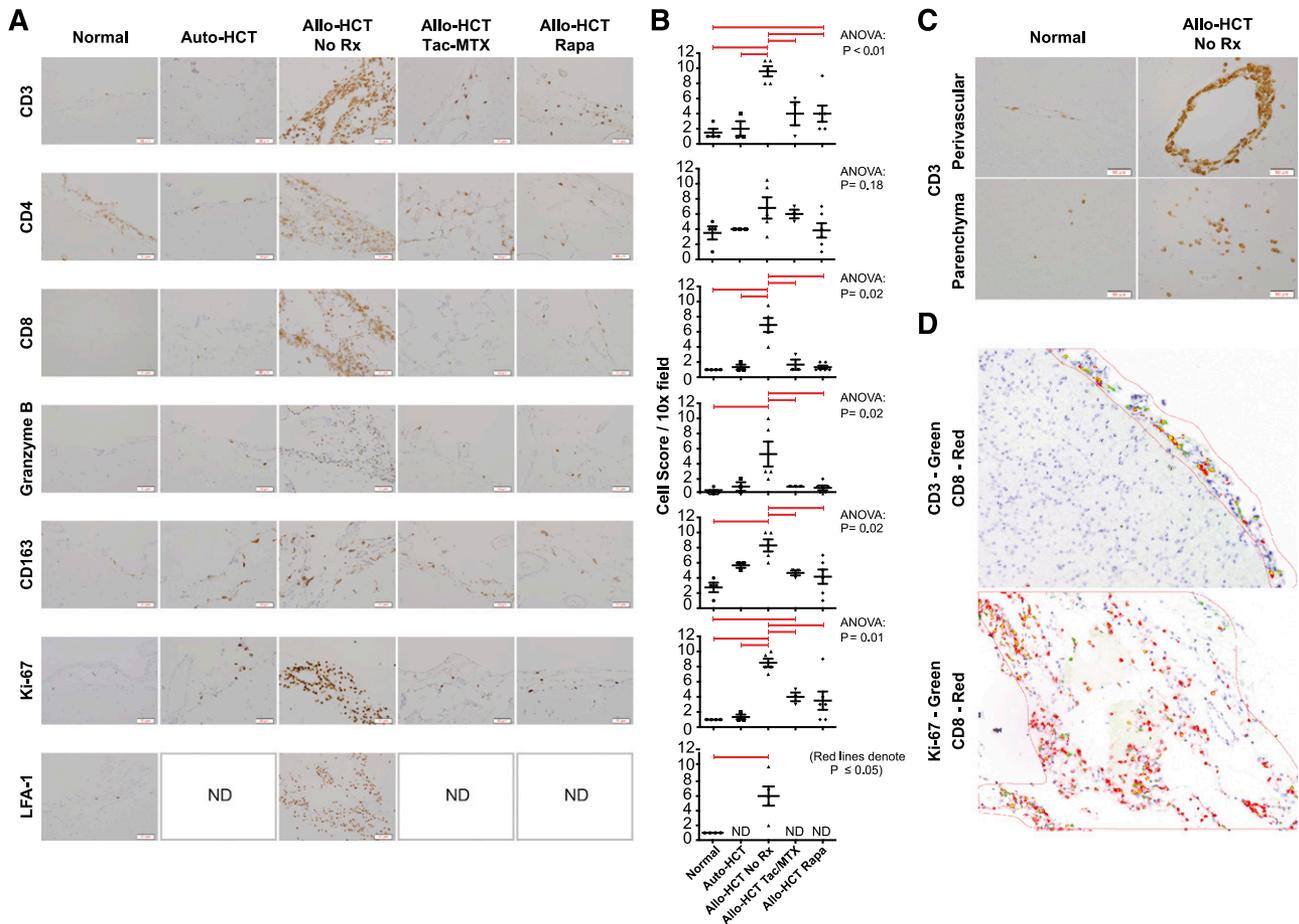
Figure 1. Immunohistochemical analysis of primate brain GVHD. Rhesus macaques used in this study were housed at the Yerkes National Primate Research Center and treated in accordance with Emory University Institutional Animal Care and Use Committee regulations. The following experimental groups were assessed: (1) healthy untransplanted controls (n = 4); (2) autologous transplanted controls (n = 3); (3) recipients of MHC-mismatched allogeneic HCT that did not receive GVHD prophylaxis (n = 5, mean survival time (MST) = 7 days); (4) allogeneic HCT recipients treated with rapamycin monotherapy (n = 6, MST = 17 days); and (5) allogeneic HCT recipients treated with tacrolimus/methotrexate (n = 3, MST = 47 days). For pretransplant preparation, all recipients received myeloablative irradiation (either 8 Gy [single fraction] or a dose equivalent [9.6 Gy] given in 2 fractions). Recipients received PBSC transplants (mean total nucleated cell dose of $6.2 \pm 0.98 \times 10^8$ cells/kg, mean $CD3^+$ T-cell dose of $1.2 \pm 0.21 \times 10^8$ cells/kg). At necropsy, brains were preserved in 10% neutral buffered formalin, and immunoperoxidase staining for various antibodies was performed on 4-μm-thick formalin-fixed paraffin-embedded sections with the use of the standard avidin-biotin peroxidase complex technique (Dako, Carperinteria, CA). No evidence for infectious etiology was found, including no viral cytopathic effect, no cytomegalovirus viremia (data not shown), and no antigenic evidence for RhLCV (the rhesus macaque Epstein-Barr virus homolog) or SV40 in any of the sections examined (data not shown). Antibodies shown in this figure include CD3 (clone PC; Dako), CD4 (clone 1F6; Vector), CD8 (clone 1A5; Vector), CD163 (clone 10D6; Thermo Scientific), Ki-67 (clone MIB-1; Dako), Granzyme B (clone GRB-7; Chemicon), and LFA-1 (clone 345913; R&D). In preparation for analysis, tissue sections were washed and developed with DAB Chromagen (Dako) and counterstained with Mayer's hematoxylin. Isotype matched relevant controls were tested on all tissues. Four to 5areas were scanned at 10×, and immunopositive cells were counted from 1 representative section to provide scores ranging from 0 to 11 according to the following strategy: no positive cells, score of 0; 1 to 5 cells, score of 1; 6 to 10 cells, score of 2; 11 to 15 cells, score of 3; 16 to 20 cells, score of 4; 21 to 25 cells, score of 5; 26 to 30 cells, score of 6; 31 to 35 cells, score of 7; 36 to 40 cells, score of 8; 41 to 45 cells, score of 9; 46 to 50 cells, score of 10; >51 cells, score of 11. The cell scores for individual markers were then analyzed statistically for significance by analysis of variance (ANOVA) and Mann-Whitney *t* test using Graph Pad Prism (La Jolla, CA). Representative photomicrographs were photographed using a ×20 objective. A 50 μM standard is included in each micrograph. (A) Representative IHC for each of the treatment groups and each of the antibodies of interest. (B) Corresponding statistical analysis of the IHC scoring for all animals. The ANOVA statistic is indicated for each comparison. Red bars indicate individual comparisons for which $P \leq .05$. (C) Representative CD3 IHC from (left) a representative normal control and (right) a representative recipient of allogeneic HCT that did not receive GVHD prophylaxis for (upper) the perivasculature and (lower) the parenchyma. (D) Representative spectral analysis from a recipient of allogeneic HCT that did not receive GVHD prophylaxis for (top) CD3/CD8 and (bottom) CD8/Ki-67, performed with an Olympus Vanox S microscope and tunable filter-based camera. Cri-Nuance software (Woburn, MA) software was used to measure the colocalization between (top) CD3 and CD8 and (bottom) CD8 and Ki67. For this analysis, CD3 and Ki-67 were assigned a green pseudo-color and CD8 was assigned a red pseudo-color. Double-stained cells are assigned a yellow pseudo-color.

model and that CNS GVHD is predominantly mediated by integrin-expressing CD8^{+} T cells.

Using immunohistochemistry (IHC), we studied the meninges, cerebral vasculature, and parenchyma from NHPs that had been transplanted according to our previously described protocol for myeloablative MHC-mismatched hematopoietic cell transplant (HCT).[5] We had previously shown that animals transplanted without posttransplant immunosuppression displayed classic stigmata of gut and liver GVHD, as well as significant lassitude, and the acute onset of behavioral depression.[5] Infiltrates were found in all 3 CNS areas (Figure 1A-C), with the most striking results occurring in the meninges. As shown in Figure 1A-B, HCT recipients with severe GVHD[5] demonstrated significant CD3^{+} lymphocytic infiltration, which was not observed in the brains of normal animals or auto-logous HCT controls. Infiltrating T cells were predominately CD8^{+}, consistent with a previous report of CD8^{+} T-cell infiltration in human CNS GVHD.[4] Although there were significantly more CD3^{+}/CD8^{+} cells (Figure 1A-B,D) in GVHD^{+} recipients vs normals, there was not a significant difference in CD4^{+} T-cell infiltration across treatment groups, and the majority of CD4^{+} cells were CD3^{−} (data not shown). These CD3^{−}/CD4^{+} cells may derive from the CD4^{+} macrophage/microglial lineage.[6,7]

The phenotype of the infiltrating CD8^{+} lymphocytes demon-strated evidence of cytotoxicity (granzyme B) and proliferation (Ki-67; Figure 1A-B,D). Additionally, lymphocyte function-associated antigen-1 (LFA-1) integrin expression was highly up-regulated (Figure 1A-B). Given the central role that LFA-1 plays in CNS leukocyte trafficking during demyelinating disease,[8] these results suggest that the mechanisms of CNS T-cell infiltration during GVHD may have important similarities to those in autoimmunity and may be amenable to similar integrin-targeting therapies.[9] In addition to CD8^{+} T cells, CD163^{+} cells (macrophage/monocytes)[10] were also observed in GVHD, suggesting that infiltration of antigen presenting cells may also characterize CNS disease.

As shown in Figure 1A-B, we observed that 2 GVHD prophylaxis regimens (either tacrolimus/methotrexate or rapamycin monother-apy) both significantly decreased the GVHD-associated CD8^{+} in-filtration. However, neither completely abrogated T-cell proliferation compared with untransplanted animals, suggesting breakthrough allo-proliferation (potentially in situ, via allorecognition of host microglia, astrocytes, and other cells) with both of these regimens. These studies also document the ability to measure this breakthrough proliferation in the primate model.

Our results suggest that, in primates as in mice,[1] the behavioral abnormalities that accompany severe GVHD may have an anatomical correlation with T-cell infiltration into the brain, and that CNS disease may significantly contribute to GVHD morbidity.[5] This infiltration is skewed toward proliferating, CD8^{+}, granzyme B^{+}, LFA-1^{+} cells. Although the current experiments were limited by the difficulty in performing confirmatory functional radiologic examination in NHP, this report represents the first study linking behavioral and histopath-ological evidence for brain GVHD in primates and suggests that this increasingly appreciated manifestation of GVHD is deserving of further clinical scrutiny.

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References

1. Hartrampf S, Dudakov JA, Johnson LK, et al. The central nervous system is a target of acute graft versus host disease in mice. *Blood.* 2013;121(10):1906-1910.

2. Kamble RT, Chang CC, Sanchez S, Carrum G. Central nervous system graft-versus-host disease: report of two cases and literature review. *Bone Marrow Transplant.* 2007;39(1):49-52.

3. Kew AK, Macaulay R, Burrell S, Rubin S, Dow G, Couban S. Central nervous system graft-versus-host disease presenting with granulomatous encephalitis. *Bone Marrow Transplant.* 2007;40(2):183-184.

4. Saad AG, Alyea EP 3rd, Wen PY, Degirolami U, Kesari S. Graft-versus-host disease of the CNS after allogeneic bone marrow transplantation. *J Clin Oncol.* 2009;27(30):e147-e149.

5. Miller WP, Srinivasan S, Panoskaltsis-Mortari A, et al. GVHD after haploidentical transplantation: a novel, MHC-defined rhesus macaque model identifies CD28- CD8+ T cells as a reservoir of breakthrough T-cell proliferation during costimulation blockade and sirolimus-based immunosuppression. *Blood.* 2010;116(24):5403-5418.

6. Funke I, Hahn A, Rieber EP, Weiss E, Riethmüller G. The cellular receptor (CD4) of the human immunodeficiency virus is expressed on neurons and glial cells in human brain. *J Exp Med.* 1987;165(4):1230-1235.

7. Unger ER, Sung JH, Manivel JC, Chenggis ML, Blazar BR, Krivit W. Male donor-derived cells in the brains of female sex-mismatched bone marrow transplant recipients: a Y-chromosome specific in situ hybridization study. *J Neuropathol Exp Neurol.* 1993;52(5):460-470.

8. Hu X, Wohler JE, Dugger KJ, Barnum SR. beta2-integrins in demyelinating disease: not adhering to the paradigm. *J Leukoc Biol.* 2010;87(3):397-403.

9. Chan AC, Carter PJ. Therapeutic antibodies for autoimmunity and inflammation. *Nat Rev Immunol.* 2010;10(5):301-316.

10. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. *Nature.* 2013;496(7446):445-455.