Brief Report: Reduced Expression of CD18 Leads to the In Vivo Expansion of Hematopoietic Stem Cells in Mouse Bone Marrow

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Key Words. Hematopoietic stem cells • Cell adhesion molecules • Stem cell-microenvironment • Neutrophil

ABSTRACT

Leukocyte adhesion deficiency type-I is a primary immunodeficiency caused by mutations in the *ITGB2* gene (CD18 leukocyte integrin) which lead to defects in leukocyte extravasation. To investigate the role of CD18 in hematopoietic stem cell (HSC) biology, we have thoroughly characterized the HSCs of CD18 *Itgb2*^{tm1bay} hypomorphic mice (CD18^{HYP}) both by flow cytometry and using in vitro and in vivo transplantation assays. Flow cytometry analyses and cultures in methyl cellulose revealed that bone marrow (BM) from CD18^{HYP} mice was enriched in hematopoietic precursors, mainly early quiescent short-term and long-term Hematopoietic progenitors cells. Strikingly, BM competition assays showed a progressive expansion of CD18^{HYP}-derived hematopoiesis in recipient mice. Additionally, we provide evidence that this HSC expansion was not caused by an increased homing capacity of CD18^{HYP} HSCs or by alterations in the hematopoietic environment of CD18^{HYP} mice due to defects in neutrophils clearance. On the contrary, our data demonstrated that the reduced expression of CD18 causes a cell-autonomous expansion in the HSC compartment, thus revealing unexpected regulatory functions for CD18 in mouse HSCs. STEM CELLS *2014;32:2794–2798*

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INTRODUCTION

Leukocyte adhesion deficiency type I (LAD-I) is a primary immunodeficiency associated with mutations in the *ITGB2* gene (β_2 common leukocyte subunit CD18). Most *ITGB2* mutations lead to reduced/null expression of β_2-integrins on the leukocytes' surface [1, 2]. Hence CD18-deficient leukocytes fail to extravasate from blood to sites of infection [3–5]. Two transgenic models have been generated: a CD18 knockout model (*Itgb2*^{tm2Bay}) [6], resembling the severe phenotype of LAD-I patients, and a CD18 hypomorphic mouse model (*Itgb2*^{tm1Bay}; CD18^{HYP}) [7] resembling the symptoms of moderate LAD-I patients (mild neutrophilia, mild hyperplasia in spleen and bone marrow (BM), and impaired inflammatory response).

Previous studies have shown a higher proportion of Lin$^-$Sca1$^+$cKit$^+$ (LSK) cells in CD18$^{-/-}$ mouse BM [8, 9]. Similarly, when CD18$^{-/-}$ and WT fetal liver cells were used together in competitive transplant, the proportion of CD18$^{-/-}$ hematopoietic cells in recipient mice was always higher compared to CD18^{WT} cells [9, 10]. Although these studies suggested a contribution of CD18 in hematopoietic stem cell (HSC) regulation, the precise role of CD18 in HSCs remains poorly characterized.

In this study, we have performed a thorough hematopoietic characterization of CD18^{HYP} mice. Our results demonstrate for the first time that the downregulated expression of CD18 confers in vivo expansion potential of HSCs in mouse BM. Moreover, our data suggest that indirect effects due to accumulation of aged CD18^{HYP} neutrophils in peripheral blood (PB) of CD18^{HYP} mice or defects in the CD18^{HYP} BM microenvironment do not account for the expansion of the HSC pool, indicating that the CD18 deficiency itself accounts for the HSC expansion.

MATERIALS AND METHODS

This section is enclosed as Supporting Information.

RESULTS AND DISCUSSION

Consistent with previously reported data [6, 7, 11], CD18^{HYP} mice developed a marked leukocytosis (Supporting Information Fig. S1) and a

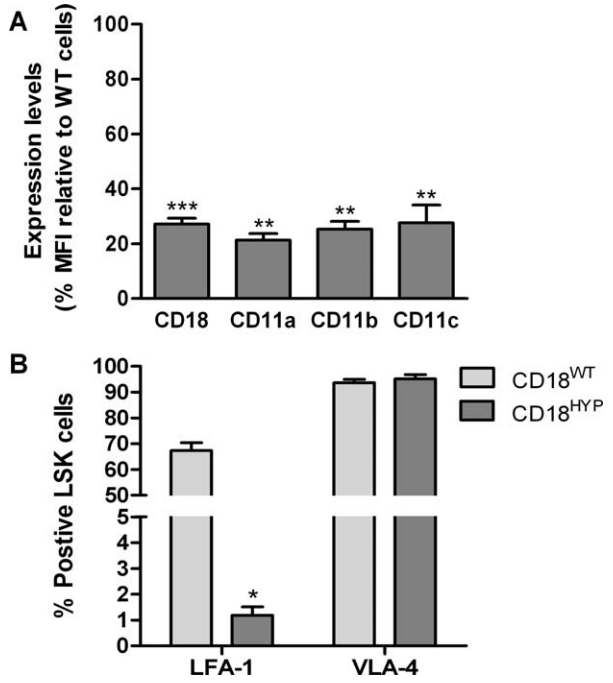

Figure 1. $CD18^{HYP}$ mice show a reduced β2-integrin expression in TBM and LSK cells. **(A):** CD18, CD11a, CD11b, and CD11c relative levels of expression in BM cells from $CD18^{HYP}$ mice. The figure shows MFI levels with respect to levels determined in BM cells from $CD18^{WT}$ mice. **(B):** Percentage of LSK cells expressing LFA-1 ($\alpha L\beta 2$ integrin dimer) and VLA-4 ($\alpha 4\beta 1$ integrin dimer). Differences were significant at: ***, $p < .001$; **, $p < .01$; *, $p < .05$. Abbreviations: $CD18^{HYP}$, CD18 hypomorphic mouse model; LFA-1, Lymphocyte Function-associated Antigen 1; LSK, $Lin^{-}Sca1^{+}cKit^{+}$; MFI, mean fluorescence intensity; TBM, Total Bone Marrow; VLA-4, Very Late Antigen 4.

severe reduction in the expression levels of CD18 and the different CD11a, CD11b, and CD11c membrane coexpressed subunits in PB leukocytes (Supporting Information Fig. S2, panels A–C).

When BM cells were analyzed, the levels of CD18 expression and of their respective CD11 membrane coexpressed subunits were also significantly decreased with respect to values observed in $CD18^{WT}$ mice (Fig. 1A). Moreover, analyses performed in BM cells of the HSC compartment showed that, in contrast to data obtained in $CD18^{WT}$ mice where 67% of the LSK cells were LFA-1^{+} (Lymphocyte Function-Associated Antigen 1; $CD18^{+}/CD11a^{+}$), only 1.2% of LSK cells from $CD18^{HYP}$ mice were LFA-1^{+} (Fig. 1B). In addition, CD18 and CD11a expression levels determined in LFA-1^{+} LSKs from $CD18^{HYP}$ mice were only around 20% and 30%, respectively, of the levels found in $CD18^{WT}$ mice (Supporting Information Fig. S2D). In contrast to this data, no differences in VLA-4 (Very Late Antigen 4) expression were detected in LSKs from $CD18^{HYP}$ (Fig. 1B; Supporting Information Fig. S2D), indicating that β_2-integrin defects in these cells are not compensated by an overexpression of β_1-integrins.

In a next set of experiments, we evaluated the content of hematopoietic progenitor cells (HPCs) in the BM of $CD18^{HYP}$ mice. While no differences in the BM cellularity were found between $CD18^{WT}$ and $CD18^{HYP}$ mice (data not shown), the proportion of LSK cells was significantly increased in $CD18^{HYP}$ mice, as compared to age-matched $CD18^{WT}$ mice (Fig. 2A). A more detailed analysis of the HPCs in the BM of $CD18^{HYP}$ mice showed that CD18 deficiency resulted in a significant increase in the proportion of long-term and short-term HPCs, as well as a nonsignificant increase in multipotent progenitors (Fig. 2B). This increase in HPCs was confirmed in clonogenic assays, which showed a significant increase in the number of colonies generated by either total BM or Lin^{-} BM cells from $CD18^{HYP}$ as compared to $CD18^{WT}$ mice (Supporting Information Fig. S3). LSK cells were then evaluated for their cell cycle and apoptosis status. A significant increase in the proportion of LSK cells in G_0 was found in $CD18^{HYP}$ mice (Fig. 2C). This indicates that CD18 deficiency leads to enrichment in quiescent HPCs, and therefore, that the characteristic leukocytosis of $CD18^{HYP}$ mice does not occur at the expense of an increased population of proliferating BM HPCs.

The role of CD18 in the regulation of apoptosis in neutrophils has been extensively studied. The engagement of β_2-integrin Mac-1 on neutrophils can either inhibit or enhance apoptosis depending on the activation state of the integrin and the presence of proapoptotic stimuli [12, 13]. While we observed that $Gr1^{+}$ BM cells from $CD18^{HYP}$ mice had reduced levels of apoptosis compared to WT cells (data not shown), we did not detect any difference in the proportion of apoptotic ($SubG_0$) LSK^{HYP} cells (Fig. 2C), suggesting that CD18 is not implicated in apoptosis regulation in mouse HPCs.

Although current data indicate that β_2-integrins do not play a direct role in HSC homing, it has been described that if the function of β_1-integrins is compromised, defects in β_2-integrin expression lead to a synergistic impairment in HSC homing [14, 15]. To determine whether the HSC homing efficiency was affected in $CD18^{HYP}$ mice, fluorescence-activated cell sorted and DiD-stained LSKs from $CD18^{HYP}$ or $CD18^{WT}$ mice were intravenously injected into lethally irradiated $CD18^{WT}$ recipients. Sixteen hours later, the proportion of DiD^{+} LSK cells that had homed into recipient BM was determined by flow cytometry. As shown in Figure 2D, no differences in the homing efficiency of $CD18^{HYP}$ and $CD18^{WT}$ LSK cells were observed in these experiments.

Recent observations in P- and E-selectin knockout (PEdKO) mice have shown an increased population of aged neutrophils with a $CD62L^{LO}$ $CXCR4^{HI}$ phenotype in PB due to an impaired extravasation of these cells to the BM, ultimately resulting in enhanced retention of HSCs in the BM [16]. Thus, we compared the proportion of aged neutrophils in PB of $CD18^{HYP}$ mice with respect to $CD18^{WT}$ and PEdKO mice. As shown in Figure 3A, $CD18^{HYP}$ mice mimicked the increased proportion of aged neutrophils characteristic of PEdKO mice, suggesting similar extravasation defects in $CD18^{HYP}$ neutrophils. Because this effect might account for the increased numbers of HPCs observed in the BM of $CD18^{HYP}$ mice, we investigated whether a 5-day infusion of ex vivo senesced $CD18^{WT}$ BM-derived neutrophils into $CD18^{HYP}$ mice could decreased the number of LSKs in $CD18^{HYP}$ BM. No significant changes in the proportion of LSKs were observed between $CD18^{HYP}$ mice treated with aged neutrophils and saline (Fig. 3B), strongly suggesting that increased numbers of HPCs in $CD18^{HYP}$ mouse BM are not produced by defects in neutrophil extravasation.

We thus considered the possibility that CD18 deficiency may induce an increased self-renewal of $CD18^{HYP}$ repopulating HSCs

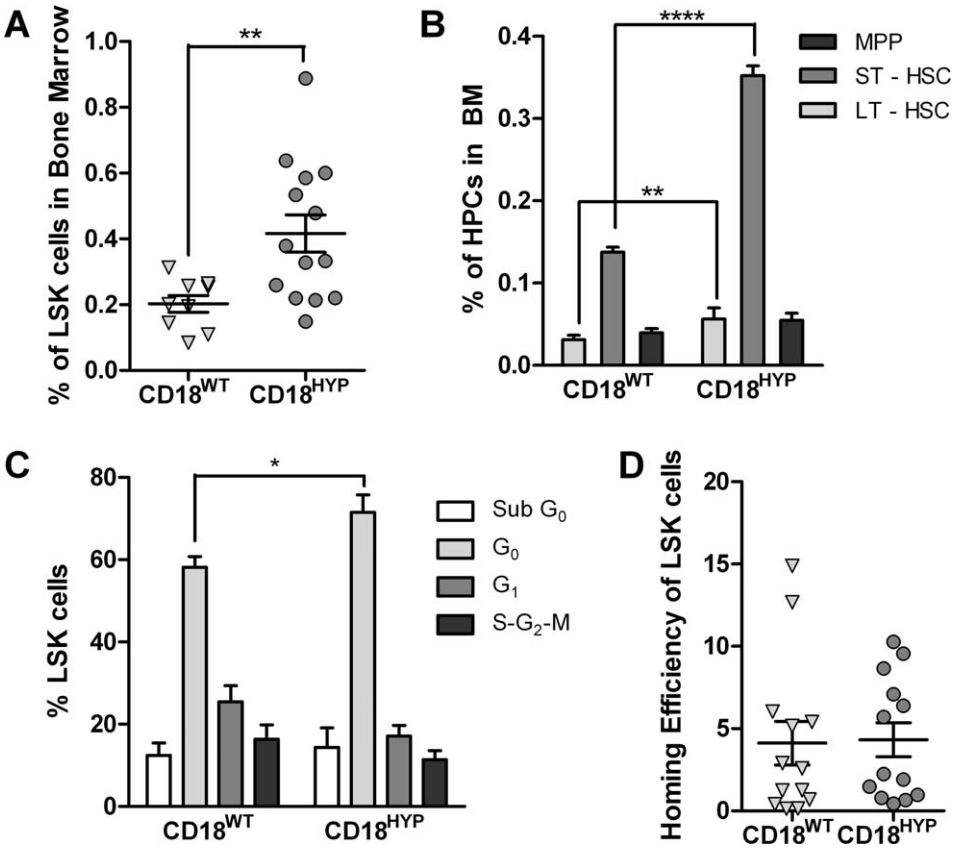

Figure 2. BM of CD18^{HYP} mice has an increased content of HPCs. **(A):** Compared analysis of LSK (Lin$^-$, Sca1$^+$, and cKit$^+$) cells in the BM of CD18^{HYP} and CD18^{WT} mice. **(B):** Flow cytometry characterization of different subpopulations of LSK cells from CD18^{HYP} and CD18^{WT} mice: LT-HSC (LSK/Flk2$^-$/CD34$^-$/CD150$^+$); ST-HSC (LSK/Flk2$^-$/CD34$^+$/CD150$^-$); MPP (LSK/IL7Rα^-/Flk2$^+$); see more details of specific markers in Supporting Information Materials. **(C):** Cell cycle analyses using Pironyn Y and Hoechst staining to distinguish LSK cells in SubG$_0$, G$_0$, G$_1$, and S-G$_2$-M phase. **(D):** Comparative analysis of the CD18^{HYP} and CD18^{WT} LSK homing; see experiment details in Supporting Information Materials. The significance of differences between groups are expressed as ****, $p < .0001$; **, $p < 0.01$; *, $p < .05$. Abbreviations: BM, bone marrow; CD18^{HYP}, CD18 hypomorphic mouse model; HPC, hematopoietic progenitor cells; LSK, Lin$^-$Sca1$^+$cKit$^+$; LT-HSC, long-term hematopoietic stem cells; MPP, multipotent progenitors; ST-HSC, short-term hematopoietic stem cells.

due to differences in their interaction with the hematopoietic environment. To investigate this hypothesis, we performed competitive repopulation assays in which BM cells from CD18^{HYP} mice (CD45.2$^+$) and from CD18^{WT} mice (CD45.1$^+$/ΔhCD4$^+$) [17] were mixed together in the same proportion and transplanted into lethally irradiated CD18^{HYP} recipients (CD45.1$^+$). Under these conditions, we could track the repopulating properties of CD18^{HYP} and CD18^{WT} HSCs in the same WT microenvironment. Control experiments showed that the repopulating ability of WT BMCs from these three different haplotypes was the same (Supporting Information Fig. S4). As shown in Figure 4A, a progressive increase of CD18^{HYP}-derived hematopoiesis was observed in transplanted recipients. Moreover, when BM from primary recipients was further transplanted into secondary recipients we found an increment of CD18^{HYP}-derived hematopoiesis in PB. This increment was also observed, even more marked, in analyses of total BM and in BM-derived colonies (Fig. 4A).

A similar BM competitive repopulation experiment was then performed mixing together a different proportion of CD18^{WT} and CD18^{HYP} BM cells (70:30), which contained the same number of CD18^{HYP} and CD18^{WT} LSK cells (Fig. 4B). In spite of the low proportion of total CD18^{HYP} BMCs transplanted in these animals, a progressive increase of CD18^{HYP}-derived hematopoietic cells could be observed in PB analyses from primary recipients. In secondary recipients, a 50% of CD18^{HYP} chimerism was observed up to 120 days post-transplantation, consistent with the initial proportion of CD18^{HYP} transplanted LSK cells. Remarkably, when BM-derived progenitor cells from secondary recipients were analyzed, the proportion of CD18^{HYP}-derived progenitors was further increased, reaching statistical significance in LSK cell analyses (Fig. 4B).

Conclusion

Our data demonstrate that downregulation of CD18 results in increased numbers of HSCs and HPCs in the BM of CD18^{HYP} mice. As no homing differences were found between CD18^{HYP} and CD18^{WT} LSK cells, our observations indicate that CD18 deficiency causes a progressive cell-autonomous in vivo expansion of the HSCs, thus uncovering a new role for CD18 in HSC homeostasis.

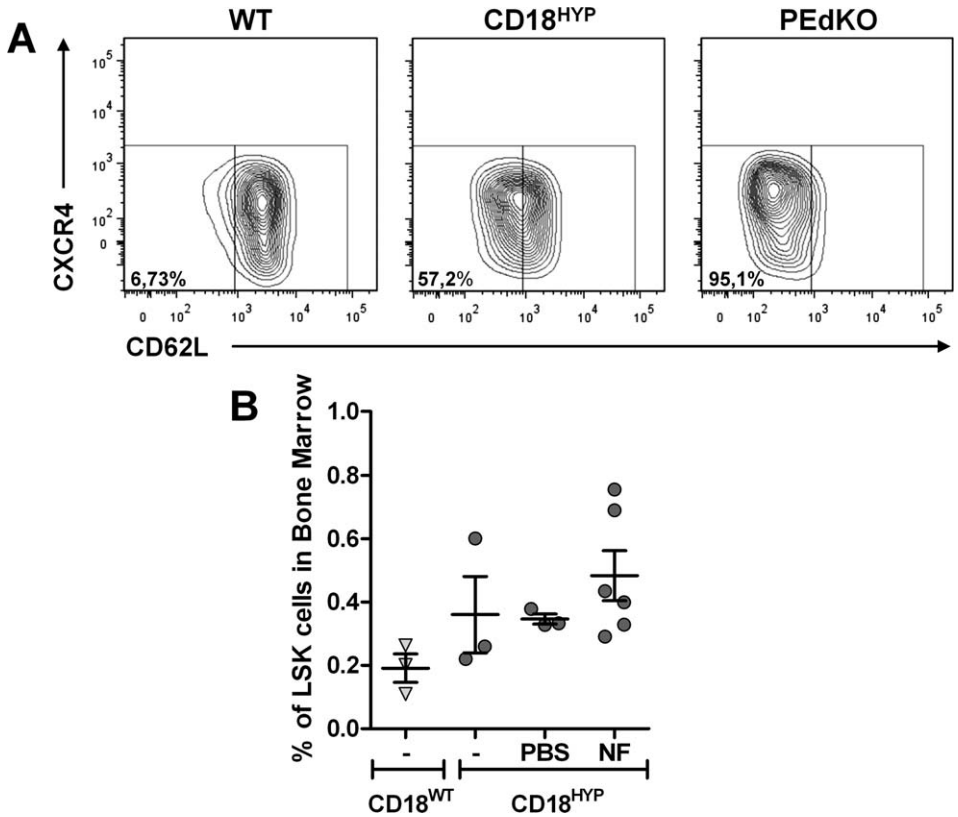

Figure 3. Clearance of aged neutrophils does not contribute to the increased LSK content in $CD18^{HYP}$ BM. **(A):** Comparative analysis of the proportion of aged neutrophils ($Ly6G^{+}CD62L^{LO}$ $CXCR4^{HI}$) in PB of $CD18^{WT}$, $CD18^{HYP}$, and PEdKO mice. **(B):** Determination of LSK content in the BM of $CD18^{HYP}$ mice treated for 5 days with $4–7 \times 10^{6}$ $CD18^{WT}$ BM-derived neutrophils senesced ex vivo. As controls, $CD18^{HYP}$ mice treated with PBS or untreated $CD18^{WT}$ and $CD18^{HYP}$ mice were used. Abbreviations: BM, Bone Marrow; $CD18^{HYP}$, CD18 hypomorphic mouse model; LSK, $Lin^{-}Sca1^{+}cKit^{+}$; NF, Neutrophils; PBS, Phosphate Buffered Saline; PB, Peripheral Blood; PEdKO, P- and E-selectin knockout.

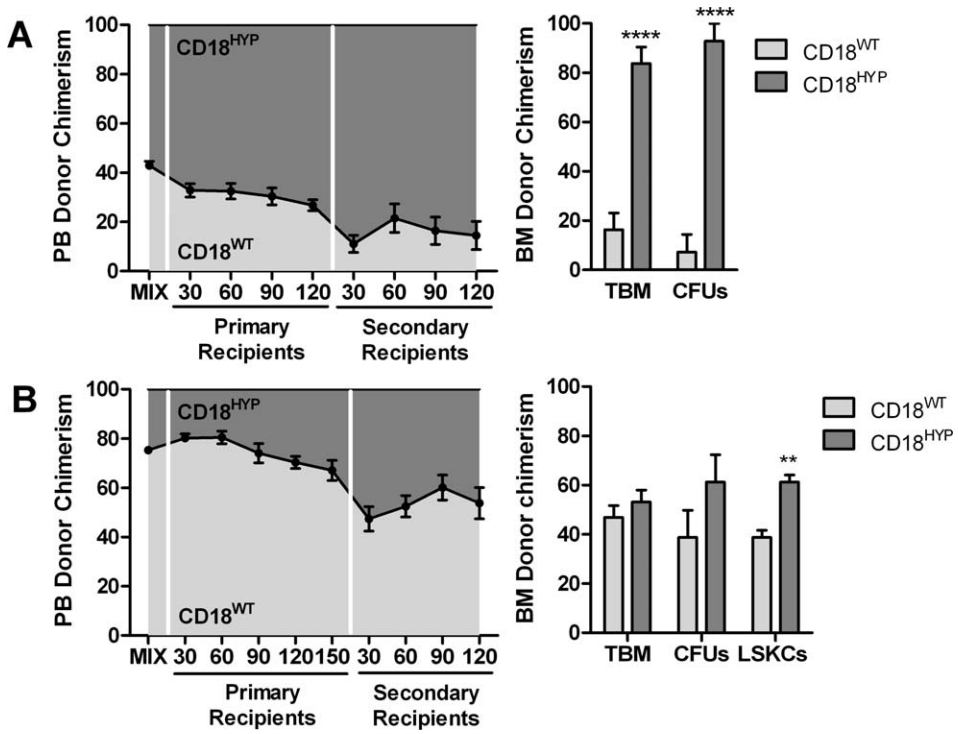

Figure 4. $CD18^{HYP}$ BM cells have an increased long-term repopulating ability. Kinetic analysis of the competitive repopulation ability of BM from $CD18^{HYP}$ mice. **(A):** BM cells from $CD18^{WT}$ ($CD45.1^{+}/\Delta hCD4^{+}$) (grey area) and $CD18^{HYP}$ mice ($CD45.2^{+}$) (black area) were mixed (50:50) and transplanted into irradiated recipients ($CD45.1^{+}$). Donor chimerism levels based on CD45.2, CD45.1, and ΔhCD4 markers were determined in PB from primary and secondary recipients during the 4-month follow-up. Chimerism levels corresponding to the BM and BM-derived colonies from primary recipients are also shown. **(B):** BM cells from $CD18^{WT}$ and $CD18^{HYP}$ mice were mixed at a ratio of 70:30 to infuse the same number of LSK cells. Donor chimerism levels were determined as in panel (A). Significance of differences between groups are expressed as ****, $p < .0001$; **, $p < .01$. Abbreviations: BM, bone marrow; $CD18^{HYP}$, CD18 hypomorphic mouse model; CFUs, Colony Forming Units; LSKCs, $Lin^{-}Sca1^{+}cKit^{+}$ cells; PB, peripheral blood; TBM, Total Bone Marrow.

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Author Contributions

D.L.R.: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing; M.A.: collection and/or assembly of data and data analysis and interpretation; R.S.: provision of analytical tools, collection and/or assembly of data, and data analysis and interpretation; J.C.S.: provision of analytical tools and data analysis and interpretation; L.W.: collection and/or assembly of data, data analysis and interpretation and manuscript writing; A.H. conception and design, data analysis and interpretation, and manuscript writing. J.A.B.: conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript; E.A.N.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no conflict of interest.

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