Normal systemic iron homeostasis in mice with macrophage-specific deletion of transferrin receptor 2

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Rishi G, Secondes ES, Wallace DF, Subramaniam VN. Normal systemic iron homeostasis in mice with macrophage-specific deletion of transferrin receptor 2. Am J Physiol Gastrointest Liver Physiol 310: G171–G180, 2016. First published November 25, 2015; doi:10.1152/ajpgi.00291.2015.—Iron is an essential element, since it is a component of many macromolecules involved in diverse physiological and cellular functions, including oxygen transport, cellular growth, and metabolism. Systemic iron homeostasis is predominantly regulated by the liver through the iron regulatory hormone hepcidin. Hepcidin expression is itself regulated by a number of proteins, including transferrin receptor 2 (TFR2). TFR2 has been shown to be expressed in the liver, bone marrow, macrophages, and peripheral blood mononuclear cells. Studies from our laboratory have shown that mice with a hepatocyte-specific deletion of Tfr2 recapitulate the hemochromatosis phenotype of the global Tfr2 knockout mice, suggesting that the hepatic expression of TFR2 is important in systemic iron homeostasis. It is unclear how TFR2 in macrophages contributes to the regulation of iron metabolism. We examined the role of TFR2 in macrophages by analysis of transgenic mice lacking Tfr2 in macrophages by crossing Tfr2−/− mice with LysM-Cre mice. Mice were fed an iron-rich diet or injected with lipopolysaccharide to examine the role of macrophage Tfr2 in iron- or inflammation-mediated regulation of hepcidin. Body iron homeostasis was unaffected in the knockout mice, suggesting that macrophage TFR2 is not required for the regulation of systemic iron metabolism. However, peritoneal macrophages of knockout mice had significantly lower levels of ferroportin mRNA and protein, suggesting that TFR2 may be involved in regulating ferroportin levels in macrophages. These studies further elucidate the role of TFR2 in the regulation of iron homeostasis and its role in regulation of ferroportin and thus macrophage iron homeostasis.

iron metabolism; hepcidin; macrophages

Iron is an essential mineral; it is required for the functioning of a variety of proteins and enzymes. A deficiency of iron leads to anemia, which has been recognized by the World Health Organization as the most common nutritional disorder affecting approximately two billion people worldwide (5, 20). Excess iron leads to hemochromatosis, which is characterized by iron deposition in the tissues and ultimately leads to organ dysfunction (4). In the absence of a known excretory mechanism it is essential for the body to regulate the amount of iron absorbed. Central to this regulation is hepcidin, a 25-amino-acid peptide produced predominantly in the liver, encoded by the hepcidin antimicrobial peptide (HAMP) gene. Hepcidin binds to and induces the internalization of ferroportin (FPN), the only known iron export protein, thus limiting the amount of iron released into the bloodstream.

Hepcidin is regulated in response to a number of external and internal stimuli, including but not limited to, body iron stores, inflammation, erythropoiesis, and hypoxia (30). These stimuli have been shown to mediate hepcidin synthesis through several different molecules, including the hemochromatosis protein (HFE), hemosiderin (HJV), transferrin receptor 2 (TFR2), interleukin-6 (IL-6), and erythropoietin (reviewed in Ref. 30). Patients (6, 33) and mice (9, 40) with mutations in TFR2 develop type 3 hemochromatosis that is characterized by inappropriate hepcidin levels in relation to body iron stores (24, 38). The mechanism by which TFR2 mediates hepcidin regulation is still unclear. Previous studies have reported that TFR2 can interact with HFE (10, 11), and that this interaction is required for the regulation of hepcidin (10). Recently it was proposed that TFR2, HFE, and the bone morphogenetic protein (BMP) coreceptor HJV can form a multiprotein complex that regulates HAMP levels in response to increased iron levels in human hematopoietic HepG2 cells (8). Contrary to this, studies using the Hfe/Tfr2 double knockout mice (37) or transgenic mice expressing myc-tagged HFE (34) have suggested that HFE and TFR2 can act independently to regulate hepcidin. Recent studies from our laboratory have also suggested that HFE and TFR2 do not interact in a stable coexpression system (29). Although the mechanism is still unclear, as a result of these studies we know that TFR2 in hepatocytes is required for an appropriate HAMP response.

TFR2 is predominantly expressed in the liver (16), but initial studies had also reported mRNA expression in extrahepatic tissues, including bone marrow, spleen, peripheral blood mononuclear cells, prostate gland (14, 16), and splenic and peritoneal macrophages (32). Mice with a hepatocyte-specific deletion of Tfr2 recapitulate the iron overload phenotype of the global Tfr2 knockout mice (9, 38–40), suggesting that the hepatic expression of TFR2 is sufficient for the systemic regulation of iron homeostasis. However, it is unclear how and whether TFR2 expressed in macrophages contributes to the regulation of iron metabolism. To examine the role of TFR2 in macrophages we generated transgenic mice lacking Tfr2 in macrophages by crossing Tfr2−/− mice with LysM-Cre transgenic mice. The role of macrophage TFR2 in systemic iron metabolism, in the presence of excess iron or an inflammatory stimulus, was also determined by feeding the mice an iron-rich diet or injecting them with lipopolysaccharide (LPS). While the results from this study indicate that macrophage TFR2 is not required for systemic iron homeostasis, they also suggest that Tfr2 expression in the macrophages may be required for the regulation of Fpn1, an essential component of the iron transport pathway.
MATERIALS AND METHODS

Generation of Tfr2\textsuperscript{−/−}/LysM-Cre\textsuperscript{−/−} mice and treatments. All animal experimentation was performed according to the guidelines and approval of the QIMR Berghofer Medical Research Institute Animal Ethics Committee. Mice were housed under a 12:12-h light-dark cycle and were provided with food and water ad libitum. Tfr2\textsuperscript{−/−} mice (40) were bred with Tfr2\textsuperscript{+/−}/LysM-Cre\textsuperscript{−/−} to generate control (Tfr2\textsuperscript{+/−}/LysM-Cre\textsuperscript{−/−}) and macrophage-specific knockout (KO) (Tfr2\textsuperscript{−/−}/LysM-Cre\textsuperscript{−/−}) mice. Control and KO male mice at 3 wk of age were fed a control (iron content: 68 mg/kg) or an iron-rich (iron content: 20 g/kg) diet for 2 wk (Speciality Feeds, Glen Forest, Western Australia). Five-week-old control and KO male mice were injected with LPS (1 μg/g body wt) (Sigma-Aldrich, Sydney, NSW, Australia) or saline (control) for 6 h, and their tissues were harvested for further analysis. All mice used in this study were bred on a C57BL/6J background.

Isolation and culture of peritoneal macrophages. Peritoneal macrophages were isolated using the peritoneal gavage method as described (43). Anesthetized control and KO mice (n = 5 and 3, respectively) were injected intraperitoneally with 10 ml of ice-cold phosphate-buffered saline. With the use of the same syringe and needle the fluid was aspirated from the peritoneum. The cells were then centrifuged at 4°C for 10 min at 400 g. After centrifugation, the cell pellet was resuspended in RPMI 1640 medium (Life Technologies, Mulgrave, Australia) supplemented with 10% fetal calf serum (FCS) (Hyclone Lab, Mordialloc, Victoria, Australia). Freshly isolated control and KO male mice were injected with LPS (1 μg/g body wt) (Sigma-Aldrich, Sydney, NSW, Australia) or saline (control) for 6 h, and their tissues were harvested for further analysis. All mice used in this study were bred on a C57BL/6J background.

Histology. Formalin (10%)-fixed tissues were processed, paraffin embedded, and sectioned by the QIMR Berghofer Histotechnology Facility. Perls’ staining was performed as described by McDonald et al. (19). Slides were scanned using the Aperio AT Turbo (Aperio, Vista, CA) at 20 magnification. The sections were analyzed using ScanScope software (Aperio).

Real-time PCR. Total RNA was isolated from bone marrow, kidney, liver, spleen, or peritoneal macrophages using TRIzol reagent (Life Technologies). The SensiFAST cDNA synthesis kit (Bioline, Sydney, NSW, Australia) was used to prepare cDNA from 1 μg of total RNA. Real-time quantitative PCR (qPCR) was performed using the SensiFAST SYBR No-Rox kit (Bioline) and the following conditions: 5 min denaturation at 95°C followed by 45 cycles of 95°C for 15 s, 60°C for 10 s, and 72°C for 15 s. The expression of all target genes was normalized to the geometric mean of three reference genes: β-actin, hypoxanthine-guanine phosphoribosyl transferase, and DNA-directed RNA polymerase II subunit RPBI. Primers sequences are listed in Table 1.

Western blotting. Liver tissue (50–100 mg) was homogenized in protein extraction buffer (previously described in Ref. 29) using the Prewittels 24 tissue homogenizer. Peritoneal macrophages were harvested directly in 200 μl of the protein extraction buffer. Tissue homogenates (25 μg protein) or cell lysates (50 μl) were electrophoresed on 12% SDS-polyacrylamide gels at 200 V for 1 h and 15 min. The proteins were then transferred onto nitrocellulose membranes (0.2 μm pore size) (Bio-Rad Laboratories, Gladesville, NSW, Australia) using the Trans-blot Turbo blotting apparatus (Bio-Rad) at 25 V, 2.5 A for 30 min in the transfer buffer. The membrane was then blocked for 2 h at room temperature (RT) with 10% nonfat milk and then probed in 1:1000 antibody dilution in 1% nonfat milk in Tris-buffered saline-Tween (TBS-T) buffer for 2 h at room temperature.

Table 1. Primers used in the study

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Fig. 1. Loss of transferrin receptor 2 (Tfr2) in macrophages leads to a reduction in ferroporin (Fpn1) mRNA. mRNA expression levels of Tfr2 (A), Fpn1 (B), divalent metal transporter 1 (Dmt1, C), hypoxia inducible factor 1α (Hif1α, D), ferritin heavy chain (Fih, E), ferritin light chain (Fli, F), superoxide dismutase 2 (Sod2, G), and Tfr2 (H) (relative to the geometric mean of 3 reference genes: β-actin, hypoxanthine-guanine phosphoribosyl transferase, and DNA-directed RNA polymerase II subunit RPBI) were measured in the peritoneal macrophages of 5-wk-old control (Tfr2\textsuperscript{+/−}, ■) and knockout (KO, Tfr2\textsuperscript{−/−}/LysM-Cre\textsuperscript{−/−}, ■) male mice (n = 3–5/group) fed a control diet. Data are shown as dot plots, showing the mean and SE. *Statistically significant differences (Student’s t-test; P < 0.05) compared with the control genotype.

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incubated with primary antibodies [anti-actin (1:40,000) (Sigma-Aldrich), anti-TFR2 (1:20,000) (38), anti-phospho (p)-mothers against decapentaplegic homolog (SMAD) 1/5 (1:2,000) (Cell Signaling Technology, Danvers, MA), or anti-FPN (1:1,000) (Alpha Diagnostics, San Antonio, TX) diluted in 10% nonfat milk] overnight at 4°C. The following day, the membranes were washed and incubated with secondary antibodies [anti-mouse/rabbit heavy- and light-chain IgG conjugated to horseradish peroxidase (1:10,000) (Invitrogen, Life Technologies) diluted in 10% nonfat milk] for 1 h at RT. After being washed the blots were incubated with chemiluminescent substrate (Lumina Forte; Merck Millipore, Kilsyth, Victoria, Australia) for 5 min and exposed to X-ray film (Fujifilm, Brookvale, NSW, Australia) for various times. Films were developed using the Minolta film processor (Konica Minolta Medical and Graphic, Tokyo, Japan). The processed films were then scanned using Scanmaker 9800 XL plus processor (Konica Minolta Medical and Graphic, Tokyo, Japan). Densitometric analysis for quantification of the proteins was performed using Genetools software (version 4.0) (Syngene, Cambridge, UK).

Statistical analyses. Statistical analyses on variables between different groups of mice were performed by using two-way ANOVA and Student's t-test. Post hoc analysis was performed to compare the differences between individual groups using Tukey's multiple-comparison tests. P values <0.05 were considered to be statistically significant. Statistical analysis was performed using the GRAPHPAD PRISM 6 software (GraphPad Software, San Diego, CA).

RESULTS

Deletion of macrophage Tfr2 leads to a reduction in ferroportin expression in peritoneal macrophages. To generate mice lacking Tfr2 specifically in macrophages we crossed mice carrying the floxed Tfr2 allele (39) with mice expressing the Cre recombinase under the lysozyme M promoter that has been shown to be active in macrophages (7). A significant reduction in Tfr2 mRNA in the peritoneal macrophages (P = 0.015) of these mice (Tfr2fl/LysM-Cre<sup>+/−</sup>, referred to KO mice here) compared with controls was shown by qPCR analysis (Fig. 1A), suggesting that the Tfr2 gene had been deleted in these cells. It was previously reported that the β-form of TFR2 may be involved in the regulation of Fpn1 specifically in macrophages, thus specifically controlling iron efflux in the spleen (32). The KO mice used in this study lack both the α- and the β-form of Tfr2, since the loxP sites were inserted flanking exon 2 to exon 6 of the Tfr2 gene (40). The Fpn1 mRNA levels in isolated peritoneal macrophages were significantly lower in the KO mice (Fig. 1B).

We also measured the mRNA expression levels of several genes known to be involved directly or indirectly in maintaining macrophage iron homeostasis. There were no significant differences between the mRNA expression levels of divalent metal transporter 1 (Fig. 1C), hypoxia inducible factor 1α (Fig. 1D), ferritin heavy chain (Fth, Fig. 1E), ferritin light chain (Ftl, Fig. 1F), superoxide dismutase 2 (Fig. 1H), or Tfr1 (Fig. 1G) in the peritoneal macrophages derived from control and KO mice.

The reduction in Fpn1 mRNA levels was also confirmed at the protein level in the peritoneal macrophages. The amount of FPN protein was significantly reduced in the peritoneal macrophages of the KO mice compared with control mice (Fig. 2).

Deletion of macrophage Tfr2 does not affect Tfr2 expression levels in the liver or systemic iron parameters. Deletion of Fpn1 in the macrophages of mice has been shown to lead to a mild tissue iron overload in the liver and spleen due to accumulation of iron in macrophages. To determine whether the reduction of Fpn1 in the macrophages of the mice lacking macrophage-Tfr2 results in iron overload we examined the liver and systemic iron parameters in the control and KO mice.

The role of macrophage TFR2 in the iron-mediated regulation of systemic iron homeostasis was investigated by measuring serum and tissue iron parameters, and feeding the mice an iron-rich diet. There were no significant differences between the serum iron (Fig. 3A), transferrin saturation (Fig. 3B), HIC (Fig. 3C), or SIC (Fig. 3D) of the control and KO mice. As expected, serum and tissue iron indexes increased significantly in the mice fed an iron-rich diet. However, there were no significant differences between the control and KO mice (Fig. 3), suggesting that macrophage-specific deletion of Tfr2 does not affect systemic iron levels.

The pattern of iron distribution was assessed by Perls' staining (Fig. 4). No differences in the morphology or the pattern of iron distribution between the control and KO mice was observed, even in mice fed an iron-rich diet. The iron distribution was in the normal periportal pattern, accumulating in hepatocytes of the mice fed an iron-rich diet, with no observable differences between the control and KO mice.
In the spleen, no stainable iron was observed in either the control or KO mice fed a control diet. However, the spleens of the mice fed an iron-rich diet showed iron loading in the reticuloendothelial cells of the red pulp (Fig. 4).

**BMP- and SMAD-mediated regulation of hamp in the liver is not affected in KO mice fed an iron-rich diet.** It is now clear that **Hamp** is central to the regulation of iron homeostasis. **Hamp** itself is regulated in response to a number of external and internal stimuli, including body iron levels. The iron-mediated regulation of **Hamp** is dependent on the BMP-SMAD pathway (1–3, 21). An increase in body iron levels induces **Bmp6** in the liver (13). The binding of BMPs to their receptors induces the phosphorylation of the receptors, which results in a signaling cascade mediating the regulation of downstream genes, including **Hamp**. We investigated the role of macrophage TFR2 in the iron-mediated regulation of **Hamp** by examining the mRNA expression of molecules involved in this pathway in the livers of the control and KO mice fed a control or an iron-rich diet.

The mRNA expression levels of **Bmp6** (Fig. 5A) and **Hamp** (Fig. 5B) and the downstream targets of BMP-SMAD signaling, **inhibitor of DNA-binding 1** (Fig. 5C) and **Smad7** (Fig. 5D), were increased in the control and KO mice fed an iron-rich diet. There were no significant differences in the levels of **Bmp6** and **Hamp** and the other downstream targets of the BMP-SMAD signaling pathway between the control and KO mice fed an iron-rich diet.
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A

Relative expression of Bmp6

Control diet | High Fe diet

B

Relative expression of Hamp

Control diet | High Fe diet

C

Relative expression of Id1

Control diet | High Fe diet

D

Relative expression of Smad7

Control diet | High Fe diet

E

Relative expression of Hfe

Control diet | High Fe diet

F

Relative expression of Hhv

Control diet | High Fe diet

G

Relative expression of Fpn1

Control diet | High Fe diet

H

Relative expression of Tfr2

Control diet | High Fe diet
and KO groups in their respective diet group (Fig. 5, A–D), suggesting that iron-mediated Hamp regulation is not affected in the mice lacking TFR2 in macrophages. In addition, we also looked at the mRNA expression of other molecules known to be involved in the iron-mediated regulation of Hamp. There were no significant changes in the mRNA levels of Hfe (Fig. 5E) and Hjv (Fig. 5F) in the control and KO mice. The levels of Fpn1 in KO mice fed an iron-rich diet were significantly lower compared with the control mice on the same diet (Fig. 5G). There were no significant differences in the mRNA levels of Tfr2 in the livers of control and KO mice, suggesting that the deletion of Tfr2 is specific to the macrophages and has not affected hepatocyte Tfr2 expression (Fig. 5H).

To analyze the regulation of BMP-SMAD signaling at the protein level, Western blotting was performed on total liver homogenates of KO and control mice fed a control or iron-rich diet. There was an increase in the levels of TFR2 protein in the mice fed an iron-rich diet (Fig. 6, A and B). This was expected, since it has been shown that an increase in holo-TF stabilizes the TFR2 protein in cells (12) and liver (31). The levels of pSMAD1/5 were increased in the livers of mice fed an iron-rich diet (Fig. 6, A and C). These results suggest there is appropriate BMP-SMAD signaling in the livers of the KO mice.

**Deletion of macrophage Tfr2 does not affect inflammation-mediated regulation of hamp.** The major function of macrophages is to maintain immune homeostasis, and one aspect of this involves regulating Hamp in response to inflammation. Inflammatory stimuli such as turpentine and LPS are known to induce Hamp in both mice and humans. This increase in Hamp is thought to be mediated by the production of IL-6 by macrophages, which then acts through the JAK-STAT pathway resulting in the production of HAMP (22, 23, 41). Mice lacking both Hfe and Tfr2 or Tfr2 alone have a reduced Hamp induction in response to inflammatory stimuli (36) compared with wild-type mice, suggesting that TFR2 could play a role in inflammation-mediated Hamp induction.

To determine whether the absence of Tfr2 in macrophages affects inflammation-mediated iron homeostasis, control and KO mice were injected with LPS (1 μg/g body wt) or saline (control) for 6 h, and their tissues were harvested for further analysis. The mice treated with LPS for 6 h had significantly lower serum iron levels compared with controls (saline treated) (Fig. 7A). These results are in agreement with previous studies which have shown that in the presence of infections or inflammatory stimuli serum iron levels decrease (23, 25). A decrease in transferrin saturation (Fig. 7B) was observed in both the control and KO mice, and no differences were observed between genotypes, suggesting that this was an LPS-specific effect and it did not require macrophage Tfr2 expression.

We investigated the role of macrophage TFR2 in the inflammation-mediated regulation of Hamp in the liver. As expected, the expression of Hamp increased significantly in the livers of mice treated with LPS (Fig. 8A), and there were no significant differences between the control and KO mice. This suggests Tfr2 expression in macrophages is not required for the inflammation-mediated Hamp response in the liver.

The mRNA levels of Fpn1 and Hjv in the livers of the control and KO mice treated with LPS were significantly lower (Fig. 8, B and C). This is in accordance with several previously mentioned studies which have shown that the expression of these genes is upregulated by LPS, leading to a decrease in their levels. These results suggest that Tfr2 expression in macrophages does not affect the regulation of these genes in response to LPS.
published studies that have reported the LPS-mediated down-regulation of Fpn1 and Hjv (17, 18, 26, 28, 42).

To determine whether macrophage-specific deletion of Tfr2 causes differences in the inflammatory response, we also measured the mRNA levels of orosomucoid 2 (Orm2) and serum amyloid A1 (Saa1) (Fig. 8, D and E), two known inflammatory markers expressed in the liver (36). The mRNA levels of these two genes were similar in the control and KO mice for both saline and LPS treatments.

**DISCUSSION**

Previous studies have suggested that Tfr2 is expressed in macrophages (15, 33). The β-form of TFR2 was suggested to...
be involved in the transcription of Fpn1 specifically in macrophages (33). To examine the molecular functions of Tfr2 in macrophages, LysM-Cre mice, which have been shown to express the Cre protein in cells of the macrophage lineage, including the peritoneal and splenic macrophages and Kupffer cells in the liver (7, 27), were crossed with Tfr2 KO mice generated in our laboratory (40). This resulted in the deletion of Tfr2 from the macrophages. Reduced expression of Tfr2 mRNA was detected specifically in peritoneal macrophages but not in the liver.

The loss of Tfr2 in the macrophages did not lead to any significant changes in the intracellular iron levels, since we did not observe any differences in the mRNA levels of Tfr1, Fth, or Ftl. The mRNA levels of other genes known to be involved in macrophage iron homeostasis did not differ in the peritoneal macrophages of the control and KO mice. These results suggested that loss of Tfr2 does not affect iron metabolism in the macrophages.

The levels of FPN mRNA and protein were significantly lower in the peritoneal macrophages of the KO mice, supporting the previous observation that Tfr2 could be influencing Fpn1 mRNA levels (32). To test this we did a correlation between Tfr2 and Fpn1 expression in the peritoneal macrophages. The Pearson correlation coefficient was R = 0.9049, and this correlation was also significant (P = 0.002). Several factors, including iron levels, inflammation, and hypoxia, have been shown to affect Fpn1 levels. We did not observe any significant differences in the mRNA levels of Tfr1, Fth, or Ftl, which have been previously used as markers of intracellular iron. This suggests that the decrease in Fpn1 is not due to altered iron levels in the macrophages. Similarly, we did not see any differences in the mRNA expression levels of the inflammatory markers Orm2 and Saa1, suggesting that the reduced Fpn1 levels are not due to increased basal inflammation. These results provide evidence that the reduced Fpn1 levels in the macrophages are due to loss of Tfr2 in the macrophages.

A reduction in FPN levels in the macrophages could lead to iron retention in these cells, which may in turn disrupt systemic iron homeostasis. To determine whether the reduced Fpn1 in macrophages of the KO mice leads to iron overload or dysregulated iron homeostasis, we examined the role of macrophage Tfr2 in iron-mediated, and inflammation-mediated, Hamp expression in the liver by either feeding the mice on an iron-rich diet or injecting them with LPS for 6 h. The deletion of Tfr2 in macrophages did not affect systemic iron metabolism, suggesting that macrophage Tfr2 is not required for the systemic regulation of body iron levels. The expression levels of genes involved in regulating iron metabolism did not differ significantly in the livers of control and KO mice. Previously it has been suggested that β-Tfr2 is required for Fpn1 transcription in macrophages (32). The KO mice generated in our studies lack both α- and β- forms of Tfr2, whereas the mice used in the study by Roetto et al. (32) lacked β-Tfr2 but expressed the α-form of Tfr2 in all other tissues. The macrophage-specific Tfr2-KO mice do not develop iron overload, similar to the mice used in the study by Roetto et al. (32), but since they lack both forms of Tfr2 in the macrophages this is a novel model to study the function of Tfr2 in the cells of macrophage lineage.

ACKNOWLEDGMENTS

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

G.R., D.F.W., and V.N.S. conceived and designed the research; G.R. and E.S.S. performed experiments; G.R., E.S.S., D.F.W., and V.N.S. analyzed data; G.R., D.F.W., and V.N.S. interpreted results of experiments; G.R. prepared figures; G.R. drafted manuscript; G.R., E.S.S., D.F.W., and V.N.S. edited and revised manuscript; G.R., E.S.S., D.F.W., and V.N.S. approved final version of manuscript.

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