Complement alternative pathway activation in the autologous phase of nephrotoxic serum nephritis

Joshua M. Thurman,1 Svetlana N. Tchepeleva,1 Mark Haas,2 Sarah Panzer,1 Susan A. Boackle,1 Magdalena J. Glogowska,1 Richard J. Quigg,3 and V. Michael Holers1

1Department of Medicine, University of Colorado School of Medicine, Aurora, Colorado; 2Department of Pathology and Laboratory Medicine, Cedars Sinai Medical Center, Los Angeles, California; and 3Department of Medicine, University of Chicago, Chicago, Illinois

Submitted 29 July 2011; accepted in final form 1 April 2012

The complement cascade is an important part of the innate immune system, but activation of this system causes tissue injury in several autoimmune and inflammatory diseases. It has been known for decades that complement is activated in the glomeruli of patients with immune complex glomerulonephritis. IgG and IgM immune complexes are typically regarded as activating the classical pathway of complement (24). Substantial complement amplification can proceed through the alternative pathway, however, even when activation is initiated by the classical or mannose-binding lectin pathway (7). Consequently, mice deficient in alternative pathway proteins are protected in several models of antibody-mediated disease, including a model of lupus-like glomerulonephritis (1, 5, 25).

Nephrotoxic serum (NTS) nephritis is a model in which rodents are passively injected with antibodies (typically generated in rabbits or sheep) to glomerular basement membrane (GBM) components. The antibodies cause acute glomerular injury, some of which is mediated by the complement system (8, 16). Acute injury after injection with NTS is sometimes referred to as the heterologous phase of injury (19). If injected mice develop antibodies against immunoglobulin from the species in which the NTS is generated, they will produce antibodies against the NTS, and heterologous immunoglobulin bound to the GBM then acts as a planted antigen. This occurs if sufficient time is allowed to elapse after injection with the NTS (referred to as the autologous phase of injury) or if the animals are presensitized to immunoglobulin from that species (accelerated NTS) (17, 19).

Because the classical pathway facilitates the clearance of injured cells and immune complexes, deficiency of early classical pathway components can actually exacerbate immune complex glomerulonephritis (4, 17). Thus, in immune complex-mediated diseases in which the alternative pathway of complement significantly contributes to tissue injury, selective inhibition of the alternative pathway may be an advantageous therapeutic strategy. Even though complement activation by NTS is initiated through the classical pathway (8), we hypothesized that the alternative pathway would be secondarily activated by NTS and that mice deficient in the alternative pathway would be resistant to injury in this model. To test this hypothesis, we induced acute heterologous NTS nephritis, accelerated NTS nephritis, and autologous NTS nephritis in mice with a targeted deletion of the gene for factor B, an essential component of the alternative pathway.

MATERIALS AND METHODS

Reagents. NTS was raised in a sheep as previously described (16), and IgG was purified from the sheep serum using protein G (Thermo-Fisher Scientific, Waltham, MA). Immunofluorescence microscopy was performed using FITC-conjugated antibodies against sheep IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), mouse C3 (MP Biomedical, Solon, OH), and mouse IgG (Jackson ImmunoResearch Laboratories). For immunofluorescence microscopy of mouse IgG1 and IgG2a, biotinylated antibodies to mouse IgG1 and IgG2a were used, and detection was accomplished with streptavidin-FITC (all from Life Technologies, Grand Island, NY). An antibody against mouse F4/80 (Life Technologies) was used to detect macrophages. Sheep IgG (Sigma Aldrich, St. Louis, MO) was used as an antigen in an ELISA to detect mouse antibodies to sheep IgG. Horseradish peroxidase-conjugated detection antibodies against mouse IgG1, IgG2a, and IgG2b (all from Invitrogen, Carlsbad, CA) were also used in the ELISA.

Mice. Factor B-deficient (fB−/−) mice were generated as previously described (13) and back-crossed seven generations on a

THE COMPLEMENT CASCADE is an important part of the innate immune system, but activation of this system causes tissue injury in several autoimmune and inflammatory diseases. It has been known for decades that complement is activated in the glomeruli of patients with immune complex glomerulonephritis. IgG and IgM immune complexes are typically regarded as activating the classical pathway of complement (24). Substantial complement amplification can proceed through the alternative pathway, however, even when activation is initiated by the classical or mannose-binding lectin pathway (7). Consequently, mice deficient in alternative pathway proteins are protected in several models of antibody-mediated disease, including a model of lupus-like glomerulonephritis (1, 5, 25).

Nephrotoxic serum (NTS) nephritis is a model in which rodents are passively injected with antibodies (typically generated in rabbits or sheep) to glomerular basement membrane (GBM) components. The antibodies cause acute glomerular injury, some of which is mediated by the complement system (8, 16). Acute injury after injection with NTS is sometimes referred to as the heterologous phase of injury (19). If injected mice develop antibodies against immunoglobulin from the species in which the NTS is generated, they will produce antibodies against the NTS, and heterologous immunoglobulin bound to the GBM then acts as a planted antigen. This occurs if sufficient time is allowed to elapse after injection with the NTS (referred to as the autologous phase of injury) or if the animals are presensitized to immunoglobulin from that species (accelerated NTS) (17, 19).

Because the classical pathway facilitates the clearance of injured cells and immune complexes, deficiency of early classical pathway components can actually exacerbate immune complex glomerulonephritis (4, 17). Thus, in immune complex-mediated diseases in which the alternative pathway of complement significantly contributes to tissue injury, selective inhibition of the alternative pathway may be an advantageous therapeutic strategy. Even though complement activation by NTS is initiated through the classical pathway (8), we hypothesized that the alternative pathway would be secondarily activated by NTS and that mice deficient in the alternative pathway would be resistant to injury in this model. To test this hypothesis, we induced acute heterologous NTS nephritis, accelerated NTS nephritis, and autologous NTS nephritis in mice with a targeted deletion of the gene for factor B, an essential component of the alternative pathway.

MATERIALS AND METHODS

Reagents. NTS was raised in a sheep as previously described (16), and IgG was purified from the sheep serum using protein G (Thermo-Fisher Scientific, Waltham, MA). Immunofluorescence microscopy was performed using FITC-conjugated antibodies against sheep IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), mouse C3 (MP Biomedical, Solon, OH), and mouse IgG (Jackson ImmunoResearch Laboratories). For immunofluorescence microscopy of mouse IgG1 and IgG2a, biotinylated antibodies to mouse IgG1 and IgG2a were used, and detection was accomplished with streptavidin-FITC (all from Life Technologies, Grand Island, NY). An antibody against mouse F4/80 (Life Technologies) was used to detect macrophages. Sheep IgG (Sigma Aldrich, St. Louis, MO) was used as an antigen in an ELISA to detect mouse antibodies to sheep IgG. Horseradish peroxidase-conjugated detection antibodies against mouse IgG1, IgG2a, and IgG2b (all from Invitrogen, Carlsbad, CA) were also used in the ELISA.

Mice. Factor B-deficient (fB−/−) mice were generated as previously described (13) and back-crossed seven generations on a
C57BL/6 background. Mice with heterozygous deficiency of the complement regulatory protein Crry were generated as previously described (26). The mice were housed and maintained in the University of Colorado Center for Laboratory Animal Care in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. All animal procedures were in adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Colorado, Denver, Animal Care and Use Committee.

**NTS nephritis models.** NTS nephritis was induced by three different methods. To induce acute, heterologous disease, we injected C57BL/6 or \( B^{-/-} \) mice via the tail vein with 0.25 or 0.375 mg of antibody purified from the serum of the immunized sheep. The mice were euthanized after 24 h, and tissues were collected. For examination of the chronic effects of NTS, mice were injected with 0.25 mg of NTS IgG and were not euthanized until 160 days after the injection. To induce accelerated autologous NTS, we immunized C57BL/6 or \( B^{-/-} \) mice with 0.5 mg of sheep IgG (Sigma Aldrich) emulsified in complete Freund’s adjuvant. After 8 days, the mice were injected with 0.25 mg of NTS IgG via the tail vein. The mice were euthanized 6 days after the injection with nephrotoxic antibody, and tissues were collected.

**Renal function.** Renal function was assessed by measurement of serum urea nitrogen (SUN) using a Beckman autoanalyzer (Fullerton, CA).

**Urinary albumin measurement.** Urine albumin was measured by an ELISA according to the manufacturer’s instructions (Bethyl Laboratories, Montgomery, TX). Urine creatinine was measured using a Beckman autoanalyzer. For normalization of urinary albumin excretion, the values are reported as micrograms of albumin per milligram of creatinine, and values <25 mg/g were considered normal (16).

**ELISA for antibodies to sheep IgG.** ELISA plates were coated overnight with 50 ng of sheep IgG at 4°C. The plates were then washed, and serum samples diluted 1:100 –1:1,600 were added to each well and incubated for 1 h at room temperature. The plates were washed again, and horseradish peroxidase-conjugated antibody diluted 1:2,000 was added to each well. After 1 h, the plates were washed, and 50 μl of 2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma Aldrich) were added to each well. The absorbance of each well at 405 nm was then measured.

**Renal histopathology and immunofluorescence microscopy.** After the kidneys were removed from the mice, sagittal sections were cut and stained with periodic acid-Schiff. To evaluate renal pathologic changes, the kidneys were examined by a renal pathologist (M.H.) in a blinded manner. For each slide, the severity of glomerulonephritis, glomerulosclerosis, crescent formation, and interstitial nephritis was graded in a semiquantitative manner (on a scale of 0–4), as described previously (2). For electron microscopy, cortical samples were fixed in 2% formaldehyde and 2.5% glutaraldehyde. The samples were processed, and images were obtained at the University of Colorado electron microscopy core.

For immunofluorescence, sagittal sections of the kidneys were snap-frozen in optimal cutting temperature compound (Sakura Finetek, Torrance, CA). Sections (4 μm) were cut with a cryostat and stored at -70°C. The slides were later fixed with acetone and stained with FITC-conjugated antibodies. For quantitative assessment of IgG and C3 immunofluorescence, high-powered images of 10–15 glomeruli for each kidney were obtained using an inverted fluorescence microscope (model T2000, Nikon). Masks were drawn around the glomeruli, and the total fluorescence was measured using SlideBook software (version 4.0, Intelligent Imaging Innovations, Denver, CO), and the results for each kidney were averaged. For assessment of macrophages, sections from four wild-type and four \( B^{-/-} \) mice were stained for F4/80 and examined. The number of positive cells per 15 high-powered fields were reported. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed using a TACS XL Blue Label Kit according to the manufacturer’s instructions (Trevigen, Gaithersburg, MD). Sections from wild-type and \( B^{-/-} \) mice were examined, and the number of positive cells in 30 glomeruli was counted in each section.

**Statistical analyses.** Statistical analyses were performed, and graphs were created using GraphPad Prism software (San Diego, CA). Comparison between two groups was performed by unpaired t-testing. \( P < 0.05 \) was considered statistically significant. Values are means ± SE.

**RESULTS**

\( B^{-/-} \) mice are not protected from acute heterologous NTS nephritis. We injected mice intravenously with NTS and harvested the kidneys after 24 h. To assess whether an intact alternative pathway is necessary for the full development of injury, we compared wild-type mice with \( B^{-/-} \) mice (13). We tested two different doses of NTS: 0.25 mg/mouse (which induces albuminuria) and 0.375 mg/mouse (which induces albuminuria and an elevation in SUN). With both of these doses, no differences in albuminuria, SUN, or histological injury were observed between the \( B^{-/-} \) mice and wild-type controls (data not shown).

It is possible that alternative pathway activation in the glomerulus does not cause significant injury in this acute model because of effective control of this complement pathway by complement regulatory proteins expressed in the glomerulus. In previous studies using the acute NTS model, it was reported that mice with deficient expression of the complement regulatory proteins factor H (15) and decay-accelerating factor (10, 20) show an increased susceptibility to injury. Homozygous deficiency of Crry is lethal in utero (26). Heterozygous mice express decreased levels of this protein in the kidney, however, and display increased susceptibility to ischemic injury of the kidney compared with wild-type controls (22). We tested whether Crry\(^{-/-}\) mice would be sensitive to NTS nephritis, but we found that the degree of albuminuria in Crry-deficient mice was not significantly different from that in wild-type controls after injection with NTS (data not shown).

\( B^{-/-} \) mice are protected from renal injury in accelerated NTS nephritis. In the accelerated model of NTS nephritis, injury is mediated, in part, by the mouse’s immune response to sheep IgG bound within the glomerulus. To test whether the alternative pathway plays a role in the accelerated model of NTS nephritis, we preimmunized \( B^{-/-} \) and wild-type mice with sheep IgG. We injected the mice with NTS 8 days after sensitization and harvested the mice 6 days after injecting them with NTS. By light microscopy, the glomeruli of some wild-type and \( B^{-/-} \) mice demonstrated focal proliferative changes, but there was no difference between the two strains (Fig. 1, A–C). Electron microscopy demonstrated similar findings in kidneys of wild-type and \( B^{-/-} \) mice, including patchy foot process effacement and small deposits (Fig. 1, D and E). Albuminuria and SUN levels were lower in \( B^{-/-} \) mice than in wild-type controls in the accelerated model, although there was also a trend toward lower SUN values in unmanipulated \( B^{-/-} \) mice (Fig. 1, F and G, Table 1). Staining for F4/80-positive cells (as a marker of macrophage infiltration) demonstrated more abundant cells in the kidneys of wild-type than \( B^{-/-} \) mice (Fig. 1H). TUNEL staining demonstrated a similar number of apoptotic cells in the glomeruli of wild-type and \( B^{-/-} \) mice (Fig. 1I).

Downloaded from http://ajprenal.physiology.org/ by 10.220.33.6 on October 21, 2017
Immunofluorescence microscopy showed granular deposition of C3 in the glomeruli of wild-type mice (Fig. 2A). Staining was seen in the \( fB^{-/-} \)/H11002/H11002 mice (Fig. 2B), but quantitative assessment of the glomerular C3 demonstrated less C3 in the glomeruli of \( fB^{-/-} \)/H11002/H11002 than wild-type mice (Fig. 2C). Deposits of mouse IgG were similar in the two strains of mice (Fig. 2, D–F).

Table 1. Albuminuria and SUN levels

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Wild-Type</th>
<th>( fB^{-/-} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Albumin/creatinine, ( \mu g/mg )</td>
<td>SUN, mg/dl</td>
</tr>
<tr>
<td>Unmanipulated</td>
<td>46.5 ± 12.0</td>
<td>19.1 ± 0.8</td>
</tr>
<tr>
<td>Accelerated NTS nephritis</td>
<td>3.460 ± 1,271</td>
<td>20.4 ± 1.5</td>
</tr>
<tr>
<td>Chronic NTS nephritis</td>
<td>161.4 ± 32.5</td>
<td>20.0 ± 2.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. SUN, serum urea nitrogen; NTS, nephrotoxic serum. *P < 0.05 vs. wild-type.

Although glomerular immune complex deposits were similar in the two strains of mice, we sought to determine whether the nature of the immune response against the sheep IgG in \( fB^{-/-} \) mice was different from that in control mice. We performed ELISAs to assess the level of mouse anti-sheep IgG in the serum of immunized mice (Fig. 3A). No significant differences in the titers of IgG1, IgG2a, and IgG2b against...
sheep IgG were detected between the two strains of mice, although there was a trend toward greater IgG1 in the ƒB/H11002/H11002 mice. Immunofluorescence microscopy for mouse IgG1 and IgG2a demonstrated linear deposition of IgG2a along the GBM of wild-type and ƒB/H11002/H11002 mice, in a pattern similar to that of the deposited sheep IgG (Fig. 3B).

ƒB/H11002/H11002 mice are protected from chronic renal injury after injection with NTS. On the basis of the relative protection in ƒB/H11002/H11002 mice observed in the accelerated NTS model, we examined whether alternative pathway deficiency would protect mice from chronic injury after injection with NTS. Mice were injected with 0.25 mg of NTS and followed for 160 days. By light microscopy, only one mouse in each group demonstrated mild focal proliferative glomerulonephritis. Glomerulosclerosis was observed in some of the wild-type and ƒB/H11002/H11002 mice, but there was no significant difference between the two strains (Fig. 4, A–C). The kidneys of wild-type mice demonstrated granular C3 deposits in the glomeruli (Fig. 4D). Some glomerular C3 was observed in ƒB/H11002/H11002 mice, although it was less than in the wild-type mice (Fig. 4E). Deposits of mouse IgG were seen in both strains of mice (Fig. 4, F and G). The degree of albuminuria was significantly less than in wild-type mice at this time point (Fig. 4H). TUNEL staining demonstrated a similar number of apoptotic cells in the glomeruli of wild-type and ƒB/H11002/H11002 mice (Fig. 4I).

ELISAs were performed to assess the level of mouse anti-sheep IgG in the serum of injected mice (Fig. 5A). No significant differences in the titers of IgG1, IgG2a, and IgG2b against sheep IgG were detected between the two strains of mice. Immunofluorescence microscopy for mouse IgG1 and IgG2a demonstrated granular deposits of both isotypes of antibody in the glomeruli of wild-type and ƒB/H11002/H11002 mice (Fig. 5B), and no differences in the pattern of immunoglobulin deposition were noted. The magnitude of the albuminuria and the abundance of IgG1 and IgG2a were relatively mild in this model, raising the possibility that the protection in ƒB/H11002/H11002 mice occurred at an earlier stage of the model and that the reduced albuminuria in ƒB/H11002/H11002 mice at day 160 was the residual effect of earlier protection.

DISCUSSION

Uncontrolled activation of the alternative pathway of complement contributes to glomerular injury in several diseases, including a model of lupus nephritis (5, 25). We found that mice that lack the alternative pathway protein factor B are not protected from heterologous injury after injection with sheep NTS, a complement-dependent model of glomerular injury. Using a model of accelerated NTS nephritis or of chronic (heterologous) NTS nephritis, however, we did see a significant difference in the degree of albuminuria between ƒB/H11002/H11002 and wild-type mice. This suggests that control of the alternative pathway within the glomerulus is subverted or overwhelmed in these models. In the accelerated NTS model, the SUN was lower in ƒB/H11002/H11002 mice than in wild-type controls, and fewer macrophages were detected within the kidneys. There was no difference in the degree of histological injury that developed...
between the strains, although the overall degree of histological injury was fairly mild.

The complement system can enhance the adaptive immune response (3) and performs an important effector function in antibody-mediated immunity. Although less glomerular C3 was seen after induction of accelerated NTS in \( fB^{-/-} \) mice than wild-type controls, glomerular IgG deposition was similar between the two strains. Furthermore, the humoral immune response of the \( fB^{-/-} \) mice against sheep IgG in the accelerated and chronic models was comparable to that of wild-type mice. Thus the protection that \( fB^{-/-} \) mice demonstrated in these models was likely due to reduced glomerular complement activation downstream of the immune complex formation and is less likely due to an altered immune response against the sheep IgG. In models of diseases such as lupus nephritis, on the other hand, the complement system is involved in the development of autoimmunity, so it can be difficult to isolate the effector functions of the complement system experimentally.

Antibodies are traditionally regarded as activating the classical pathway of complement (24). The alternative pathway is secondarily activated by the classical and mannose-binding lectin pathways and forms an amplification loop. Thus, even when complement is activated through one of the other pathways, amplification through the alternative pathway contributes to the generation of proinflammatory fragments. Studies have demonstrated that the contribution of the amplification loop in immune complex-mediated complement activation is greater than was originally thought (7). This may explain why the alternative pathway plays such an important role in some immune complex-mediated diseases. On the other hand, complement regulatory proteins can limit amplification through the alternative pathway on some surfaces upon which the classical pathway is activated (6). Therefore, immune complexes may engage the alternative pathway if local regulation of this pathway is lost.

Our study provides another example in which antibody-induced glomerular injury is mediated, or at least exacerbated, by the alternative pathway of complement. This suggests that targeting the alternative pathway may be a promising therapeutic strategy for autoimmune diseases involving the complement system.
by engagement of the alternative pathway. Our findings also suggest that the glomerular environment is capable of suppressing alternative pathway activation in the acute heterologous model. The relatively unimportant role of the alternative pathway in this acute model may be due to effective local control of the pathway by alternative pathway regulatory proteins expressed within the glomerulus. We did not find that mice that underexpress Crry develop more severe injury, but

Fig. 4. $fB^{-/-}$ mice are protected from development of chronic, autologous NTS nephritis. Wild-type and $fB^{-/-}$ mice were injected with NTS. Tissues and urine were collected 160 days after injection. A–C: light microscopy shows glomerulosclerosis in some of the wild-type (A) and $fB^{-/-}$ (B) mice, and overall degree of histological injury was similar in the two strains of mice (C). D and E: immunofluorescence microscopy demonstrates C3 deposition in glomeruli of wild-type (D) and $fB^{-/-}$ (E) mice. F and G: mouse IgG deposits are also detected in glomeruli of wild-type (F) and $fB^{-/-}$ (G) mice. Glomeruli are indicated with arrowheads. H: urine albumin/creatinine levels were lower in $fB^{-/-}$ mice than wild-type controls. I: TUNEL staining demonstrates no significant difference in number of apoptotic cells between the two strains ($n = 4$ for each group). Original magnification $\times200$ (A, B, and D–G).
other groups reported that mice lacking factor H or decay-accelerating factor develop more severe injury after injection with NTS (10, 15). It is possible, therefore, that these other regulatory proteins are more important for controlling complement activation in the glomerular capillary wall. The susceptibility of mice to alternative pathway-mediated injury in the accelerated and chronic models may also indicate that the mechanisms by which the alternative pathway is controlled can be bypassed over time or may be impaired by glomerular injury. We also previously showed that the alternative pathway contributes to glomerular injury in adriamycin-induced injury (a model of toxin-induced glomerular damage) (9). Together, these previous studies and the current work indicate that complement regulatory proteins effectively inhibit alternative pathway amplification of acute antibody deposition within the glomerulus but that various insults or chronic persistence of the antibodies may impair local complement regulation, permitting alternative pathway-mediated amplification of injury.

A therapeutic complement inhibitor, eculizumab, has been used in a number of patients with renal disease (11, 12, 14, 27). Agents that selectively block the alternative pathway of complement have also been developed (21, 23). Such agents could potentially block alternative pathway-mediated tissue injury without impairing some potentially beneficial effects of the classical pathway or impeding the adaptive immune response. Indeed, mice deficient in the early classical pathway component C1q develop more severe injury than wild-type controls in the accelerated NTS model (17), and deficiency of factor B appears to be more protective than deficiency of C3 in a mouse model of lupus nephritis (18, 25). Deficiency of factor B did not fully protect the mice in any of the protocols tested, indicating that other mechanisms of injury were engaged in all these settings. Our findings do, however, provide additional evidence that the alternative pathway is an important mediator of immune complex-mediated injury. Future studies will advance our understanding of how the glomerulus loses the

Fig. 5. \( \beta B^{-/-} \) and wild-type mice with chronic, autologous NTS nephritis generate similar levels of IgG1, IgG2a, and IgG2b antibodies against sheep IgG. A: serum levels of IgG1, IgG2a, and IgG2b antibodies against sheep IgG were similar in the two strains of mice. B: immunofluorescence microscopy of kidneys for IgG1 and IgG2a demonstrates granular deposits of IgG1 and IgG2a in glomeruli of wild-type and \( \beta B^{-/-} \) mice. Glomeruli are indicated with arrowheads. At least 20 glomeruli were examined in 4 kidneys of each genotype. Representative images are shown. Original magnification ×400.
ability to control this pathway and will delineate the role of alternative pathway inhibitors in the treatment of glomerular disease.

GRANTS
This work was supported in part by National Institutes of Health Grants DK-076690 (J. M. Thurman), AI-052441 (S. A. Boackle), DK-048173 (R. J. Quigg), T32 AR-07534 (V. M. Holers), and AI-31105 (V. M. Holers).

DISCLOSURES
J. M. Thurman and V. M. Holers are consultants for Alexion Pharmaceuticals, Inc.

AUTHOR CONTRIBUTIONS
J. M.T., S. N. T., S. A. B., R. J. Q., and V. M. H. are responsible for conception and design of the research; J. M. T., S. N. T., M. H., S. P., and M. J. G. performed the experiments; J. M. T., S. N. T., M. H., S. P., S. A. B., and V. M. H. analyzed the data; J. M. T., M. H., S. P., S. A. B., and V. M. H. interpreted the results of the experiments; J. M. T. prepared the figures; J. M. T. drafted the manuscript; J. M. T., S. A. B., R. J. Q., and V. M. H. edited and revised the manuscript; J. M. T. and S. P. approved the final version of the manuscript.

REFERENCES