Ischemic Preconditioning in the Liver Is Independent of Regulatory T Cell Activity

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Abstract

Ischemic preconditioning (IPC) protects organs from ischemia reperfusion injury (IRI) through unknown mechanisms. Effector T cell populations have been implicated in the pathogenesis of IRI, and T regulatory cells (Treg) have become a putative therapeutic target, with suggested involvement in IPC. We explored the role of Treg in hepatic IRI and IPC in detail. IPC significantly reduced injury following ischemia reperfusion insults. Treg were mobilized rapidly to the circulation and liver after IRI, but IPC did not further increase Treg numbers, nor was it associated with modulation of circulating pro-inflammatory chemokine or cytokine profiles. We used two techniques to deplete Treg from mice prior to IRI. Neither Treg depleted FoxP3.LuciDTR mice, nor wildtype mice depleted of Tregs with PC61, were more susceptible to IRI compared with controls. Despite successful enrichment of Treg in the liver, by adoptive transfer of both iTreg and nTreg or by in vivo expansion of Treg with IL-2/anti-IL-2 complexes, no protection against IRI was observed. We have explored the role of Treg in IPC and IRI using a variety of techniques to deplete and enrich them within both the liver and systemically. This work represents an important negative finding that Treg are not implicated in IPC and are unlikely to have translational potential in hepatic IRI.

Introduction

Ischemia reperfusion injury (IRI) is the cause of considerable morbidity and mortality across a wide range of common diseases. In liver resection surgery, ischemic insults cause post-operative transaminasemia and failure of synthetic function. In liver transplantation, IRI causes a spectrum of graft dysfunction, ranging from biochemical abnormalities through to primary non-function of the transplanted organ [1].

Ischemic preconditioning (IPC) is a manoeuvre in which brief interruption, then reinstatement of an organ’s blood supply protects that organ from subsequent injury. IPC has been shown to protect the liver from subsequent IRI in animals and humans [2,3]; its mechanism of action has yet to be fully elucidated.

In recent years, IRI has been thought of as an example of sterile inflammation, and the contribution of lymphocytes has been examined extensively. A transient T cell influx has been described, occurring during the first few hours of reperfusion, and resolving by 24 hours [4,5]. Nude [6,7], RAG1 knockout [8], SCID [9], TCRβ−/− [10], and CD4−/− animals [6] have all been shown to be protected from IRI. Experiments in which the protected phenotype of nude mice has been reversed by adoptive transfer of CD4+, but not CD8+ lymphocytes, have been published in both renal [6] and liver ischemia [11] models. Inhibition of lymphocyte chemotaxis through manipulation of the sphingosine-1-phosphate (S1P) pathway has also been used to ameliorate IRI [5,12,13].

Since this data suggests that effector T cells contribute to IRI, CD4+CD25+FoxP3+T regulatory cells (Treg) have become a putative therapeutic target. Treg depletion/reconstitution experiments in renal ischemia [8,14], and pharmacotherapies designed to enhance Treg recruitment [15] have suggested a protective role for Treg, while adoptive transfer of induced Treg showed protective potential in models of renal [14] and hepatic ischemia [16,17]. In addition to experiments, which have directly examined the potential of Treg as a cellular therapy, several molecules and pathways linked with Treg activity have also been associated with IRI: IL-10 was shown to be over-expressed in renal [14] and hepatic IRI, which conversely was abolished by IL-10 neutralizing antibodies [18]. Receptor mediated purinergic signalling with adenosine has long been implicated in protection induced by IPC [19]. Degradation of interstitial ATP to adenosine is catalysed by the ectonucleotidase CD39, which is expressed by Treg and implicated in their suppressive function [20,21]. CD39−/− animals are susceptible to hepatic ischemic injury, whereas administration of soluble CD39 attenuated hepatic injury: furthermore, CD39 was shown to be induced by IPC [22].

The potential of immunomodulation as a therapeutic strategy for hepatic IRI was supported by our previous work, in which we showed that hemeoxygenase-1 (HO-1) protected the liver from

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ischemic injury by modulation of antigen presenting cell (APC) differentiation to an anti-inflammatory phenotype [23]. Subsequent work by others showed that FoxP3+Treg mediated immune suppression to requires APC HO-1 [24].

Here we have used a range of approaches to study the contribution of Treg to IPC in vivo, and to establish whether this cell type is likely to have translational potential in the context of hepatic ischemia. We demonstrate that in our model, Treg do not modulate ischemic injury, and are unlikely to contribute to IPC.

Materials and Methods

Ethics Statement

All procedures and animal care were approved by the University of Edinburgh Ethical Review Committee and performed in accordance with UK Home Office licensing regulations (Animal Scientific Procedures Act 1986).

Animals, Surgical Models, and Injury Assessment

C57 BL/6j animals with insertion of a BAC containing a cassette encoding enhanced GFP (eGFP), the human DTR, and CBGr99 luciferase within exon 3 of the FoxP3 gene (FoxP3.LuciDTR) mice were made available by G. Hammerling, Heidelberg [25]. Foxp3 GFP reporter mice on the C57BL/6j background were provided by Dr. A. Rudensky, Seattle [26].

IRI animals underwent surgical occlusion of the portal pedicle (hepatic artery and portal vein) supplying the left hepatic lobe for 50 minutes under isoflurane anaesthesia as described by previously [23,27,28]. IPC mice, underwent portal pedicle occlusion for 15 minutes followed by 15 minutes reperfusion prior to the 50 minute ischemic insult. Core temperature was maintained at 36°C throughout. Animals were culled at various times post-operatively. All experiments were internally controlled.

Severity of ischemic injury was assessed by measurement of serum ALT utilising a commercial kit (Alpha Laboratories Ltd., Eastleigh, UK) adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd, Welwyn Garden City, UK). Histological injury was evaluated on haematoxylin and eosin stained sections. Percentage area necrosis was estimated on representative fields selected at random by isotropic uniform random sampling (Image pro-plus software, Media Cybernetics Inc, CA, USA) according to the manufacturer’s instructions. Antibodies were incubated at 1:100 dilution for 30 minutes.

Flow cytometry was performed using a LSR Fortessa cytometer (Becton Dickinson, UK) and 5 ug anti-IL-2 Ab (clone JES6/A12, eBioscience) according to the manufacturer’s instructions. Data was collected using an LSR Fortessa cytometer (Becton Dickinson, UK) and analysed with Flowcytomix Pro v2.4 software (eBioscience).

Immunohistochemistry and Cell Counting

Tissue samples were formalin fixed prior to paraffin embedding. Sections were stained with rabbit anti mouse CD3 (Sigma-Aldrich, Poole, Dorset, UK) at 1:1000 dilution. Staining was detected with diaminobenzidine with haematoxylin counterstaining. Numbers of stained cells were counted on 400x fields selected at random by isotropic uniform random sampling (Image pro-plus software, Media Cybernetics Inc, CA, USA) using a Zeiss Axios A1 (Carl Zeiss Ltd, Cambridge, UK) stereology microscope.

Measurement of Circulating Cytokines

Serum samples were analysed for IP-10, GM-CSF, IL-1β, IL-2, IL-6, IL-10, IL-17, CXCL-1/KC, MIP-1α, MIP-1β and IFNγ using a multiplex FlowCytomix kit (eBioscience) according to the manufacturer’s instructions. Data was collected using an LSR Fortessa cytometer (Becton Dickinson, UK) and analysed with Flowcytomix Pro v2.4 software (eBioscience).

Preparation of Cells for Adoptive Transfer

iTreg were generated by Miltenyi Macs negative selection of CD4+ cells from FoxP3 GFP mouse spleens (Miltenyi Biotec, UK). CD4+CD62L high GFP- naive T cells were subsequently purified by FACS sorting. These were plated into anti-CD3/anti-CD28 coated plates in the presence of 5 ng/ml TGFβ and 100 U/ml IL-2 and cultured for 5 days [30]. iTreg were purified using FACS sorting for CD4+GFP+ prior to adoptive transfer of 1–2 million cells 24 hours prior to ischemic insult. Successful engraftment of donor cells was confirmed by flow cytometry of recipient blood, liver and spleen.

nTreg were obtained by Miltenyi Macs negative selection of CD4+ cells from FoxP3 GFP mouse spleens, followed by FACS sorting. FoxP3 expression was previously shown to be more stable in CD4+CD62L high FoxP3+ cells [31], therefore this subgroup was selected for ex-vivo expansion. Around 2–3×10⁶ nTreg were recovered from each donor mouse, which underwent ex vivo expansion in the presence of Dynal CD3/CD28 activation beads (Life Technologies, Paisley, UK) and high dose (2000U/ml) IL-2 in order to produce around 10–20 fold expansion at 21 days.
Figure 1. Treg mobilization during reperfusion is not enhanced by IPC. Mice (n = 5–8 per group) were subjected to ischemia reperfusion injury with or without ischemic preconditioning and killed at 3 or 24 hours. Ischemic preconditioning protected the liver from injury both in terms of ALT release [a] and histological injury score [b] measured at 24 hours. CD4+FoxP3+ cells were mobilized into the circulation during reperfusion [c]. Hepatic CD4+FoxP3+ cells increased over the same time period [d]. Total CD3+ lymphocytes were stable throughout reperfusion [e]. FlowCytomix was used to profile circulating chemo/cytokines. Rises were detected in CXCL-10/IP-10 [f], CXCL-1/KC [g], IL-6 [h] and GM-CSF [i]. Other analytes (IL-1α, IL-1β, IL-2, IFNγ, IL-17 and IL-17F, and the Treg cytokine IL-10) were not detected.

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Statistical Analysis
Data were collected in Microsoft Excel for Mac and analysed using Prism version 5 (Graphpad, San Diego, USA). Data are presented as mean±/− standard error. Comparison of means was with 2 tailed t test or ANOVA as appropriate.

Results

Treg Mobilization during Reperfusion
IPC protected the liver from injury both in terms of ALT release and histological injury score measured at 24 hours [Figure 1a,b]. Early reperfusion was accompanied by a significant mobilization of FoxP3+ Tregs into the circulation, rising to between 10 and 15% of circulating CD4+ cells at 3 hours, and remaining elevated at later time points [Figure 1c]. In turn, the liver accumulated Tregs, rising to 5–10% of hepatic CD4+ cells at 24 hours [Figure 1d]. Total numbers of CD3+ lymphocytes detected on immunohistochemistry was stable throughout reperfusion [Figure 1e].

Importantly, despite the significant protection from injury offered by IPC, numbers of Treg in the circulation and enrichment within the liver was similar between IRI and IPC mice [Figure 1c, d]. Thus enrichment with Treg was not associated with a protected phenotype.

To further evaluate whether IPC might be associated with Treg activity, serum chemokine and cytokine profiles were compared between animals subject to IRI and IPC. Cytokines IL-1α, IL-1β, IL-2, IFNγ, IL-17 and IL-17F, and IL-10 were not detected in the serum of either IRI or IPC animals. There were rises in CXCL10/IP-10 [Figure 1f], KC/CXCL-1 [Figure 1g], IL-6 [Figure 1h] and GM-CSF [Figure 1i].

Apart from a small difference in levels of serum CXCL-10/IP-10, and a trend towards lower KC/CXCL-1 in IPC mice (p = 0.06), chemokine and cytokine profiles did not significantly differ between IRI and IPC groups [Figure 1f, g, h, i]. Specifically, IPC was not associated with significant reductions in pro-inflammatory cytokines or an excess of IL-10, which may have implicated a role for Treg.

Effect of T Regulatory Cell Depletion
If Treg were responsible for modulating ischemic injury, then Treg deficient animals would be more susceptible to injury. To test this hypothesis, we depleted animals of Treg using two complementary approaches.

Foxp3.LuciDTR mice or wildtype littermates received diphtheria toxin 25 ng/g 24 hours prior to IRI. Loss of CD4+FoxP3+ cells, from blood [Figure 2c,d], spleen [Figure 2e],

Figure 2. Treg depletion in the FoxP3.LuciDTR mouse does not increase susceptibility to IRI. FoxP3.LuciDTR mice or wildtype littermates received diphtheria toxin 25 ng/g 24 hours prior to ischemic insult. ALT release [a] and histological injury score [b] did not differ between groups. DT treatment of DTR animals effected almost total depletion of Treg from the circulation [c,d], spleen [e] and liver [f].

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and liver [Figure 2f] was profound. Despite this, injury severity was equal between Treg depleted Foxp3.LuciDTR and DT treated Treg intact wildtype littermate control animals at 24 hours of reperfusion [Figure 2a, b].

Since DT treatment is toxic, and exacerbated IRI in these experiments, we verified the absence of Treg mediated protection using antibody depletion. Mice received two doses of the monoclonal anti CD25 antibody PC61 or PBS control 7 days and 2 days prior to surgery, as described previously by our laboratory [32]. During protocol optimization, this regimen reduced numbers of CD25+ cells to around one third of their original number, and approximately halved the proportion of splenic FoxP3+CD4+ cells [Figure 3c, d]. PC61 Treg depleted mice were no more susceptible to IRI in terms of ALT or histological injury score than Treg intact animals [Figure 3a, b].

FlowCytomix was used to profile circulating chemo/cytokines. No differences were detected in post-operative rises in CXCL-10/IP-10 [e], KC/CXCL-1 [f], IL-6 [g] and GM-CSF [h] between Treg intact and depleted animals. Other analytes (IL-1α, IL-1β, IL-2, IFNγ, IL-17 and IL-17F, and IL-10) were not detected.

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Figure 3. Treg antibody depletion does not increase susceptibility to IRI. Wildtype mice received the CD25 depleting antibody pc61 7 and 2 days or PBS (n = 5–8 per group) prior to ischemia reperfusion injury. Injury severity was no different between Treg intact and Treg depleted animals in terms of ALT [a] or histological injury score [b]. PC61 treatment resulted in significant depletion of CD4+CD25+[c] and CD4+FoxP3+[d] cells. FlowCytomix was used to profile circulating chemo/cytokines. No differences were detected in post-operative rises in CXCL-10/IP-10 [e], KC/CXCL-1 [f], IL-6 [g] and GM-CSF [h] between Treg intact and depleted animals. Other analytes (IL-1α, IL-1β, IL-2, IFNγ, IL-17 and IL-17F, and IL-10) were not detected.

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FlowCytomix was used to measure cytokine and chemokine levels in serum from animals undergoing antibody mediated Treg depletion prior to ischemic insult. No differences were detected between Treg depleted and vehicle treated animals [Figure 3e, f, g, h], specifically, the relative absence of Treg did not result in
elevated levels of circulating pro-inflammatory cytokines at 24 hours of reperfusion. Cytokines IL-1α, IL-1β, IL-2, IFNγ, IL-17 and IL-17F, and IL-10 were not detected in the serum of Treg intact or Treg depleted animals at all [data not shown]. Post-operative rises in CXCL-10/IP-10 [Figure 3e], KC/CXCL-1 [Figure 3f], IL-6 [Figure 3g] and GM-CSF [Figure 3h] did not differ between groups. Of note, secretion of pro-inflammatory chemokines and cytokines was not exacerbated in Treg depleted animals.

The lack of increased susceptibility of Treg depleted mice to ischemic injury suggests that Treg do not have a physiological role in modulating injury, and are unlikely to be responsible for the protected phenotype observed in IPC.

Testing the Therapeutic Potential of Transferred or Expanded Pre-activated Treg

If Treg were capable of ameliorating ischemic injury, expansion of Treg numbers might be protective. To test this hypothesis we supplemented animals with additional activated Treg using techniques shown to protect animals from other inflammatory pathologies within our laboratory.

Activated FoxP3 GFP iTreg were generated in vitro using techniques previously published by our laboratory [30], and purified to >99% by FACS sorting, before adoptive transfer of 1–2 million cells [Figure 4a, b, c, d]. 24 hours later, animals were subjected to hepatic ischemic insult. Successful engraftment of donor cells was confirmed by flow cytometry of recipient spleen [Figure 4e] and liver [Figure 4f]. Cells were prepared and transferred using procedures and reagents shown to confer protection in other models in reports from our laboratory [30]. No difference was detected in injury severity between iTreg supplemented and PBS treated control animals at 3 or 24 hours of reperfusion (n = 4–5 per group) [g].

Since previous reports have shown iTreg to produce IFNγ when stimulated [30], and FoxP3 GFP reporter iTreg have been shown to exhibit reduced stability of FoxP3 expression and reduced suppressive ability [33], experiments were repeated using adoptively transferred nTreg. 1–2 million cells were transferred into recipient mice prior to ischemic injury [Figure 5a, b, c]. Despite engraftment of nTreg [Figure 5d,e], mice were not protected from ischemic injury in terms of ALT at 3 hours of reperfusion [Figure 5f].

A possible explanation of the failure of adoptive transfer experiments to protect animals from ischemic injury was that insufficient Treg had been transferred to ameliorate injury. IL-2/anti-IL-2 complexes have been shown to induce Treg expansion, protecting experimental models from airway inflammation [34], experimental autoimmune encephalomyelitis (EAE) [29], and conferring long term tolerance to pancreatic allografts [29]. Prior to ischemia reperfusion insults, animals received three consecutive daily injections of IL-2/anti-IL-2 complexes resulting in substantial Treg expansion in spleen [Figure 6a], blood [Figure 6b] and liver [Figure 6c].

Despite a two to threefold expansion in Treg, including in the liver, animals were not protected from injury in terms of ALT release or histological injury score at 24 hours of reperfusion [Figure 6d,e].

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Figure 4. Adoptive transfer of pre-activated iTreg does not protect animals from IRI. iTreg were generated in vitro from sorted CD62L high, FoxP3- naive T cells cultured in the presence of TGFβ and IL-2 for 5 days before flow sorting to maximize purity [a-d]. Successful transfer was confirmed by detection of FoxP3 GFP+cells in spleen [e] and liver [f]. No difference was detected in injury severity between iTreg supplemented and PBS treated control animals at 3 or 24 hours of reperfusion (n = 4–5 per group) [g].

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Serum cytokine/chemokine profiles showed increased levels of IL-2 in keeping with exogenous administration [Figure 6f]. Interestingly, CXCL-10/IP-10 was elevated in complex treated animals compared with PBS treated controls [Figure 6g], as was IL-6 [Figure 6h]. KC/CXCL-1 [Figure 6i], and GM-CSF [Figure 6j] did not differ between groups. Other analytes (IL-1α, IL-1β, IL-10, IL-17, IL-17F, IFNγ, MIP-1α) were not detected in either group.

Discussion

These experiments, which employed a range of strategies of Treg supplementation/expansion previously shown to ameliorate other inflammatory disorders, demonstrate that Treg are unlikely to represent a useful therapeutic target in amelioration of hepatic IRI.

We have shown a rapid mobilization of Treg into the circulation and accumulation in the liver during the first 24 hours of reperfusion. This mobilization and accumulation was not exaggerated by IPC, and did not inversely correlate with severity of ischemic injury, as would have been predicted had they had a significant role in injury modulation.

Profound depletion of Treg in the FoxP3 LuciDTR mouse did not affect injury compared with appropriate DT treated control animals. Antibody depletion of CD25+ cells (which led to partial loss of FoxP3+ Treg) also did not adversely affect outcome of ischemia reperfusion insults. If Treg were protective, injury would have been more severe in Treg deficient animals than Treg intact controls.

Conversely, if Treg were implicated in IPC, supplementation or expansion of these cells would be expected to recapitulate the protected preconditioned phenotype. We employed three putative therapeutic strategies of either Treg transfer or expansion, in attempts to ameliorate injury. Pre-activated iTreg have previously been used in our laboratory to ameliorate experimental autoimmune encephalomyelitis (EAE) [30]. In our ischemia model, we were unable to demonstrate protection. We hypothesized that a protective effect might be undermined by potentially pathological IFNγ production by iTreg [30]. We also noted recent data demonstrating impaired suppressive capability and FoxP3 stability in FoxP3 GFP iTreg [33]. Thus, we undertook further experiments using transferred ex vivo expanded nTreg [31] which again did not protect the liver from ischemic injury.

A limitation of adoptive transfer experiments is their inability to significantly expand circulating or tissue Treg numbers. We transferred similar numbers of iTreg and nTreg to those shown to protect animals from other inflammatory disorders, and transferred cells which were pre-activated, increasing their likely suppressive potency. Nonetheless, 1–2 million Treg remains a small proportion of the total number within the host mouse, and donor cells were detected in the liver and spleen at around 0.5% CD4+ cells.

Figure 5. Adoptive transfer of pre-activated nTreg does not protect animals from IRI. nTreg were obtained from FoxP3 GFP mouse spleens by flow sorting followed by expansion in vitro for 21 days and further flow-sorting for purity [a–c]. Flow cytometry of spleen and liver confirmed successful transfer [d,e]. There was no difference in injury severity between nTreg supplemented and control animals at 3 hours of reperfusion (n=4 per group) [f].

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To address this issue, we utilised IL-2/anti IL-2 complexes, which have been shown to ameliorate airway inflammation [34], and EAE, as well as inducing long term tolerance to transplanted pancreatic islets [29]. In common with previously published reports, this technique produced major Treg expansion in the spleen, circulation and liver. Despite 25% of hepatic CD4+ cells being FoxP3+, the ischemic preconditioned phenotype was not recapitulated by in vivo Treg expansion.

It would be possible to ascribe a lack of protective effect in Treg transferred or expanded animals to those cells’ polyclonal TCR repertoire. Previous work using transgenic TCR restricted models highlighted that Treg recognising a disease-relevant autoantigen are more effective than polyclonal Treg in prevention of EAE [31]. In contrast, ischemic injury is likely to result in unveiling of a wide range of antigens as the contents of lysing cells are ‘spilt’ into the interstitium. As such, TCR restricted models are not only unavailable, but they are also unlikely to be therapeutically relevant in the context of IRI.

In these experiments, we have used a number of complementary approaches to establish whether Treg have a role in modulating hepatic IRI, and have found no data to support this hypothesis. Other published Treg depletion experiments have had mixed results using the PC61 anti-CD25 antibody, with some showing increased susceptibility [8,14,35], and others unable to demonstrate an effect [15,36] in IRI models. Results of Treg supplementation experiments from other laboratories contrast with our own data. Transfer of iTreg cells into mice [16] and rats [17] have been shown by others to be protective against hepatic IRI. RAG1 knockout mice, which underwent reconstitution with FoxP3−/− lymphocytes were highly vulnerable to ischemic injury compared with recipients of wildtype lymphocytes [8]. In a study of late phase preconditioning (in which the preconditioning insult occurred 7 days prior to index ischemia), the same group showed that Treg accumulated during resolution of injury, and that these partially reduced the severity of subsequent ischemic insults [14].

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**Figure 6.** *In vivo* expansion of Treg does not protect animals from IRI. Mice received IL-2/JES6-A12 complexes or PBS for three consecutive days prior to hepatic ischemia reperfusion insults (n = 8 per group). Animals were culled at 24 hours of reperfusion, and tissues analysed for Treg frequency. There was significant expansion of splenic [a], circulating [b] and hepatic [c] Treg. Injury severity was similar between Treg expanded and control animals, in terms of ALT and histological injury [d,e]. FlowCytomix was used to profile circulating chemokines. Levels of IL-2 [f], CXCL-10/IP-10 [g], and IL-6 [h] were elevated. Rises in KC/CXCL-1 [i], and GM-CSF [j] did not differ between groups. Other analytes (IL-1α, IL-1β, IL-10, IL-17, IL-17F, IFNγ, MIP-1α) were not detected.

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The work presented here demonstrates an important negative finding, contrary to our original hypothesis, that Treg are unlikely to be responsible for IPC. Clearly this cell type represents an important therapeutic target for the treatment of a wide range of immune mediated disorders, but it is unlikely to be of clinical value in the treatment of ischemia reperfusion injury.

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Author Contributions

Conceived and designed the experiments: LRD JAR RO'C SMA SEMH. Performed the experiments: LRD JAR GB SC AFH. Analyzed the data: LRD. Contributed reagents/materials/analysis tools: R O'C SMA. Wrote the paper: LRD JAR RO'C AFH SMA SEMH.

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