EXTENDED REPORT

B cell OX40L supports T follicular helper cell development and contributes to SLE pathogenesis

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ABSTRACT

Objectives TNFSF4 (encodes OX40L) is a susceptibility locus for systemic lupus erythematosus (SLE). Risk alleles increase TNFSF4 expression in cell lines, but the mechanism linking this effect to disease is unclear, and the OX40L-expressing cell types mediating the risk are not clearly established. Blockade of OX40L has been demonstrated to reduce disease severity in several models of autoimmunity, but not in SLE. We sought to investigate its potential therapeutic role in lupus.

Methods We used a conditional knockout mouse system to investigate the function of OX40L on B and T lymphocytes in systemic autoimmunity.

Results Physiologically, OX40L on both B and T cells contributed to the humoral immune response, but B cell OX40L supported the secondary humoral response and antibody affinity maturation. Our data also indicated that loss of B cell OX40L impeded the generation of splenic T follicular helper cells. We further show that in two models of SLE—a spontaneous congenic model and the graft-versus-host-induced model—loss of B cell OX40L ameliorates the autoimmune phenotype. This improvement was, in each case, accompanied by a decline in T follicular helper cell numbers. Importantly, the germline knockout did not exhibit a markedly different phenotype from the B cell knockout in these models.

Conclusions These findings contribute to a model in which genetically determined increased OX40L expression promotes human SLE by several mechanisms, contingent on its cellular expression. The improvement in pathology in two models of systemic autoimmunity indicates that OX40L is an excellent therapeutic target in SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterised by autoantibodies against nuclear antigens along with the deposition of immune complexes. As with most other autoimmune diseases, environmental and genetic factors contribute to the risk of developing SLE. Genome-wide association studies have revealed over 50 susceptibility loci. TNFSF4 (tumour necrosis factor ligand family, member 4, CD252) is an established susceptibility gene for SLE and for several other autoimmune diseases. Fine-mapping of this locus in SLE identified two independent association signals upstream of TNFSF4 in multiple ancestries. These two signals align with separate expression quantitative trait loci, each one associated with elevated expression of TNFSF4 in Epstein Barr virus (EBV) lymphoblastoid cell lines, suggesting that TNFSF4 transcription is upregulated in individuals harbouring risk alleles.

TNFSF4 encodes the costimulatory molecule, OX40L, a type II transmembrane protein expressed on several immune cell types on activation, including antigen presenting cells (APCs), such as dendritic cells (DCs), B cells and macrophages, activated T cells, and mast cells and vascular endothelial cells. In contrast, its only known receptor, OX40, is expressed mainly on activated CD4+ T cells. Nevertheless, how these various functions relate to the cell types expressing OX40L is still unclear. Constitutive expression of OX40L on T cells has been shown to induce spontaneous autoimmunity in C57BL/6 mice. A recent study showed that OX40L expression on a subset of myeloid DCs is implicated in the pathogenesis of SLE. The beneficial effect of blocking the OX40L–OX40 signalling pathway has been shown in several different mouse models of autoimmune diseases, but experimental evidence of its efficacy in SLE is unknown.

We sought to understand the function of OX40L using CD4+ T cell and B cell conditional knockout mice. We investigated the role of OX40L using immunisation and we went on to determine how the loss of OX40L affected the pathology in two different SLE mouse models.

MATERIALS AND METHODS

Mice

A bacterial artificial chromosome (BAC) clone encoding the extracellular domain and 3′-untranslated region of Tnfsf4 was obtained from a C57BL/6-derived genomic library. The Tnfsf4 conditional targeting vector was constructed using recombinering, as described in online supplementary figure S1A. The mice (Tnfsf4Δ/Δ) were made according to a standard gene targeting approach in A9 embryonic stem cells (ES). We used (129xC57BL/6)F1 ES; therefore, microsatellite analyses were undertaken to confirm that the targeting vector had recombined on the C57BL/6 chromosome. The mice were backcrossed for eight generations on the C57BL/6 background. Tnfsf4Δ/Δ mice were crossed with β-actin-cre, CD4-cre and CD19-cre (Jackson Laboratories) to...
obtain Tnfsf4<sup>−/−</sup>, Tnfsf4<sup>fl/fl</sup>/CD4-cre and Tnfsf4<sup>fl/fl</sup>/CD19-cre, respectively. Before each experiment mice were genotyped by PCR. The primers and expected PCR product size are listed in supplementary table S1. B6.Sle16 mice were bred-in-house and B6.Sle16.Tnfsf4<sup>−/−</sup> were generated by crossing them with Tnfsf4<sup>−/−</sup> mice. B6-H2<sup>bom12</sup> mice were purchased from the Jackson Laboratories (B6.C.H2-Ab1<sup>bom12</sup>/KhEgJ; strain no. 001162, https://www.jax.org/strain/001162). The mice used were female, 8–12 weeks old and housed in specific pathogen-free conditions. All animal procedures were performed in accordance with institutional guidelines and approved by the UK Home Office.

In vitro analysis of OX40L expression
To assess OX40L expression in vitro, different cell subsets were purified from mouse spleen using LS Columns and MACS Technology (Miltenyi Biotec) and stimulated as described before. Briefly, single cell suspensions were obtained from collagenase-treated spleens, and B cells, DCs and T cells were then purified incubating the splenic cell suspension with anti-CD43 (Ly-48) microbeads, anti-CD11c microbeads or CD4+ T cell isolation kit, respectively, following the manufacturers’ protocols. The purity of each subpopulation was tested routinely by fluorescence-activated cell sorting (FACS) and a value >95% was measured for each purification. Purified B cells and DCs were stimulated for 72 hours with anti-CD40 (Clone/23 at 2.5 µg/mL) plus F(ab')2 anti-mouse IgM (10 µg/mL) or anti-CD40 alone, respectively. T cells were stimulated with anti-CD3 (0.005 µg/mL), IL-2 (100 U/mL) and IL-12 (10 ng/mL) for 7 days. After stimulation, the cells were harvested and analysed by FACS for OX40L expression.

Flow cytometry
Flow cytometry was performed using a five-colour or six-colour staining protocol and analysed with a BD FACSVersa (BD Biosciences, San Jose, California, USA). The following Abs were used: anti-CD4 (GK.5), anti-CXCR5 (L138D7), anti-PD1 (29F.1a12), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-B220 (RA3-6B2), anti-CD4 (GK.5), anti-CXCR5 (L138D7), anti-PD1 (29F.1a12), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-B220 (RA3-6B2), anti-CD43 (Ly-48), anti-CD11c, anti-GL7 (GL7), anti-CD138 (281-2) and anti-IgD (11–26 c.2a). Abs were purchased with AP-conjugated goat anti-mouse IgG (-chain specific) (Sigma-Aldrich) or IgM (Southern Biotechnology Associates). The results were expressed as AEU relative to a standard positive sample derived from an MRL/Mp<sup>fl/fl</sup> mouse pool.

Total serum IgG and IgM levels
Total serum IgM and IgG levels were assayed by capture ELISA as previously described. IgG, IgM and C3 kidney deposition
Fluorescein (FITC)-conjugated goat Abs against mouse total IgG (1/400 dilution; Sigma-Aldrich), mouse total IgM (1/200 dilution; Biocisense) and mouse C3 (1/50 dilution; ICN Pharmaceuticals) were used on snap-frozen kidney sections. The staining with FITC-conjugated Abs was quantified as previously described and expressed as arbitrary fluorescence units.

Statistical analysis
Where appropriate either the Student’s t-test, two-way analysis of variance (ANOVA) or one-way ANOVA followed by Fisher’s least significant difference (LSD) multiple comparison test was performed using GraphPad Prism V6.00 for Windows (GraphPad Software, La Jolla, California, USA).

RESULTS

Generation of Tnfsf4 conditional knockout strains
We generated a floxed Tnfsf4 mouse (Tnfsf4<sup>fl/fl</sup>) on the C57BL/6 genetic background (see online supplementary figure S1A,B) to avoid the confounding effects caused by epistatic interactions between 129 and C57BL/6 genes that promote an autoimmune phenotype. Germline knockout (KO) mice were obtained by crossing Tnfsf4<sup>fl/fl</sup> with the β-actin Actb-cre mouse strain. Conditional T cell Tnfsf4<sup>fl/fl</sup>CD4<sup>−/−</sup> and B cell Tnfsf4<sup>fl/fl</sup>CD19<sup>−/−</sup> specific knockout mice were created by crossing with CD4<sup>−/−</sup> and CD19<sup>−/−</sup> mice, respectively. Lack of OX40L was observed in all cell types from Tnfsf4<sup>−/−</sup> mice, while a cell-specific deletion was confirmed in the conditional knockouts (see online supplementary figure S1C).

B cell OX40L promotes antibody affinity maturation
Conflicting data have been reported on the importance of the OX40L-OX40 pathway in controlling T-dependent antibody responses. Thus, we explored this response by immunising...
the three KO strains and a control group with NP-CGG, a well-studied T cell-dependent antigen. All three strains showed significantly lower titres of low-affinity IgG2a and IgG2b antibodies against NP25-BSA compared with wild-type mice (figure 1A). In contrast, the IgG1 response was hardly affected by the lack of OX40L. Affinity maturation during the primary response was also assessed by measuring antibody against NP4-BSA on day 28 and by calculating the affinity maturation index (ratio of high-affinity to low-affinity antibody responses). All three knockout strains displayed lower titres of high-affinity IgG2a and IgG2b (figure 1B,C) compared with wild-type animals and a lower affinity maturation index (figure 1D), which suggested OX40L contribution in the antibody affinity maturation process.

OX40L is essential for T cell activation
As the impaired humoral response could be a consequence of defective T cell activation, we decided to investigate the splenic T cell composition (see online supplementary figure S2A) of immunised mice on days 14 and 42 (figure 2). By day 14, Tnfsf4−/− and Tnfsf4−/−(CD4)−/− had a markedly lower proportion of effector T and effector/memory CD4+ T cells. In contrast, Tnfsf4−/−(CD19)−/− mice showed only a reduction in the proportion of effector/memory T cells, indicating that B cell OX40L may not play a major role in priming naïve T
cells. On day 42, all three knockout strains had fewer T effector cells than control mice (figure 2). Interestingly, Tnfsf4−/− and Tnfsf4β0/0(CD19)−/− mice also showed a small, but statistically significant, increment in the frequency of central memory T cells, suggesting that OX40L may regulate the balance between effector and central memory T cells during the recall response (figure 2). Our data confirmed the previous reported role of OX40-OX40L signalling in T cell activation and development of T effector memory cells.18 22 23 36 The explanation for the difference in the secondary humoral response between Tnfsf4β0/0(CD4)−/− and Tnfsf4β0/0(CD19)−/− mice was not evident. We therefore decide to investigate further analysing the extent of the germinal centre (GC) reaction in the immunised mice.

OX40L on B cells supports plasma cell development
All three groups of immunised KO mice showed no difference in the GC B cell population (see online supplementary figure S2B) on day 14 (figure 3A), although during the secondary response, 1 week after the rechallenge, Tnfsf4−/− mice showed a smaller proportion of GC B cells (figure 3A). Similarly, no differences were detected in the plasma cell frequency on day 14, but Tnfsf4−/− and Tnfsf4β0/0(CD19)−/− mice showed a significantly lower percentage of plasma cells on day 42 (figure 3B).

B cell OX40L is essential for $T_{FH}$ maturation
Having demonstrated the importance of OX40L in T cell activation and in plasma cell development, we investigated its possible role in T follicular helper cell ($T_{FH}$) maturation. We identified the GC $T_{FH}$ population as a subset of CD4+ T cells expressing CXCR5 and high levels of PD-1 (CXCR5+PD-1hi) (figure 3C,F), and in figure 3G the frequencies of splenic GC T cells (as a proportion of CD4+ T cells) following immunisation are illustrated. There were fewer GC T cells in the spleens of Tnfsf4−/− and Tnfsf4β0/0(CD19)−/− compared with wild-type mice during both the primary and secondary responses. In contrast, no differences were observed between controls and Tnfsf4β0/0(CD4)−/− mice. Both Tnfsf4−/− and Tnfsf4β0/0(CD19)−/− mice showed a reduction in the expression levels of PD1 at both time points, and importantly displayed a greater frequency of CXCR5+PD1low cells ($T_{FH}$ precursors) in the CD4+ population compared with control mice on day 42 (figure 3D,F). Interestingly, Tnfsf4−/− and Tnfsf4β0/0(CD19)−/− mice also revealed a reduced number of CXCR5+ cells during the primary (day 14) but not the secondary response (day 42) (figure 3E).

Lack of OX40L reduces $T_{FH}$ number and ameliorates the lupus phenotype
In view of the genetic association of TNFSF4 and SLE and the functional results outlined above, we investigated the effect of loss of OX40L in SLE using two different mouse models: a congenic model and a graft-versus-host model. Tnfsf4−/− mice were crossed with B6.Sle16 lupus-prone mice, which are characterised by development of humoral autoimmunity associated with splenomegaly, high level of total IgG and IgM, autoantibodies production and glomerulonephritis linked to Ig and C3 deposition in the kidney.31 32 The resultant B6.Sle16.Tnfsf4−/− female animals were monitored for 9 months (figure 4). The absence of OX40L was associated with a marked reduction in splenomegaly (figure 4A,B) and a lower serum level of total IgG and IgM (figure 4C). No detectable levels of IgG anti-DNA were observed either in the knockout or the B6.Sle16 control group. However, when we analysed IgM anti-ssDNA autoantibodies, a significant lower titre was observed in mice lacking OX40L compared with the B6.Sle16 group (figure 4D). To investigate the effect of loss of OX40L on target organs, we quantified glomerular IgG, IgM and complement C3. As expected fluorescent quantification revealed significantly lower amount of IgG and IgM deposition in the glomerular in the absence of OX40L; in contrast a similar level of C3 deposition was observed (see online supplementary figure S3). Mice lacking OX40L had less T cell activation and higher proportions of central memory and naive T cells (CD62L+ CD44low) (figure 4E). Consistent with the immune response data (figure 3), the B6.Sle16. Tnfsf4−/− showed a fivefold reduction, relative to the B6.Sle16 mice, in the proportion of CD4+ T cells (figure 4F), along with a dramatic reduction of PD-1 expression on CD4+ cells (figure 4G). Furthermore, the percentage of plasma cells and GC B cells (B220+ IgDGL7+) was also significantly lower in the absence of OX40L (figure 4H).

We then used the I-A$\alpha^{m12}$ chronic graft-versus-host-disease (cGVHD) mouse model, in which an alloimmune interaction of T and B cells expressing different major histocompatibility complex (MHC) class II (I-A) induces an SLE-like phenotype.37 38 As shown in figure 5A, Tnfsf4−/− and Tnfsf4β0/0(CD19)−/− mice injected with B6.H2-Ab1$\alpha^{m12}$ splenocytes developed a lower titre of IgG anti-dsDNA compared with controls. In addition, both knockout groups showed a lower percentage of effector/memory T and $T_{FH}$ cells (figure 5B,C). A trend towards a lower percentage of plasma...
and GC B cells was observed in both OX40L-deficient groups (figure 5D,E). Of note, no differences were seen between Tnfsf4−/− and Tnfsf4(CD19)−/− mice, indicating that the observed differences are primarily due to the lack of OX40L on B cells.

**DISCUSSION**

The Tnfsf4 locus (that encodes OX40L) shows association with several autoimmune diseases; it has one of the most consistent and strongest genetic risk factors in SLE. OX40L has a well-established role in the activation and maintenance of T cell-mediated...
immune responses. However, the diversity of cells that express OX40L is such that a pathogenic mechanism relating the genetic findings to disease has not been clearly established. In this study, we generated B and CD4+ T cell OX40L conditional knockout mice, alongside a complete OX40L knockout, to explore and compare the function of OX40L on these cells.

Although a role for OX40L in the T-dependent antibody response has been suggested, conflicting results using different OX40L-deficient mice have been reported. The contradictory results may be partly explained by variability in genetic background. Our conditional knockout mice were on a pure C57BL/6 background and, in accord with the one study.
Figure 5 OX40L deficiency diminishes anti-dsDNA antibody production in the cGVHD model. Female wild-type controls, Tnfsf4<sup>−/−</sup> and Tnfsf4<sup>(CD19)<sup>−/−</sup> mice were injected intraperitoneally with 5×10<sup>7</sup> splenocytes from B6.H2<sup>−/−</sup> female mice. Sera were collected on days 14, 28, 42 and 56. On day 56, spleens were collected and analysed by FACS. (A) Titre of IgG anti-dsDNA in the sera of injected mice at different time points. (B) Quantification of naïve (T<sub>n</sub>) (CD4+, CD62L+, CD44<sup>lo</sup>), (T<sub>eff</sub>) effector (CD4+, CD62L<sup>low</sup>, CD44<sup>hi</sup>), (T<sub>em</sub>) effector/memory (CD4+, CD62L<sup>high</sup>, CD44<sup>lo</sup>) and (T<sub>cm</sub>) central/memory (CD4+, CD62L+, CD44<sup>lo</sup>) T cells expressed as percentage of CD4+ cells. (C) Quantification of GC T<sub>FH</sub> cells (CD4+, CXCR5+, PD-1<sup>hi</sup>) presented as frequency among the CD4+ population. (D) Quantification of GC B cells (B220<sup>hi</sup>, GL7<sup>hi</sup>, IgD<sup>lo</sup>) presented as frequency among the CD19<sup>hi</sup> population. (E) Percentage of plasma cells (B220<sup>lo</sup>, CD138<sup>hi</sup>). Each symbol represents data from an individual mouse. Bars indicate the mean±SEM. N.S., not significant; *p<0.05, **p<0.01 and ***p<0.001 (one-way analysis of variance). cGvHD, chronic graft-versus-host-disease; dsDNA, double-stranded deoxyribonucleic acid; FACS, fluorescence-activated cell sorting; GC, germinal centre.

Our Tnfsf4<sup>−/−</sup> mice showed a reduced primary and secondary antibody response. However, while the Tnfsf4<sup>(CD4)<sup>−/−</sup> mice showed the same phenotype as the Tnfsf4<sup>−/−</sup> mice, the Tnfsf4<sup>(CD4)<sup>−/−</sup> mice had a normal secondary response, indicating that only OX40L expression by B cells is essential for the generation of an effective secondary humoral response and by implication B cell memory. We then investigated whether this defective humoral response was due to impaired T cell activation; as expected, Tnfsf4<sup>−/−</sup> mice showed lower percentage of T effector and T effector memory cells (figure 2). The same defect, although at a lower extent, was also found by both conditional knockouts, despite the normal secondary response in Tnfsf4<sup>(CD4)<sup>−/−</sup> mice. These results suggest that B cell OX40L may be involved in biological processes that promote memory responses that are independent of T cell activation.

T cell-dependent B cell immune response involves both an early wave of low-affinity antibody production, and a GC response, which gives rise to long-lived plasma or memory cells and a later wave of high-affinity antibodies. OX40L has been previously suggested to be essential for the development of high-affinity Ig-producing plasma cells; however, no further evidence has been subsequently reported. In our study, alongside an impaired memory response (figure 1C), there were fewer plasma cells on day 42 in Tnfsf4<sup>−/−</sup> and Tnfsf4<sup>(CD19)<sup>−/−</sup> mice (figure 3B), which suggests that B cell OX40L contributes to an effective GC reaction.

We show that Tnfsf4<sup>−/−</sup> and Tnfsf4<sup>(PD-1)<sup>−/−</sup> mice have a lower percentage of GC T<sub>FH</sub> cells than their Tnfsf4<sup>−/−</sup> counterparts. The development of mature GC T<sub>FH</sub> cells which characteristically expresses CXCR5, along with high levels of the surface receptors ICOS, CD40 ligand (CD40L), PD-1 and importantly OX40L<sup>−/−</sup> includes two stages: after activation, a fraction of CD4+ T cells migrate towards B cell follicles by upregulating the chemokine receptor CXCR5, and these T<sub>FH</sub> precursors then interact with antigen-presenting B cells at the border of the B cell follicle and T cell zone and fully mature into functional GC T<sub>FH</sub> cells. In particular OX40L has been shown to be essential for the expression of CXCR5 and the consequent migration of T cells to the T/B border of B cell follicles, providing the first evidence of the role of OX40L in this process. Our results corroborate this finding; we found that fewer CXCR5<sup>+</sup> T cells were generated during the primary response in Tnfsf4<sup>−/−</sup> and Tnfsf4<sup>(PD-1)<sup>−/−</sup> mice (figure 3E). Whether OX40L-OX40 signal is responsible for the induction, maturation, or maintenance of T<sub>FH</sub> cells and which cell types expressing OX40L are necessary is still unclear; however, a recent work by Tahiliani and colleagues<sup>8</sup> shows a markedly diminished humoral response and production of fewer T<sub>FH</sub> cells in OX40 KO mice following immunisation with vaccinia virus. In particular the authors show a direct association between OX40<sup>+</sup> T<sub>FH</sub> cells and OX40L-expressing DCs and B cells at the T/B borders and GC providing supportive evidence to how a sustained OX40L-OX40 signal on T<sub>FH</sub> cells is necessary for the induction of T<sub>FH</sub> cells and their...
maturation to maintain a proper GC reaction. In our study, the reduced numbers of T<sub>H</sub>~ cells in Tnfsf4<sup>−/−</sup> and Tnfsf4<sup>F<sub>H</sub>−/−(CD19)−/−</sup> mice were accompanied by an increase in CXCR5<sup>+</sup>PD1<sup>+</sup> cells during the secondary response (figure 3F,G). Since low levels of cell-surface PD1 have been shown to characterise T<sub>H</sub>~ precursor cells, our data suggest a novel role for OX40L on B cells: after activation by DCs, immature T<sub>H</sub>~ cells migrate towards the T/B borders of the B cells follicles, where activated antigen presenting B cells induced their maturation into the GC T<sub>H</sub>~ resident state and their maintenance by sustaining OX40L-OX40 signalling. Tnfsf4 has been reproducibly associated with SLE. A recent important study from Jacquemin and colleagues demonstrated that stimulation through OX40 induced T cells to express T<sub>H</sub>~ cells-specific genes such as Bcl6 and CXCR5. They also observed a positive correlation between disease activity, percentage of blood T<sub>H</sub>~ cells and frequency of OX40L+myeloid APC, suggesting OX40L-OX40 axis as a contributor factor in the aberrant T<sub>H</sub>~ response observed in SLE. However, the ability to study tissue T<sub>H</sub>~ cells in humans is limited. In our study, although in a murine model, the generation of T<sub>H</sub>~ cells in the spleen is similarly impeded in the B cell conditional knockout and in the germine Tnfsf4 knockout, indicating the importance of B cell OX40L. In the human study, there was no correlation between blood B cells expressing OX40L and T<sub>H</sub>~ cells. However, this lack of correlation could be a consequence of the compartmentalisation of activated B cells expressing OX40L in the secondary lymphoid organs rather than an evidence of their lack of involvement in the development of pathogenicic T<sub>H</sub>~ cells in SLE.

In our study, to elucidate the role of OX40L in SLE, we used two different SLE mouse models, and in particular the GrnHd model was chosen to investigate the role of OX40L on B cells during the B-T cell interaction. In both models of systemic autoimmunity, the lack of OX40L-OX40 signalling was associated with amelioration of the disease phenotype, as shown by a reduced production of anti-dsDNA autoantibodies and Ig kidney deposition together with reduced numbers of GC T<sub>H</sub>~ cells (figures 4F and 5C) and plasma cells (figures 4I and 5E). These data suggest that OX40L supports the expression of the disease phenotype as well as autoantibody production. This conclusion is further strengthened by the observation that blockade of OX40L reduces degree of proteinuria associated with glomerulonephritis in an accelerated murine model.

The results presented in this paper support a mechanism by which genetically determined elevated expression of OX40L predisposes to SLE via increased B cell expression, which in turn supports T<sub>H</sub>~ development. In light of the argument that genetic factors augment the likelihood of success with a drug target, our data strongly support exploration of this therapeutic strategy. It is potentially important for optimal treatment to know which OX40L-expressing cell types should be targeted, and the defined risk alleles at Tnfsf4 further raise the possibility that genetic screening may identify individuals most likely to benefit from OX40L inhibition.

Correction notice This article has been corrected since it published Online First. The first author’s name has been corrected to Deborah S Cunninghame Graham.

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Contributors AC designed, performed and analysed experiments and wrote the manuscript. UE performed experiments, helped with the statistical analysis and discussed the data. THM performed experiments. DSCG discussed the data and edited the manuscript. MB and TJV designed experiments, discussed the data and wrote the manuscript.

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