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B cell-intrinsic requirement for STK4 in humoral immunity in mice and humans
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<td>Corresponding Author:</td>
<td>Tri Phan, MBBS, FRACP, FRCPA, PhD Garvan Institute of Medical Research Sydney, NSW AUSTRALIA</td>
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<td>Imogen Moran</td>
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Abstract:
Humoral immune defects are described in 9 patients from 5 families with STK4 deficiency. A mouse model carrying the novel p.Y88del show that these defects are intrinsic to the B cells.
B cell-intrinsic requirement for STK4 in humoral immunity in mice and humans

Imogen Moran, PhD1,2, Danielle T. Avery, BSc1, Kathryn Payne, BSc1, Helen Lenthall, MMSc1, E. Graham Davies, MD3, Siobhan Burns, MD PhD4,5, Winnie Ip, MD3, Matïffedas M. Oleastro, MD6, Ismail Reisli, MD7, Sukru Guner, MD7, Seygi Keles, MD7, Luigi Notarangelo, MD8, Elissa K. Deenick, PhD1,2, Christopher C. Goodnow, PhD1,2, David Zahra, PhD1, Robert Brink, PhD1,2, CIRCA9, Melanie Wong, PhD10,11*, Stuart G. Tangye, PhD11,2*, Cindy S. Ma, PhD1,2*, Tri Giang Phan, PhD1,2*

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Conflicts of interest: The authors have no conflicts of interest to declare.

Key finding: Patients with STK4 deficiency have humoral immune defects due to intrinsic defects in B cell development and differentiation.

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To the Editor:

Biallelic loss-of-function mutations in serine threonine kinase 4 (STK4), also known as mammalian sterile 20-like 1 (MST1), are associated with a combined immunodeficiency characterised by recurrent bacterial, fungal and viral infections (1-6). Most patients have intermittent neutropenia, T and B lymphopenia and, paradoxically, specific antibody defects despite the reported presence of elevated levels of serum IgG, A and E antibodies and antibody-mediated autoimmune cytopenias (1-6). While there have been numerous studies of neutrophil, macrophage, dendritic cell and T cell function in Stk4 knock-out mice (7-11), the nature and basis of the underlying B cell dysregulation in STK4-deficient patients remains poorly characterised. To address this, we investigated the B cell phenotype and function in 9 patients from 5 unrelated families with STK4 deficiency due to 5 different mutations, 2 of which are novel (Table E1 and Fig E1, A), and in CRISPR/Cas9 gene-edited mice carrying either the novel p.Y88del 3 base pair in-frame deletion (Y88del) in the kinase domain of STK4 present in 2 of these patients, or a premature stop codon (Fig E1, B-C). Notably, we did not observe elevated immunoglobulins, with the exception of IgE in our patients (Figure E1, D). Indeed, the dysglobulinemia in our STK4-deficient patients more closely resembles that observed in patients with DOCK8 deficiency.

STK4 protein expression was decreased in Stk4<sup>Y88del/Y88del</sup> and Stk4<sup>+/−</sup> mice (Fig E1, E), confirming the p.Y88del mutation affects protein stability, resulting in STK4 deficiency. Stk4<sup>Y88del/Y88del</sup> and Stk4<sup>+/−</sup> mice phenocopy the two patients with the mutation with elevated IgE and decreased peripheral blood naïve T cells (Table E1, Fig 1, A and E1, F-G). Immunization with sheep red blood cells (SRBCs) demonstrated that, similar to STK4-deficient patients, mice had specific antibody defects (Fig 1, B). STK4-deficient patients have decreased proportions of circulating memory B cells (B<sub>mem</sub>) and increased transitional B cells compared to age-matched healthy controls (Fig 1, C and refs. 1-3, 5-6). Therefore, we examined B cell development in Stk4<sup>Y88del/Y88del</sup> mice. While early B cell development in the bone marrow was relatively normal (Fig E2, A), there was a decrease in the number of splenic mature follicular B cells, an absence of splenic marginal zone B cells, and a decrease in the proportion of peritoneal cavity B1a cells in Stk4<sup>Y88del/Y88del</sup> and Stk4<sup>+/−</sup> mice compared to WT mice (Fig 1, D-F), similar to observations made previously in other strains of Stk4-deficient mice (9, 12-14). Mixed bone marrow radiation chimeras demonstrated that these defects in peripheral B-cell differentiation were cell-intrinsic, and not secondary to T cell or myeloid cell defects (Fig E2, B-E).

Naïve B cells from STK4-deficient patients were cultured in vitro to determine their capacity to proliferate and differentiate into antibody-secreting cells (ASCs). This revealed a mild proliferative
defect in response to mimics of BCR engagement, but not T-cell help induced by CD40L and IL-21 (Fig 2, A). Interestingly, while naïve B cells from patients and healthy controls generated similar numbers of ASCs (Fig 2, B), STK4-deficient ASCs secreted less antibodies in response to CD40L and IL-21 stimulation (Fig 2, C). Notably, while CD19 expression has been reported to be reduced on B cells from Stk4 knock-out mice (12), the level of CD19 was not found to be significantly decreased in both STK4-deficient patients and gene-targeted mice (Fig E3, A-B). Accordingly, there were no differences in BCR-mediated upregulation of CD69 and CD86 following in vitro stimulation with cognate antigen of STK4-deficient mouse B cells (Fig 2, D).

Stk4Y88del/Y88del mice were immunized with SRBCs and this showed a decreased number of germinal centre (GC) B cells, B_mems and plasma cells compared to Stk4+/+ mice (Fig 2, E). STK4-deficient patients have decreased circulating memory Tfh cells (Fig E4, A), and immunized mice have decreased Tfh cells in the spleen (Fig E4, B). Mixed bone marrow chimeras suggested that, despite the Tfh cell defect in intact mice, the defective humoral immune response was B cell-intrinsic (Fig E4, C-E). This was confirmed by adoptive transfer experiments in which SWHEL B cells (15) were used to track affinity maturation to cognate antigen (16). This showed that, in a system where the immune system was otherwise completely intact, while the response of STK4-gene-targeted SWHEL B cells on day 5 was comparable to wild-type SWHEL B cells, STK4 mutant SWHEL B cells failed to expand and sustain the GC response, which rapidly contracted by day 9. Notably, there was also a relative reduction in the proportion of dark zone GC B cells (Fig 2, F and E5, A). Short-term labeling with BrdU showed that this failure to sustain the GC reaction was due to defective proliferation rather than increased cell death as there was no difference in caspase-3 staining (Fig 2, G and E5, B). Nevertheless, somatic hypermutation, affinity maturation, and class switch recombination were unaffected (Fig E5, C-D).

We next examined the capacity of SWHEL B cells with STK4 mutations to differentiate into B_mems and plasma cells in vivo. Similar to immunization with SRBCs, there was defective generation of Stk4Y88del/Y88del B_mems (Fig 2, H). However, the residual Stk4Y88del/Y88del B_mems were functional, as shown by their ability to generate recall responses in immune mice, albeit to a greatly reduced extent compared to wild-type B_mems (Fig E6, A-C). Consistent with this, the few B_mems present in STK4-deficient patients were capable of differentiating into ASCs in vitro, albeit at reduced levels (Fig E6, D). Interestingly, despite the impaired specific antibody secretion in mice with Stk4Y88del/Y88del B cells, STK4 deficiency did not quantitatively impact the ability of these SWHEL B cells to generate plasma cells in vivo (Fig 2, I-K). Thus, similar to B cells from STK4-deficient
patients, Stk4<sup>Y88del/Y88del</sup> B cells are able to differentiate into plasma cells, but these plasma cells fail to secrete adequate amounts of specific antibody.

STK4 is a multifunctional kinase that phosphorylates multiple cellular proteins, including those in the Hippo signaling pathway (17, 18). Many of these substrates are also phosphorylated by its paralog STK3, suggesting STK3 may functionally compensate for STK4 deficiency. Indeed, B cell defects are more readily observed in Stk3/Stk4 double knockout mice (14). Interestingly, STK4 has been shown to phosphorylate FOXO1 and promote its nuclear localization (19), and FOXO1 was recently shown to be required for dark zone formation and GC maintenance (20-22). However, while FOXO1 levels were decreased in Stk4<sup>Y88del/Y88del</sup> and Stk4<sup>-/-</sup> GC B cells, we could not rescue the GC defect by retroviral overexpression of Foxo1 (Fig E7, A-B), suggesting that other mechanisms might also be involved. Another limitation of our study is the small number of patients involved which prevents any firm conclusion, especially regarding differences in the serum immunoglobulin levels in our cohort of 9 patients and the previously reported 14 patients. Nevertheless, our data establishes a B cell-intrinsic requirement for STK4 in humoral immunity in mice and humans.

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References:


Figure No. 1 - Unmarked

**Moran et al. Figure 1 2018**

**A**
- Serum IgM (μg/mL) vs. IgG (μg/mL) vs. IgE (μg/mL) vs. IgA (μg/mL)
- Stk4+/+ (blue) vs. Stk4Y88del/Y88del (red) vs. Stk4/- (green)
- Healthy Controls (yellow) vs. STK4-deficient patients (purple)

**B**
- Kappa LC: Anti-SRBC MFI vs. IgM vs. IgG1
- SRBC day 7: Stk4+/+ vs. Stk4Y88del/Y88del vs. Stk4/-

**C**
- % B cells vs. Transitional, Naive, Memory

**D**
- Cells/spleen (x 10^6)
- Immature vs. T1 vs. T2 vs. T3 vs. Mature vs. FoB vs. MZ B

**E**
- Stk4+/+ vs. Stk4Y88del/Y88del
- MZ vs. CD3

**F**
- % CD19+ B cells vs. B-2, B-1, B-1a, B-1b
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JACI-D-19-00027, B cell-intrinsic requirement for STK4 in humoral immunity in mice and humans

Figure Legends

Figure 1. STK4-deficient mice and patients have B cell-intrinsic defects in peripheral B cell development. (A) Serum immunoglobulins in unimmunized Stk4^{Y88del/Y88del}, Stk4^{-/-} and Stk4^{+/+} mice. Data are representative of >2 independent experiments with 7 mice per group. (B) Serum kappa light chain (LC), IgG1 and IgM antibodies against SRBCs 7 days after immunization. Data are representative of 2 independent experiments with 3-4 mice per group. (C) Proportion of circulating transitional, naïve and memory B cells in the peripheral blood of STK4-deficient patients and healthy donors. (D) Number of immature, transitional (T1-T3), mature, follicular (FoB) and marginal zone B cells (MZ B) in the spleen of Stk4^{Y88del/Y88del}, Stk4^{-/-} and Stk4^{+/+} mice. Combined data from 2 independent experiments with 4-5 mice per group. (E) Immunohistochemistry showing MAdCAM (blue), B220 (red) and CD3 (green) in splenic sections from Stk4^{Y88del/Y88del} and Stk4^{+/+} mice. Data are representative of 3 independent experiments with 2 mice per group. (F) Proportion of B-2, B-1, B-1a and B-1b cells in the peritoneum of Stk4^{Y88del/Y88del}, Stk4^{-/-} and Stk4^{+/+} mice. Data are combined from 2 independent experiments with 4-5 mice per group. ** p < 0.01, ****p < 0.0001.

Figure 2. STK4-deficient mice and patients have B cell-intrinsic defects in humoral immunity. Naïve B cells from STK4-deficient patients and healthy controls were sorted and cultured in vitro to assess (A) CFSE dilution, (B) plasma cell differentiation, and (C) immunoglobulin secretion after 4-5 days. (D) B cells from from Stk4^{Y88del/Y88del}, Stk4^{-/-} and Stk4^{+/+} SWHEL mice were stimulated overnight to assess BCR signaling. (E) Humoral immune response in Stk4^{Y88del/Y88del} and Stk4^{+/+} mice immunized 7 days earlier with SRBC. (F) Kinetics of the GC B cell response of adoptively transferred Stk4^{Y88del/Y88del} and Stk4^{+/+} SWHEL B cells. (G) Decreased proliferation of Stk4^{Y88del/Y88del} compared to Stk4^{+/+} SWHEL GC B cells. Data combined from 2 independent experiments with 4-5 mice per group on day 5. Kinetics of (H) B_{mems} and (I) plasma cell response of adoptively transferred Stk4^{+/+} or Stk4^{Y88del/Y88del} SWHEL B cells. (J) Serum anti-HEL (total) antibodies on 7 day and (K) anti-HEL^{2X} (high affinity) antibodies on 14 day. Unless otherwise stated, mouse data is representative of at least 2 independent experiments with >4 mice per group per time point. * p < 0.5, *** p < 0.001, ****p < 0.0001.
Table E1. Clinical phenotype and laboratory findings in 9 STK4-deficient patients from 5 families.

Extended figure legends

Figure E1. Mutations in a cohort of 8 patients with STK4 deficiency and generation of a novel mouse model of STK4 deficiency. (A) Model of STK4 protein showing position of known mutations in STK4-deficient patients. Novel Y88del and R115* mutations in this report are shown in red; previously described mutations are in black. AID, autoinhibitory domain; SARAH, Sav/Rassfl/Hpo domain. (B) Model of STK4 showing position of Y88 residue in the ATP binding site. (C) Generation of CRISPR/Cas9 gene-edited mice with deletion of the Y88 amino acid residue (Stk4<sup>Y88del/Y88del</sup>) or a knockout with a premature stop codon (Stk4<sup>-/-</sup>). (D) Serum immunoglobulin levels and age-matched reference ranges (grey shading) of 9 patients with STK4 deficiency. (E) Western blot STK4 protein levels in Stk4<sup>+</sup>/+, Stk4<sup>Y88del/Y88del</sup> and Stk4<sup>-/-</sup> murine splenocytes. (F) Proportion of naïve, effector memory and central memory CD4 and CD8 T cells in peripheral blood of healthy donors or STK4-deficient patients. (G) Proportion of naïve, effector memory and central memory CD4 and CD8 T cells in peripheral blood of Stk4<sup>Y88del/Y88del</sup>, Stk4<sup>-/-</sup> and Stk4<sup>+/+</sup> mice. Data representative of 2 independent experiments with 4-5 mice per group. * p < 0.05, ** p < 0.01, *** p < 0.001, ****p < 0.0001.

Figure E2. B cell-intrinsic defect in peripheral B cell development in STK4 deficient mice. (A) Number of pre-pro, pro, pre, immature and mature B cells in the bone marrow of Stk4<sup>Y88del/Y88del</sup>, Stk4<sup>-/-</sup> and Stk4<sup>+/+</sup> mice. Data are combined from 2 independent experiments with 4-5 mice per group. (B) Experimental design of mixed bone marrow radiation chimera mice that were reconstituted with 50% CD45.1<sup>+</sup> Stk4<sup>-/-</sup> and 50% CD45.2<sup>+</sup> Stk4<sup>Y88del/Y88del</sup> or Stk4<sup>-/-</sup> bone marrow. Reconstitution ratios of B cell subsets in (C) bone marrow, (D) spleen and (E) peritoneal cavity of mixed chimera mice with 50% CD45.1<sup>+</sup> Stk4<sup>-/-</sup> and 50% CD45.2<sup>+</sup> Stk4<sup>Y88del/Y88del</sup> or Stk4<sup>-/-</sup>. Data are combined from 2 independent experiments with 3-6 mice per group. *** p < 0.001, ****p < 0.0001.

Figure E3. Normal expression of CD19 in human and mouse STK4 deficient B cells. (A) Expression of BCR co-receptor CD19 on human peripheral blood B cell subsets from healthy donors and STK4 deficient patients. (B) Expression of BCR co-receptor CD19 on splenic B cells Stk4<sup>Y88del/Y88del</sup>, Stk4<sup>-/-</sup> and Stk4<sup>+/+</sup> mice. Data representative of 3 independent experiments with >3 mice per group. * p < 0.05.

Figure E4. B cell-intrinsic defect in humoral immune response. (A) Measurement of circulating memory Tfh cells in the blood of STK4-deficient patients. (B) Tfh cell numbers in the spleen of STK4-deficient mice on day 7 after SRBC immunization. Reconstitution ratios on day 7 of SRBC immunization in spleen for (B) GC B cells, (C) B<sub>mems</sub> and (D) plasma cells of mixed chimera mice
with input of 50% CD45.1+ Stk4+/+ and 50% CD45.2+ Stk4Y88del/Y88del or Stk4+/+. Data are representative of 2 independent experiments with 3-6 mice per group. ****p < 0.0001.

Figure E5. Altered composition of the GC but normal cell death, somatic hypermutation and class switching in STK4-deficient B cells. (A) Proportion of dark zone (DZ) and light zone (LZ) GC B cells from day 7 of Stk4+/+ or Stk4Y88del/Y88del SWHEL B HEL2X-SRBC response. Data are representative of 2 independent experiments with 5 mice per group. (B) Proportion of active caspase-3 cells in the GC in mice adoptively transferred with Stk4+/+ or Stk4Y88del/Y88del SWHEL B cells challenged with HEL2X-SRBC. Data is combined from 2 independent experiments with 4-5 mice per group on day 5. (C) Sequencing analysis of Ig heavy chain genes showing proportion of GC B cells with affinity increasing mutations, including the canonical Y53D mutation, in mice adoptively transferred with Stk4+/+ or Stk4Y88del/Y88del SWHEL B cells challenged with HEL3X-SRBC. n shows number of GC B cells sequenced and number mutations/cells shows total number of mutations in sequenced region. Representative of 2 independent experiments with 4-5 mice per group. (D) Proportion of IgG1+ B cells in the GC in mice adoptively transferred with Stk4+/+ or Stk4Y88del/Y88del SWHEL B cells challenged with HEL3X-SRBC. Data are representative of 2 independent experiments with 5 mice per group per time point. ***p < 0.001.

Figure E6. Memory B cells were functional and able to generate a secondary response. (A) Experimental design to setup a B_mem lymph node response, where Stk4+/+ or Stk4Y88del/Y88del SWHEL B cells and wild-type OT2 CD4 T cells were adoptively transferred into recipient mice and challenged with HEL-OVA. (B) Number of donor (CD45.1+) B_mems in draining lymph node. (C) Number of donor B cells in the recall response in draining lymph node. Lymph node data are representative of 2 independent experiments with 4-5 mice per group per time point. (D) Immunoglobulin secretion of cultured sorted memory B cells from STK4-deficient patients and healthy donors. *p < 0.5, ****p < 0.0001.

Figure E7. STK4 deficiency results in decreased FOXO1 expression but overexpression of FOXO1 does not rescue germinal centre defect. (A) Intracellular FACS analysis of FOXO1 expression in GC B cells and total B cells 7 days after SRBC immunization of Stk4Y88del/Y88del, Stk4−/− and Stk4+/+ mice. (B) Proportion of donor lymphocytes following retroviral overexpression in mice adoptively transferred with Stk4+/+ or Stk4Y88del/Y88del SWHEL B cells challenged with HEL3X-SRBC. All data are representative of 2 independent experiments with 3-4 mice per group per time point. **p < 0.01, ****p < 0.0001.
METHODS

Human blood samples
Buffy coats from healthy donors were purchased from the Australian Red Cross Blood Service. Pediatric blood samples were collected from individuals either attending clinic for non-immunological conditions, or for genetic testing due to a family history of disease, but were found not to carry the mutation. Whole blood was collected, PBMCs were isolated and cryopreserved as single-cell suspensions, shipped to the Garvan Institute on dry ice, and then stored in liquid nitrogen until use. Approval for this study was obtained from the relevant hospital human research ethics committees. Informed consent was obtained from all participants for human experiments described in this study.

Human lymphocyte phenotyping
PBMCs were incubated with following mAbs: BUV395-anti CD20, PE-Cy7-anti CD27, BV786-anti CD27, APC-anti CD10, BV421-anti CD3, BUV737-anti CD4, BUV395-anti CD8, PE-Cy7-anti CCR7, BV605-anti CD45RA, APC-anti CD38, BV711-anti CD19. The proportions of CD20⁺ CD27⁻ CD10⁺ (transitional), CD20⁺ CD27⁻ CD10⁻ (naïve), and CD20⁺ CD27⁺ CD10⁻ (memory) B cells, and T cell subsets (CD4 or CD8) naïve (CCR7⁺ CD45RA⁻), central memory (CCR7⁺ CD45RA⁻), effector memory (CCR7⁻ CD45RA⁻) and for CD8⁺ cells terminally differentiated effector memory T cells expressing CD45RA (CCR7⁻ CD45RA⁺) was determined by flow cytometry (LSRII, Becton Dickinson) and analysed using FlowJo software (Tree Star).

Isolation and in vitro activation of human B cell subsets
PBMCs were labeled with mAbs against CD20, CD27, and CD10 and naïve (CD20⁺ CD10⁻ CD27⁻) B cells were then sorted using a FACS Aria III (Becton Dickinson). Purity of the recovered populations was >90%. Naïve B cells were then cultured as previously described (23). B cell viability was determined using the Zombie Aqua Viability dye (BioLegend) and proliferation determined by CFSE (eBioscience) dilution after 4-5d of in vitro culture. Differentiation of B cells to plasmablasts was assessed by determining the frequency of naïve B cells acquiring a CD38⁺ CD27⁺ phenotype during in vitro culture by flow cytometry (LSRII, Becton Dickinson) and analyzed using FlowJo software (Tree Star).

Human Ig ELISAs
Secretion of IgM, IgG and IgA by in vitro cultured human transitional and naive B cells was
determined using Ig heavy-chain specific ELISAs, as described previously (24).

Mice

SW<sub>HEL</sub> mice expressing a knock-in BCR against hen egg lysozyme (HEL) (15) were maintained on a C57BL/6J or C57BL/6-SJL.Ptprc<sup>a/a</sup> congenic background. Thy1.1 congenic mice (000406; B6.PL-<sup>T</sup>Ty1<sup>a</sup>/CyJ) (25) were crossed to OT2 TCR transgenic mice (B6.Cg-TcraTcrb425Cbn/J) (26), and maintained on a C57BL/6 background. C57BL/6 and C57BL/6-SJL.Ptprc<sup>a/a</sup> congenic mice were purchased from Australian BioResources (Moss Vale, Australia).<br>

<sub>Stk4</sub><sup>Y88/Y88del</sup> and <sub>Stk4</sub><sup>+/+</sup> mice were produced by the Mouse Engineering Garvan/ABR (MEGA) Facility using CRISPR/Cas9 gene targeting in C57BL/6J mouse embryos following established molecular and animal husbandry techniques. A single guide RNA (sgRNA) was employed that targeted Cas9 to exon 4 of <sub>Stk4</sub>, adjacent to the Y88 codon (CCTCACGTAGTCAAGTATTTGG: Y88 codon italicized, protospacer-associated motif = PAM underlined). A solution consisting of sgRNA (15ng/μl), polyadenylated <i>S.pyogenes</i> Cas9 mRNA (30ng/μl) and a 150 base, single-stranded, deoxy-oligonucleotide homologous recombination substrate lacking the Y88 codon (54 bases 5’ plus 96 bases 3’, 10ng/μl) was prepared and microinjected into the nucleus and cytoplasm of C57BL/6J zygotes. Microinjected embryos were cultured overnight and those that underwent cleavage introduced into pseudo-pregnant foster mothers. Pups were screened by PCR across the target site and Sanger sequencing of PCR products used to detect mice carrying (1) a 2bp frame shift insertion or (2) specific removal of the Y88 codon which were then bred on a C57BL/6J background to establish the <sub>Stk4</sub><sup>+/+</sup> and <sub>Stk4</sub><sup>Y88del/Y88del</sup> lines, respectively. <sub>Stk4</sub><sup>Y88del/Y88del</sup> and <sub>Stk4</sub><sup>+/+</sup> mice were crossed to SW<sub>HEL</sub> mice on a C57BL/6-SJL.Ptprc<sup>a/a</sup> congenic background. For bone marrow chimeras, C57BL/6-SJL.Ptprc<sup>a/a</sup> (CD45.1<sup>+</sup>) mice were irradiated in two doses, 6 hours apart, with 425 Rad (X-RADA 320 Biological Irradiator, PXI) and injected with 2 x 10<sup>6</sup> bone marrow cells (50:50 mixture of wild-type C57BL/6 CD45.1<sup>+</sup> bone marrow and either <sub>Stk4</sub><sup>Y88del/Y88del</sup> or <sub>Stk4</sub><sup>+/+</sup> CD45.2<sup>+</sup> marrow). They were allowed to reconstitute for 8-10 weeks before analysis or immunisation. All mice were bred and maintained in specific-pathogen free conditions at Australian BioResources (Moss Vale) and the Garvan Institute Biological Testing Facility. Animal experiments were approved by the Garvan Institute of Medical Research/St Vincent’s Hospital Animal Ethics Committee.

Immunisations and adoptive cell transfer

SRBC immunisation
For SRBC immunisation, mice were given i.v. injection of $2 \times 10^8$ SRBCs (Alsevers) in 200μL. Splenocytes were harvested at d7 post immunisation and analysed by flow cytometry. To detect anti-SRBC antibodies in the serum, $2 \times 10^6$ SRBCs were plated in individual wells of a 96 well plate and SRBCs then incubated with serum dilutions from SRBC immunised mice. Serum from non-SRBC immunised mice was included as a negative control. Anti-SRBC antibodies were detected with anti-kappa biotin and SA-A647. Samples were acquired on a CytoPlate (Beckman Coulter).

**HEL-SRBC immunisation**

Purified hen egg lysozyme (HEL) was purchased from Sigma-Aldrich. Recombinant mutant HEL$^{2X}$ and HEL$^{3X}$ proteins with intermediate and low affinity for the HyHEL10 BCR were grown ns in yeast (Pichia pastoris) and purified from culture supernatants as described (27). For adoptive transfers, spleen cells from donor SW$_{HEL}$ mice containing $3 \times 10^6$ HEL-binding B cells were transferred i.v. into wild-type recipient mice together with $2 \times 10^8$ HEL$^{2X}$-SRBC or HEL$^{3X}$-SRBC, conjugated as previously describe (27).

**HEL-OVA immunisation**

OT2 T cells were enriched by negative depletion with biotinylated antibodies for anti-B220 clone RA3-6B2, anti-CD11b clone M1/70, anti-CD11c clone HL3, anti-CD8 clone, and Stk4$^{+/+}$, Stk4$^{Y88del/Y88del}$ or Stk4$^{-/-}$ SW$_{HEL}$ B cells were enriched by negative depletion with biotinylated antibodies for anti-CD11b, anti-CD11c, anti-CD4 clone GK1.5, anti-CD43 clone S7 (all from BD Bisociences) and MACs anti-biotin magnetic beads (Miltenyi). Purity of CD4$^+$ Vα2$^+$ OT2 T cells was typically 70-80% and B220$^+$ HEL-binding SW$_{HEL}$ B cells >99% as determined by FACs analysis. 2.5 $\times 10^5$ CD4$^+$Vα2$^+$ OT2 T cells and B220$^+$ HEL-binding SW$_{HEL}$ B cells were adoptively transferred into age and sex matched 6-9 week old recipient mice. Recipient mice were immunised the next day by subcutaneous injection with 20μg HEL-OVA in Sigma Adjuvant System (SAS) in the lower flank and base of tail. For memory responses, mice that had been immunised were rested for at least 28 days and then re-challenged with 40μg HEL-OVA in SAS injected subcutaneously in the lower flank and base of tail. HEL was conjugated to OVA$^{323-339}$ peptide (CGGISQAVHAHAEINEAGR) (Mimotopes/Genscript) using the SMPH cross-linking agent Succinimidyl-6-[(β-maleimidopropionamido) hexanoate] (Thermo Fisher Scientific).

**BrdU incorporation**
1mg bromodeoxyuridine (BrdU) (Sigma) was injected i.v. into recipient mice, and harvested 1hr post injection. Splenocytes were surface stained, then samples fixed, permeabilised and stained with anti-BrdU-FITC using the BrdU flow kit (BD Biosciences) as per the manufacturers protocol.

**FACS analysis of mouse cells**

Spleen and inguinal lymph nodes were harvested, dissected free of fat and fascia, and lymph nodes teased apart with microforceps and mashed through a 70µm filter. Bone marrow cells were harvested from tibia and femur by centrifugation and peritoneal cavity cells were harvested by peritoneal lavage. Blood was collected by cardiac puncture for FACS analysis into ~50uL heparin solution, or allowed to clot at room temperature and collected serum stored at -20°C for future ELISAs. Spleen, bone marrow and blood samples were RBC lysed. Single cell suspensions were then washed and Fc receptors blocked with unlabeled anti-CD16/32 clone 2.4G2 before staining. To detect HEL-binding B cells, cells were stained with saturating levels of HEL at 200 ng/ml, followed by HyHEL9 Alexa Fluor 647. For detection of HEL-binding IgG1⁺ B cells, anti-IgG1 staining was performed first and followed by blocking with 5% mouse serum before subsequent staining for HEL-binding with HyHEL9, a mouse IgG1 monoclonal antibody. Antibodies used for surface staining are shown in Table 1. For intracellular staining, cells were fixed with Fixation/Permeabilization buffer and antibodies stained in Permeabilization buffer (eBioscience). Antibodies used for intracellular staining were: anti-FOXO1 (C29H4, CST) detected with anti-rabbit FITC (Southern Biotech) and anti-active caspase-3 (C92-605, BD Biosciences). Cells were filtered using 35 µm filter round-bottom FACS tubes (BD Biosciences) immediately before data acquisition on either an LSR II SORP or Fortessa (BD) and data analysed using FlowJo software (Tree Star, Inc.).
<table>
<thead>
<tr>
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<th>CONJUGATION</th>
<th>SOURCE</th>
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<td>BrdU</td>
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<td>Caspase-3 (active)</td>
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<td>-</td>
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<td>Vα2</td>
<td>B20.1</td>
<td>FITC, APC</td>
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</table>
Mouse SHM analysis

SW$_{\text{HEL}}$ GC B Cells (B220$^+$ CD45.1$^+$ Fas$^+$ CD38$^-$ IgD$^-$) were sorted from recipient mice using FACS Aria III (BD Biosciences) 13d after transfer and immunisation with HEL$^{3X_\text{SRBC}}$ and deposited as single cells in 96 well plates. The SW$_{\text{HEL}}$ heavy chain variable region was amplified from genomic DNA by nested PCR and products were sequenced and analysed.

Mouse ELISAs

Isotype specific polyclonal antibody levels from unimmunised mice and anti-HEL antibody levels in sera from immunised mice were analysed by ELISA. In brief, 384 well flat bottom plates (Nunc) were coated overnight at 4°C with specific isotype for unimmunised mice or HEL, HEL$^{2x}$ or HEL$^{3x}$ at 10μg/mL for HEL$^{2x/3x}$ immunized mice. The wells were then blocked with 1% BSA/PBS and serial dilutions of sera added together with appropriate standards. Biotinylated anti-kappa for unimmunised mice or IgG1 from HEL$^{2X/3X}$ immunised mice in 0.1% BSA/1% skim milk powder/PBS was used to detect bound antibody. SA-alkaline phosphatase in 0.1% BSA was then added and visualized with the substrate p-nitrophenyl phosphate (1mg/mL) in NPP buffer. Absorbance at 405nm was read and the concentration of isotype specific polyclonal antibodies or anti-HEL antibodies calculated from the standard curve.

Mouse epifluorescence microscopy

Spleens were snap frozen in cryomolds with OCT (Tissue Tek). 7μm sections were cut using a CM3050S cryostat (Leica), transferred to PolySine glass slides and air-dried. Cut sections were fixed in ice-cold acetone, dried and blocked with 30% horse serum (Invitrogen), 3% BSA in PBS. Sections were subsequently stained with antibodies described and visualized on a Leica DM5500 microscope. Images were compiled and brightness and contrast adjusted in Adobe Photoshop.

Mouse in vitro B cell stimulation

Lymph node cells were cultured overnight at 37°C with or without HEL (200ng/mL) in B cell medium and activation surface marker expression analysed 18 hours later by FACS analysis.

Retroviral transduction
Anti-CD40 mAb (BioXCell) and IL-4 (R & D systems) cultured SW_{HEL} spleens were retrovirally transduced with genes encoding FOXO1 or empty cassette, transferred into recipient mice and immunised with HEL^{3X}-SRBC. Donor response was analysed by flow cytometry as described above.

**Western blot**

Red blood cell lysed mouse spleenocytes were washed in chilled PBS then cell lysed with NP40 buffer with protease inhibitors, reduced with reducing buffer for 10 minutes at 70°C and western blot for STK4 (CST, 14946) and GAPDH (Santa Cruz, SC-32233) protein levels performed.

**Statistical Analysis**

Data was analysed with Prism software (GraphPad). For comparison between two normally distributed groups a one-tailed unpaired Student’s *t*-test with Welch’s correction was used, and for more than two groups we used one-way ANOVA with Tukey’s correction for multiple comparisons. Non-parametric data was analysed by Mann-Whitney *U* test. Differences between multiple paired measurements were analysed by the Wilcoxon signed-rank test. * *p < 0.05, ** *p < 0.01, *** *p < 0.001 and **** *p < 0.0001.

**Extended References**


A

Healthy Controls

STK4-deficient patients

cTfh cells (% of memory CD4+ T cells)

B

Stk4+/+

Stk4Y88del/Y88del

Tfh cells/spleen (x10^3)

C

SRBC day 7:

Ratio CD45.2/CD45.1

GC B

****

D

SRBC day 7:

Ratio CD45.2/CD45.1

MBCs

****

E

SRBC day 7:

Ratio CD45.2/CD45.1

Mixed chimeras:
50% CD45.1+ Stk4+/+
50% CD45.2+:

Stk4+/+

Stk4Y88del/Y88del

PCs

0.0

0.2

0.4

0.6

0.8

1.0
**Figure E5**

**A**

HEL\(^{2x}\)-SRBC day 7:

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<tr>
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<th>GC B cells (%)</th>
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<tbody>
<tr>
<td>DZ</td>
<td>60 ± 5</td>
<td></td>
</tr>
<tr>
<td>LZ</td>
<td>40 ± 5</td>
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</table>

**B**

HEL\(^{3x}\)-SRBC day 5-6:

<table>
<thead>
<tr>
<th></th>
<th>Caspase-3(^{+})</th>
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</thead>
<tbody>
<tr>
<td>GC</td>
<td>4.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>DZ</td>
<td>3.5 ± 0.2</td>
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</tr>
<tr>
<td>LZ</td>
<td>3.2 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

SW\_HEL donor B cells:
- Stk4\(^{+/+}\): blue
- Stk4\(^{Y88del/Y88del}\): red

**C**

HEL\(^{3x}\)-SRBC day 13:

SW\_HEL, donor B cells:
- Stk4\(^{+/+}\): orange
- Stk4\(^{Y88del/Y88del}\): blue

- IgG1
  - \(\times 10^{6}\) % GC B cells

**D**

IgG1 (% GC B cells vs. Days post HEL\(^{3x}\)-SRBC immunisation)

- Orange: Stk4\(^{+/+}\)
- Blue: Stk4\(^{Y88del/Y88del}\)
- Green: No affinity ↑ mutations
- Red: Y53D
- Black: Y53D + additional affinity ↑ mutations
- Light blue: Y53D + additional affinity ↑ mutations

Legend:
- Y53D
- Y53D + additional affinity ↑ mutations
- Y53D + additional affinity ↑ mutations
- No affinity ↑ mutations
A

SRBC day 7:

![Bar graph showing FOXO1 MFI with groups labeled Stk4 +/-, Stk4 Y88del/Y88del, and Stk4 -/- for GC B and Total B.]

B

HEL^3X-SRBC day 7:

![Bar graph showing donor % lymphocytes with groups labeled WT, Y88, WT, and Y88 for SW^HEL donor.]

Transduction:

- Empty vector
- FOXO1