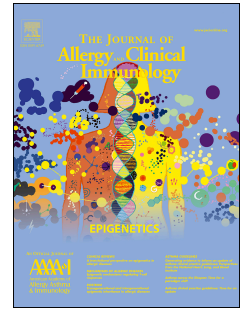


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B cell-intrinsic requirement for STK4 in humoral immunity in mice and humans

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Title page:**B cell-intrinsic requirement for STK4 in humoral immunity in mice and humans**

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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.

Capsule summary: 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.

36 **Key words:** STK4 deficiency; combined immunodeficiency disease; CRISPR/Cas9; mouse model;
37 humoral immunity; germinal centre; plasma cells; memory B cells.
38 **Abbreviations:** STK4, serine-threonine kinase 4; MST1, mammalian sterile 20-like 1; B_{mem},
39 memory B cells; GC, germinal centre; ASC, antibody-secreting cell; BCR, B cell receptor; HEL,
40 hen-egg lysozyme.

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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*^{Y88del/Y88del} and *Stk4*^{-/-} mice (Fig E1, E), confirming the p.Y88del mutation affects protein stability, resulting in *STK4* deficiency. *Stk4*^{Y88del/Y88del} and *Stk4*^{-/-} 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*_{mems}) 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*^{Y88del/Y88del} 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*^{Y88del/Y88del} and *Stk4*^{-/-} 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).

Stk4^{Y88del/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 SW_{HEL} 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 SW_{HEL} B cells on day 5 was comparable to wild-type SW_{HEL} B cells, STK4 mutant SW_{HEL} 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 SW_{HEL} 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 *Stk4*^{Y88del/Y88del} B_{mems} (Fig 2, H). However, the residual *Stk4*^{Y88del/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 *Stk4*^{Y88del/Y88del} B cells, STK4 deficiency did not quantitatively impact the ability of these SW_{HEL} B cells to generate plasma cells in vivo (Fig 2, I-K). Thus, similar to B cells from STK4-deficient

patients, *Stk4*^{Y88del/Y88del} 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*^{Y88del/Y88del} and *Stk4*^{-/-} 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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197

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 *Stk4*^{Y88del/Y88del}, *Stk4*^{-/-} and *Stk4*^{+/+} SW_{HEL} 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*^{+/+} SW_{HEL} B cells. (G) Decreased proliferation of *Stk4*^{Y88del/Y88del} compared to *Stk4*^{+/+} SW_{HEL} 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} SW_{HEL} 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.

Patient	P1 (Family 1)	P2 (Family 1)	P3 (Family 2)	P4 (Family 2)	P5 (Family 3)	P6 (Family 3)	P7 (Family 4)	P8 (Family 5)	P9 (Family 5)
Country	Australia	Australia	England	England	Turkey	Turkey	Turkey	Argentinian	Argentina
Genetics	homozygous	homozygous	homozygous	homozygous	homozygous	homozygous	homozygous	homozygous	homozygous
Mutation	p.Y88del		c.349C>T; p.R117X		c.1245delA; p.P416Lfs*4		homozygous c.1103delT; p.M368fsX369	c.343C>T; p.R115*	
Consanguinity	yes	yes	yes	yes	yes	yes	yes	no	no
Demographic	15M	10F	15F	21M	12M	14F	8F	4F	16M
Clinical features									
Eczema and skin infections	yes	yes	yes	yes	yes	yes	yes	no	no
Recurrent sinopulmonary infection	no	yes	yes	yes	yes	yes	yes	no	no
Oral candidiasis	no	no	no	yes	no	yes	no	no	no
Staphylococcal pneumonia	yes	no	no	no	no	no	no	no	no
Cryptosporidiosis	no	no	no	no	no	no	no	no	no
Allergic disease	no	yes	no	yes	yes	yes	no	no	no
Viral infections									
EBV	yes	no	yes	yes	no	no	no	yes	no
CMV	yes	no	no	no	no	no	no	no	no
Molluscum contagiosum	yes	yes	yes	yes	no	no	no	no	no
Varicella zoster virus (VZV)	yes	yes	no	yes	no	no	no	no	yes - encephalitis
Influenza	no	yes	no	no	no	no	no	no	no
Herpes simplex virus (HSV)	no	yes	no	no	yes	yes	yes	no	no
Human papillomavirus (HPV)	no	yes	no	no	no	no	no	no	yes
Autoimmunity									
ITP	yes	no	no	no	no	no	no	no	no
AIHA	yes	no	no	no	no	no	no	yes	yes
Other	no	no	no	no	no	no	no	no	no
Immunophenotyping (cells/mL)									
Total lymphocyte count	0.1	0.5	1.84	0.87	0.7	0.8	1.003	1.3	1.33
Total T cells	0.05	0.22	1.23	0.53	0.415	0.512	0.403	0.351	1.004
CD4 T cells	0.03	0.16	0.2	0.17	0.147	0.158	0.13	0.156	0.401
Naïve CD4 T cells	ND	ND	0.01	0.04	0.0966	0.0688	0.081	0.032	0.12
CD8 T cells	0.02	0.1	0.79	0.33	0.203	0.296	0.247	0.169	0.575
Naïve CD8 T cells	ND	ND	0.06	0.09	0.558	0.408	0.47	0.063	ND
B cells	0.1	0.2	0.39	0.18	0.14	0.144	0.388	0.806	0.228
Transitional B cells (% B cells)	ND	ND	17	13	25	36.5	8.5	ND	ND
Memory B cells (% B cells)	ND	ND	0.8	7	8.8	3	1.3	ND	0.147
Neutrophils	0.1	3	4.27	2.4	1.4	4.1	3.2	ND	1276
NK cells	0.04	0.06	0.18	0.06	0.077	0.144	0.265	0.074	0.08
Serum Ig levels									
IgG (g/L)	15	10	14.6	13.6	1.29	1.32	2.39	1.32	2.77
IgA (g/L)	3.5	7	0.151	0.112	0.272	0.215	0.34	0.039	0.738
IgM (g/L)	1.2	0.5	0.159	0.1	0.111	0.081	0.177	0.277	0.064
IgE (IU/L)	283	480	ND	ND	1280	569	1600	ND	1700
Specific antibody responses									
Pneumococcus	poor	intact	intact	no response	low	intact	intact	poor	absent
Tetanus toxoid	intact	intact	intact	no response	intact	poor	intact	poor	absent
Hemophilus	intact	intact	ND	ND	ND	ND	ND	ND	ND
Diphtheria toxin	intact	intact	ND	ND	ND	ND	ND	ND	ND
Isohemagglutinin	ND	ND	ND	ND	present	present	present	absent	absent
Hepatitis B surface antigen	intact	intact	ND	ND	absent	absent	absent	absent	absent
Treatment	HSCT	HSCT	IVIg	IVIg	SCIg	SCIg	SCIg	IVIg	IVIg

IVIG pre-transplant

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Bactrim
and fungal
prophylaxis

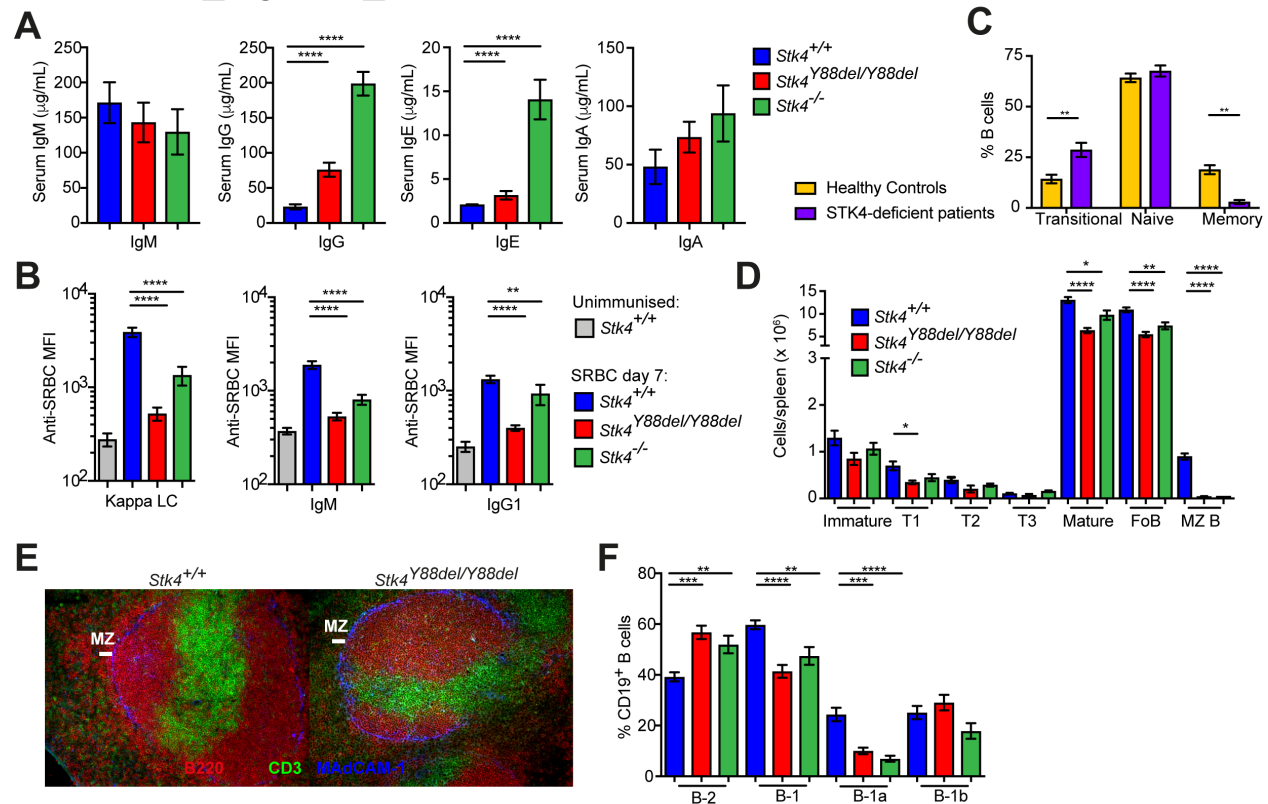
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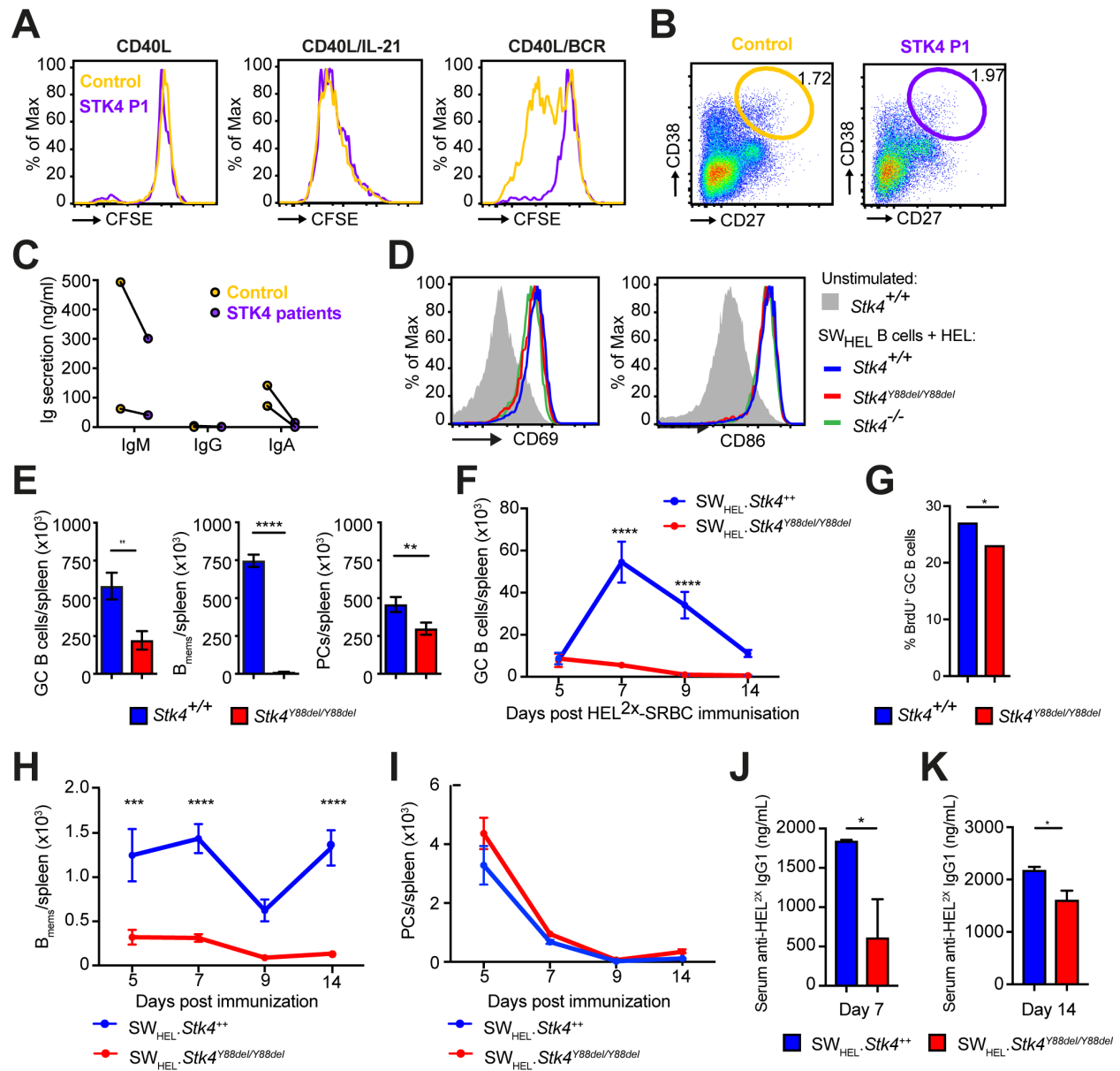
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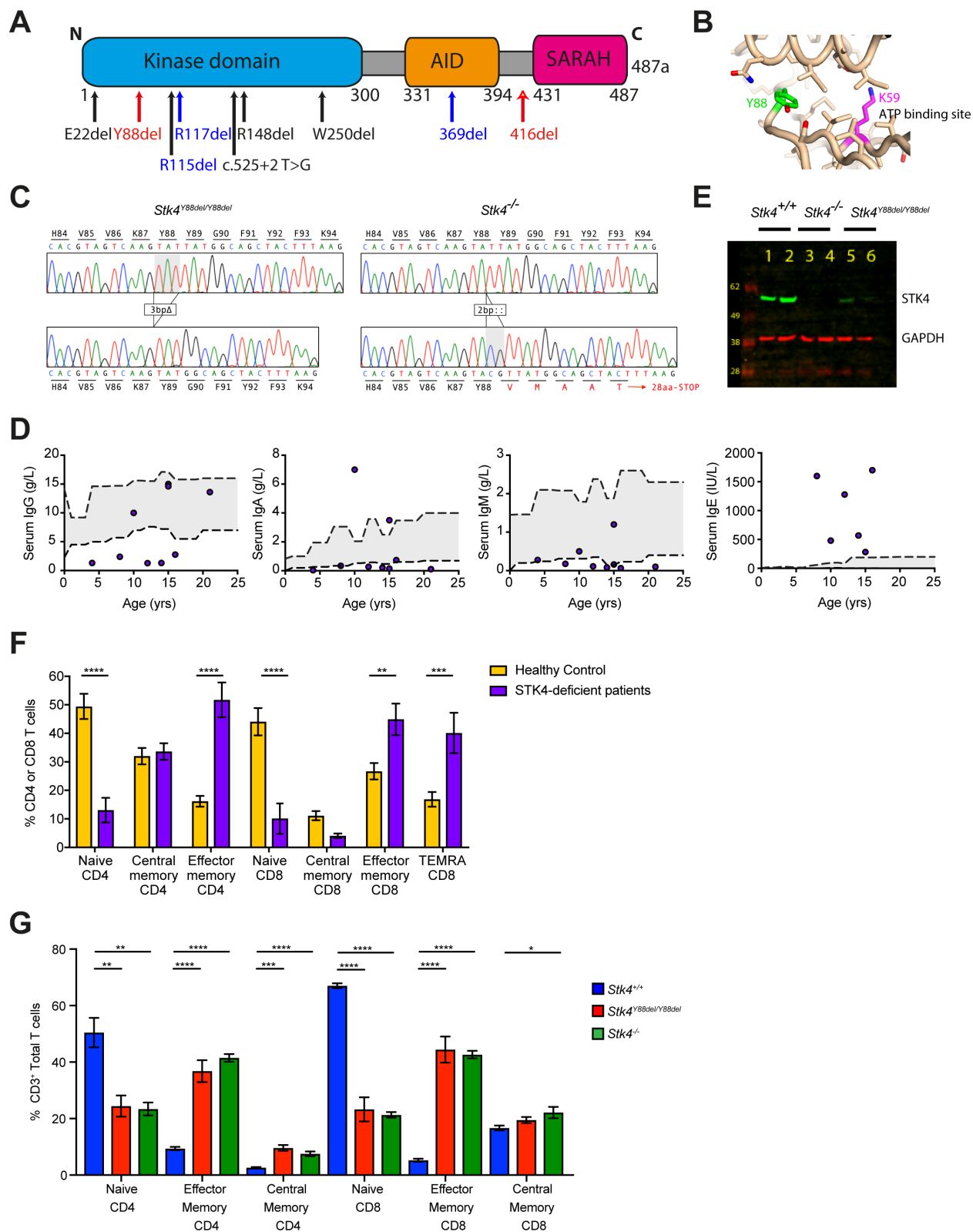
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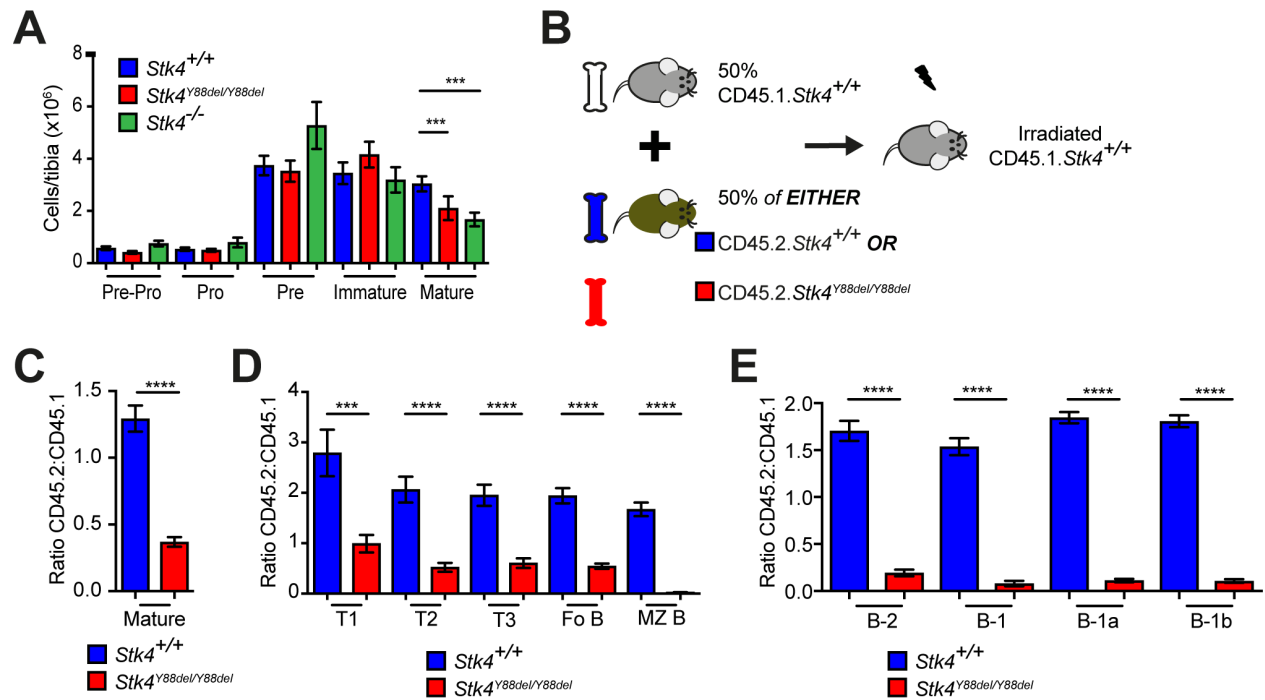
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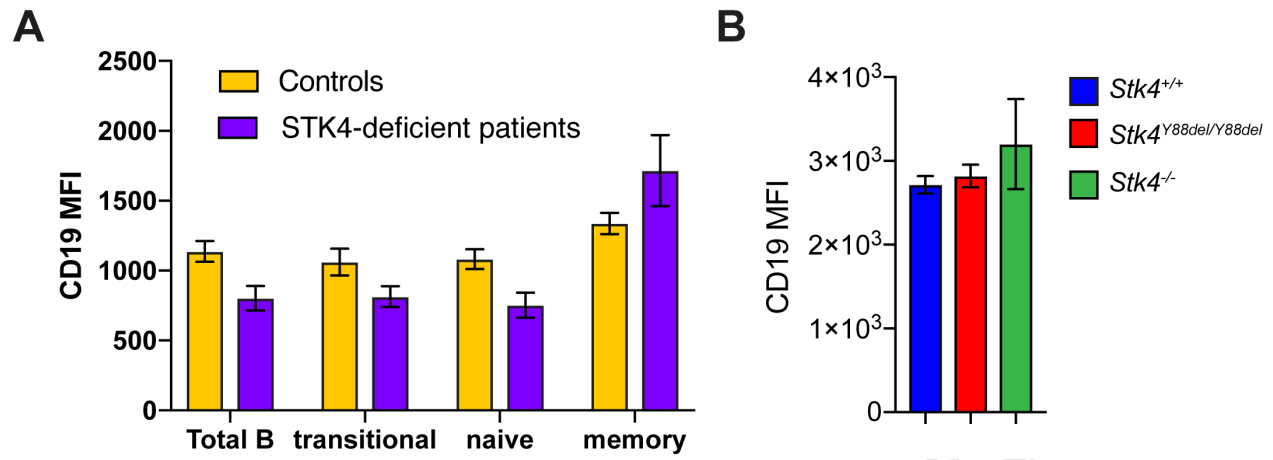
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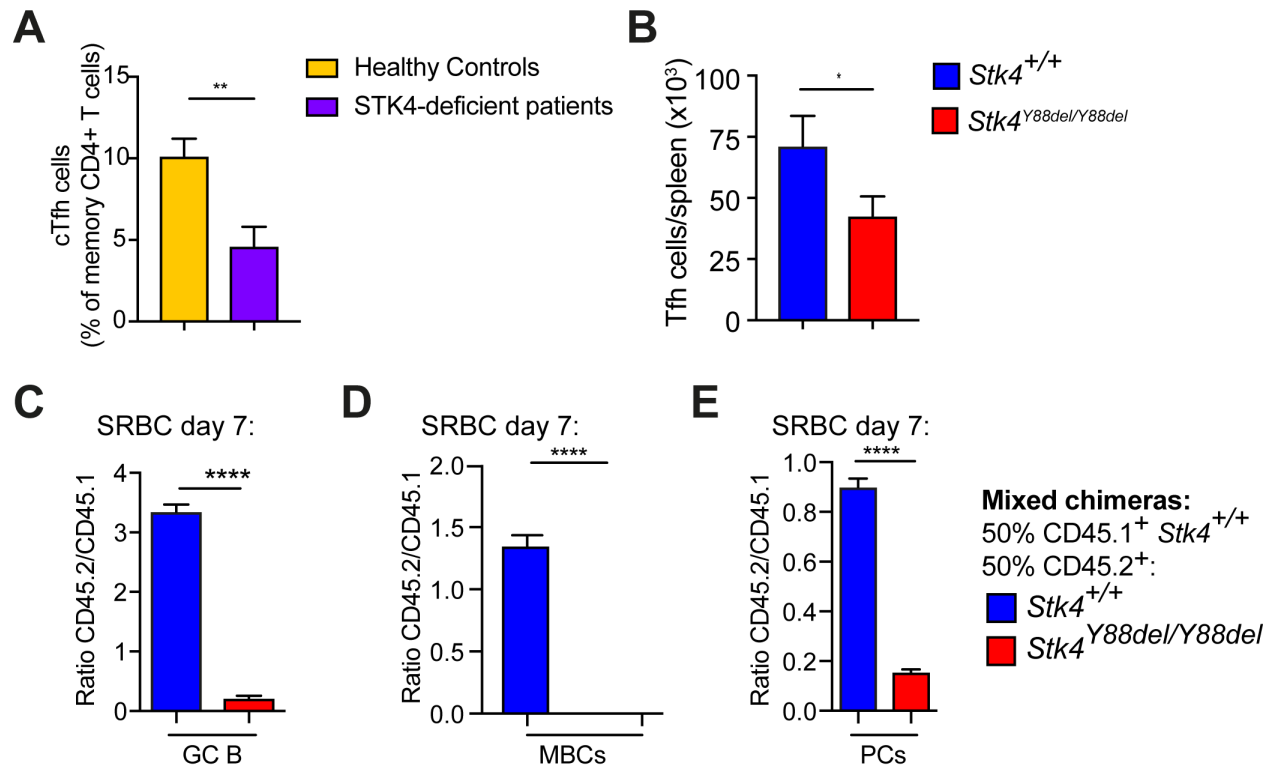


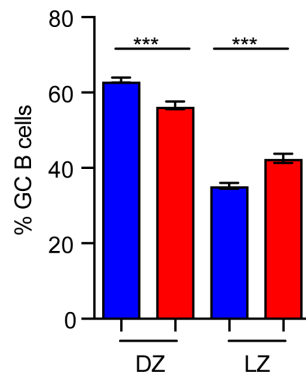
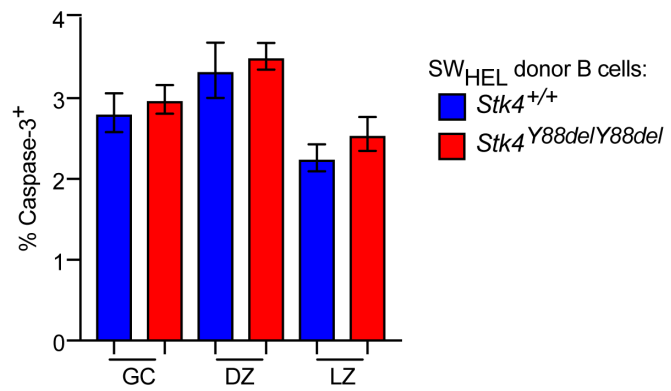
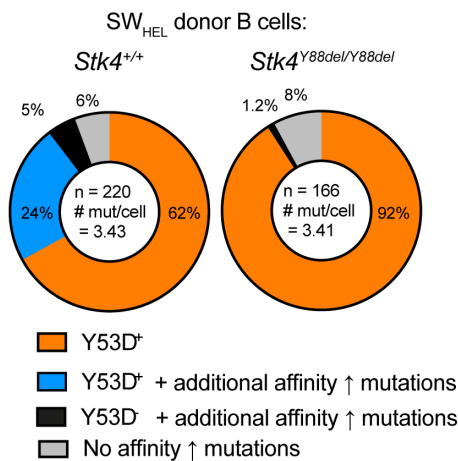
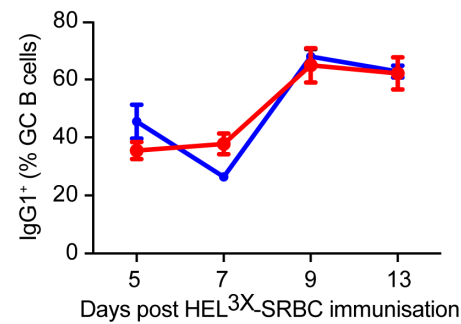


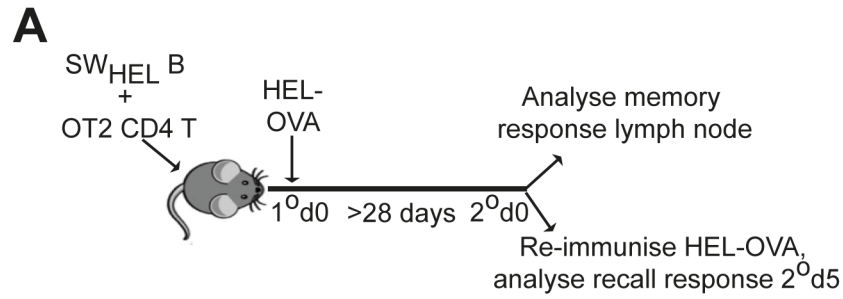




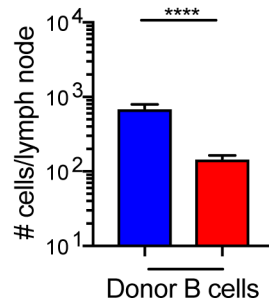




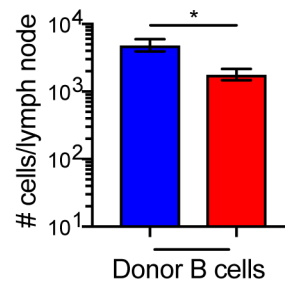
AHEL^{2X}-SRBC day 7:**B**HEL^{3X}-SRBC day 5-6:**C**HEL^{3X}-SRBC day 13:**D**



B HEL-OVA memory Lymph node:



C HEL-OVA 2⁰ day 5 Lymph node:



SW_{HEL} donor B cells:

■ *Stk4*^{+/+} ■ *Stk4*^{Y88del/Y88del}

D Memory B cell culture 7 days, CD40L/IL-21

