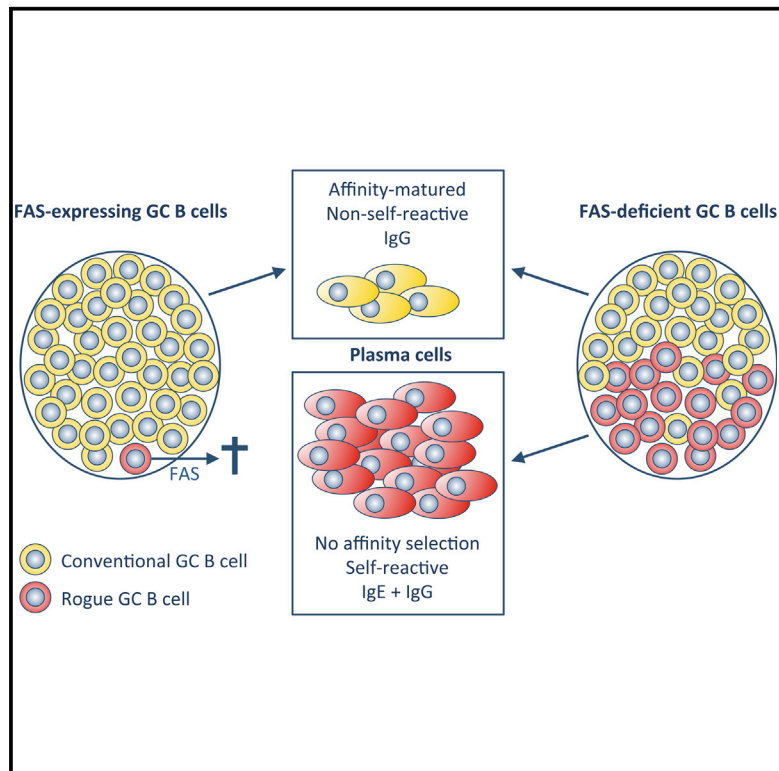


Immunity

FAS Inactivation Releases Unconventional Germinal Center B Cells that Escape Antigen Control and Drive IgE and Autoantibody Production

Graphical Abstract



Authors

Danyal Butt, Tyani D. Chan, ...,
V. Koneti Rao, Robert Brink

Correspondence

r.brink@garvan.org.au

In Brief

FAS deficiency is associated with autoimmunity. Brink and colleagues show that FAS prevents development of “rogue” germinal center (GCr) B cells that escape normal regulatory controls and produce high amounts of IgE and autoantibodies, which might explain the presence of such antibodies in autoimmune disease.

Highlights

- FAS is not required to eliminate conventional self-reactive GC B cells
- FAS is required to prevent development of rogue GC (GCr) B cells
- GCr B cells escape normal selection and show extensive plasma cell differentiation
- GCr B cells might explain the production of IgE and autoantibodies in ALPS and SLE



FAS Inactivation Releases Unconventional Germinal Center B Cells that Escape Antigen Control and Drive IgE and Autoantibody Production

Danyal Butt,^{1,2,6} Tyani D. Chan,^{1,2,6} Katherine Bourne,¹ Jana R. Hermes,¹ Akira Nguyen,¹ Aaron Statham,¹ Lorraine A. O'Reilly,^{3,4} Andreas Strasser,^{3,4} Susan Price,⁵ Peter Schofield,¹ Daniel Christ,^{1,2} Antony Basten,^{1,2} Cindy S. Ma,^{1,2} Stuart G. Tangye,^{1,2} Tri Giang Phan,^{1,2} V. Koneti Rao,⁵ and Robert Brink^{1,2,*}

¹Immunology Division, Garvan Institute of Medical Research, 384 Victoria St., Darlinghurst, NSW 2010, Australia

²St. Vincent's Clinical School, UNSW Australia, Darlinghurst, NSW 2010, Australia

³Molecular Genetics of Cancer Division, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, VIC 3052, Australia

⁴Department of Medical Biology, The University of Melbourne, Parkville, VIC 3010, Australia

⁵Molecular Development Section, Laboratory of Immunology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892, USA

⁶Co-first author

*Correspondence: r.brink@garvan.org.au

<http://dx.doi.org/10.1016/j.immuni.2015.04.010>

SUMMARY

The mechanistic links between genetic variation and autoantibody production in autoimmune disease remain obscure. Autoimmune lymphoproliferative syndrome (ALPS) is caused by inactivating mutations in *FAS* or *FASL*, with autoantibodies thought to arise through failure of FAS-mediated removal of self-reactive germinal center (GC) B cells. Here we show that FAS is in fact not required for this process. Instead, FAS inactivation led to accumulation of a population of unconventional GC B cells that underwent somatic hypermutation, survived despite losing antigen reactivity, and differentiated into a large population of plasma cells that included autoantibody-secreting clones. IgE⁺ plasma cell numbers, in particular, increased after FAS inactivation and a major cohort of ALPS-affected patients were found to have hyper-IgE. We propose that these previously unidentified cells, designated “rogue GC B cells,” are a major driver of autoantibody production and provide a mechanistic explanation for the linked production of IgE and autoantibodies in autoimmune disease.

INTRODUCTION

Production of pathogenic antibodies by B lymphocytes contributes significantly to inflammatory disease. Autoantibodies, typically of the immunoglobulin G (IgG) class, are involved in many human autoimmune diseases including systemic lupus erythematosus (SLE), bullous pemphigoid (BP), and autoimmune lymphoproliferative syndrome (ALPS). Additionally, airborne or ingested proteins can trigger the production of IgE, with resulting induction of allergic diseases such as asthma and food allergies. The processes that regulate autoantibody and allergic IgE production

are often considered separately because of the distinct nature of the antigens targeted (self versus foreign). Nevertheless, the presence of IgE as well as IgG autoantibodies in autoimmune diseases has been recognized since the 1970s (Permin and Wiik, 1978; Provost and Tomasi, 1974) and strong evidence exists that IgE autoantibodies contribute to disease pathogenesis in BP (Messingham et al., 2014) as well as human and murine SLE (Dema et al., 2014a, 2014b). However, the mechanisms underlying the production of IgE together with IgG autoantibodies remain unknown.

A key component of adaptive humoral immunity is the germinal center (GC) reaction. GCs are transient microanatomical structures in which B cells activated by foreign antigen undergo iterative cycles of proliferation and somatic hypermutation (SHM) of their Ig variable region genes to yield the high-affinity antibodies required for long-term immunity (Victora and Nussenzweig, 2012). Because of the random nature of SHM, the development of self-reactive B cells within the GC is inevitable (Nossal, 1988). We showed recently that self-reactive B cells are rapidly removed from the GC and thus prevented from undergoing differentiation into autoantibody-producing plasma cells (PCs) (Chan et al., 2012). However, this process is compromised under some circumstances as indicated by the fact that GCs are a major source of pathogenic IgG autoantibodies (Brink, 2014). In particular, mutations affecting the expression and/or function of molecules involved in the removal of self-reactive GC B cells are likely to make significant contributions to autoimmune diseases such as SLE, BP, and ALPS.

The pro-apoptotic TNF receptor superfamily member FAS is highly expressed on GC B cells (Liu et al., 1995; Smith et al., 1995). Mutations in the genes encoding FAS or its ligand (FASL) result in ALPS in humans (Fisher et al., 1995; Rieux-Laucat et al., 1995; Wu et al., 1996) and model ALPS and SLE in mice (Andrews et al., 1978; Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992). Significantly, the selective removal of FAS from activated B cells results in ALPS-like disease and autoantibody production (Hao et al., 2008). These findings have led to a widely accepted view that the autoimmune predisposition of individuals with *FAS*/*FASL* mutations derives at least in part from a

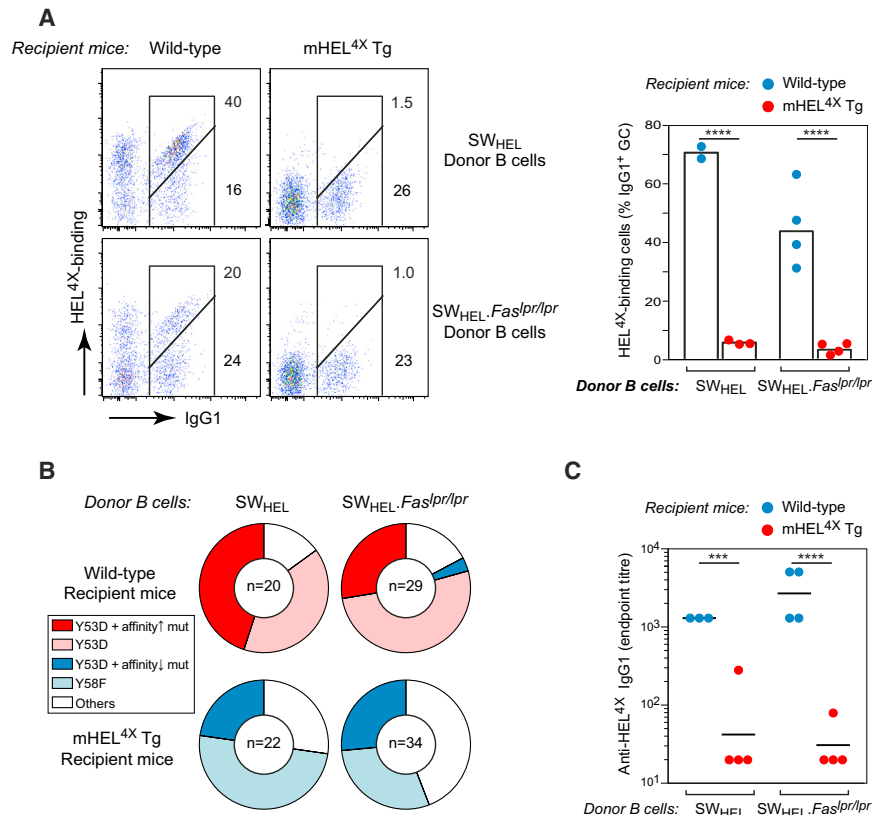


Figure 1. FAS-Mediated Apoptosis Is Not Required to Remove Conventional Self-Reactive GC B Cells

SW_{HEL} or SW_{HEL}.Fas^{Ipr/Ipr} B cells were transferred into wild-type or mHEL^{4X} Tg recipient mice, challenged with HEL^{3X}-SRBCs, and analyzed on day 21. Data are representative of three independent experiments.

(A) Left: Flow cytometric analysis of donor-derived splenic GC B cells (CD45.1⁺CD45.2⁻B220⁺CD38^{lo}). Profiles are concatenated from four mice per group. Right: Summary data from one experiment indicating the proportion of HEL^{4X}-binding cells among IgG1⁺ GC B cells using the windows shown on the flow cytometry panels.

(B) Single-cell SHM analysis of donor-derived splenic GC B cells. Mutations that increase or decrease the affinity of Y53D for HEL^{4X} are S31R/Y58F and L4F/N94K, respectively (see Figure S1). n = number of clones analyzed. Data were obtained from three pooled wild-type or mHEL^{4X} Tg recipient mice. Data from wild-type donor SW_{HEL} B cells have been previously published (Chan et al., 2012).

(C) Endpoint titers of HEL^{4X}-binding IgG1 determined by serum ELISA.

failure to eliminate self-reactive GC B cells (Ait-Azzouzene et al., 2010; Hao et al., 2008). Nevertheless, direct evidence that FAS carries out this function in the GC has remained elusive.

Here we utilize a high-resolution in vivo murine model to unambiguously identify the role of FAS in controlling GC B cells. FAS was found to be dispensable for the removal of conventional self-reactive GC B cells. FAS expression by responding B cells was instead found to be essential for preventing the emergence of a population of unconventional “rogue” GC (GCr) B cells that persist and undergo SHM and PC differentiation independent of normal antigen-dependent regulation. Surprisingly, PCs derived from GCr (PCr) B cells included a large fraction of IgE-producing cells, leading us to identify a large cohort of non-atopic human ALPS patients with hyper-IgE. A proportion of PCr B cells also developed autoreactivity and produced high titers of serum autoantibodies. The identification of GCr B cells explains how B-cell-expressed FAS safeguards against autoantibody responses. It also provides a mechanism for the previously unexplained link between IgG and IgE autoantibody production in antibody-mediated autoimmune diseases.

RESULTS

FAS Expression Is Not Required for the Deletion of Self-Reactive GC B Cells

To test whether FAS is required for the elimination of self-reactive GC B cells, we compared the in vivo responses of antigen-specific B cells that differed only in their ability to express FAS. B cells from wild-type or FAS-deficient (Fas^{Ipr/Ipr}) SW_{HEL} mice (expressing the

hen egg lysozyme [HEL] specificity of the HyHEL10 mAb) (Phan et al., 2003) were transferred into CD45 congenic recipient mice (CD45.1⁺ cells into CD45.2⁺ mice) and challenged with antigen in the form of recombinant HEL^{3X} protein (HEL^{R21Q,R73E,D101R}) coupled to sheep red blood cells (HEL^{3X}-SRBCs) (Paus et al., 2006). SW_{HEL} B cells bind HEL^{3X} with an affinity (K_a) of $\sim 10^7$ M⁻¹ but cannot recognize the closely related HEL^{4X} protein (HEL^{3X,K97R}) (Chan et al., 2012). In wild-type recipient mice, SW_{HEL} GC B cells acquire cross-reactivity with HEL^{4X} as they undergo affinity maturation to HEL^{3X} (Figure 1A). In membrane HEL^{4X} transgenic (mHEL^{4X} Tg) recipients, however, self-tolerance prevents such cells from accumulating (Figure 1A; Chan et al., 2012). When SW_{HEL}.Fas^{Ipr/Ipr} donor B cells were used, self-reactive HEL^{4X}-binding GC B cells still failed to develop in mHEL^{4X} Tg recipients (Figure 1A), and clones carrying the HEL^{4X}-reactive Y53D Ig heavy chain substitution were strongly counter-selected or edited with complementary, deleterious somatic mutations (Figures 1B and S1; Chan et al., 2012). Accordingly, anti-HEL^{4X} serum antibody was absent from mHEL^{4X} Tg recipients on day 21 of the response regardless of whether or not the donor SW_{HEL} B cells expressed FAS (Figure 1C). Thus, contrary to previous assumptions (Ait-Azzouzene et al., 2010; Hao et al., 2008), the expression of FAS by self-reactive B cells generated within the GC is not required for their elimination.

FAS-Deficient Mice and Humans Produce Large Amounts of IgE Antibodies

Although self-reactive GC B cells were removed normally, the response of SW_{HEL}.Fas^{Ipr/Ipr} B cells to HEL^{3X}-SRBCs did exhibit aberrant features. Notably, splenic basophils from recipient mice (CD45.2⁺B220⁻c-Kit⁻CD49b⁺) acquired high HEL-binding activity in recipients of SW_{HEL}.Fas^{Ipr/Ipr} B cells but not in recipients of

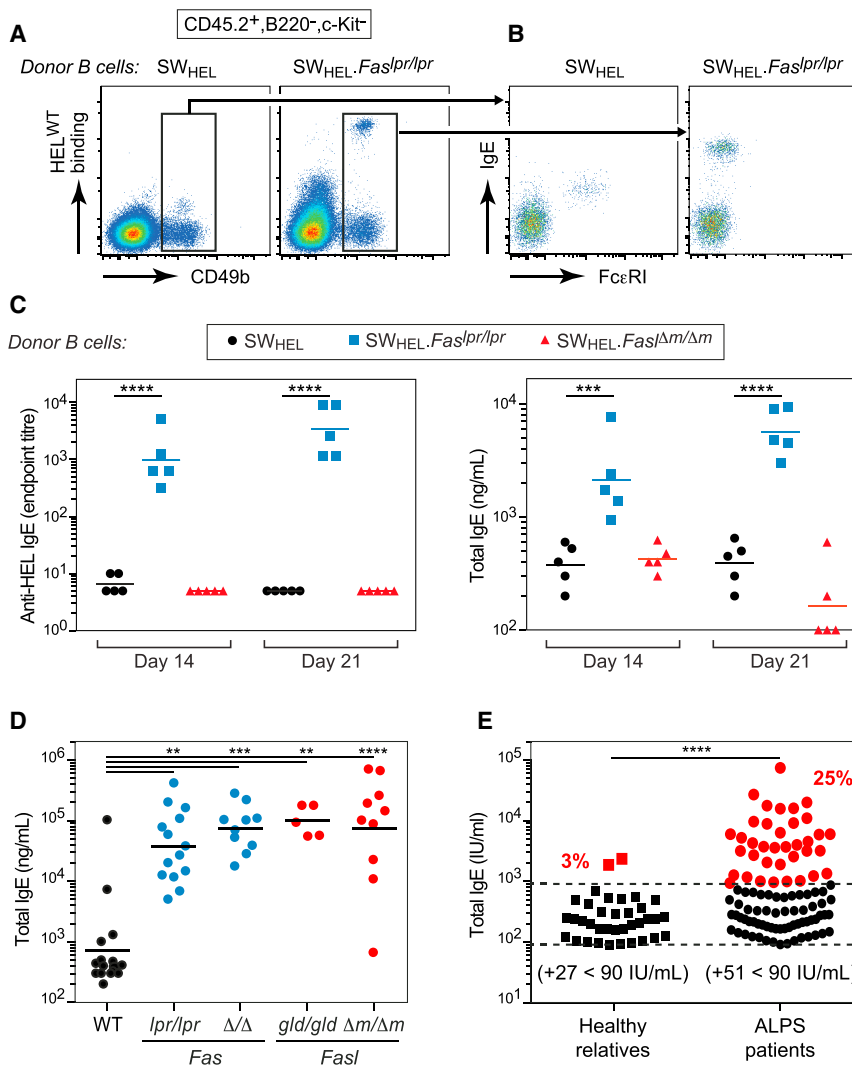


Figure 2. FASL-FAS Suppress IgE Antibody Production in Mice and Humans

(A and B) Flow cytometric analysis of endogenous splenic basophils (CD45.2⁺B220⁺c-Kit⁻CD49b⁺) in wild-type recipients of SW_{HEL} or SW_{HEL}.Fas^{lpr/lpr} B cells. Note that the anti-FcεRI mAb is blocked by receptor-bound IgE. Data are representative of two independent experiments.

(C) SW_{HEL}, SW_{HEL}.Fas^{lpr/lpr}, or SW_{HEL}.Fas^{Δm/Δm} B cells were transferred into wild-type recipient mice and challenged with HEL^{3X}-SRBCs, and sera were taken at days 14 and 21 and analyzed by ELISA. HEL^{WT}-binding IgE (left) was determined by endpoint titer and total IgE was also measured (right). Data are representative of two independent experiments.

(D) Total serum IgE in 16- to 18-week-old wild-type C57BL/6 mice compared to those lacking functional FAS (Fas^{lpr/lpr}, Fas^{Δ/Δ}) or FASL (Fas^{gld/gld}, Fas^{Δm/Δm}).

(E) Total serum IgE in ALPS patients and healthy relatives. Data points < 90 U/ml not shown.

For all figures, significant differences are indicated by asterisk(s). A full explanation of statistical analyses is available in the [Experimental Procedures](#).

wild-type SW_{HEL} B cells (Figure 2A). This occurred regardless of the recipients' expression of mHEL^{4X} (data not shown). Flow cytometric analysis via a mAb that is blocked from binding the high-affinity IgE receptor (FcεRI) by receptor-bound IgE revealed that the FcεRI on these basophils were almost completely saturated with anti-HEL IgE (Figure 2B), suggesting that SW_{HEL}.Fas^{lpr/lpr} B cells were generating large amounts of IgE antibody when challenged with HEL^{3X}-SRBCs.

To test this directly, serum antibody responses in wild-type recipient mice were analyzed on day 21 after transfer and challenge of SW_{HEL} or SW_{HEL}.Fas^{lpr/lpr} B cells. An additional group received SW_{HEL} B cells lacking the membrane form of FASL (SW_{HEL}.Fas^{Δm/Δm} B cells) (O'Reilly et al., 2009). SW_{HEL} and SW_{HEL}.Fas^{Δm/Δm} B cells challenged with HEL^{3X}-SRBCs in wild-type recipient mice failed to mount serum IgE responses (Figure 2C). In contrast, SW_{HEL}.Fas^{lpr/lpr} B cells initiated a strong response, with anti-HEL serum IgE reaching 5–10 μg/ml by day 21 (Figure 2C), verifying that B cells lacking FAS (but not membrane FASL) produce anomalously high IgE antibody responses.

A link between FAS deficiency and IgE production has been suggested from observations in Fas^{lpr/lpr} BALB/c mice (Takaha-

shi et al., 2013). We confirmed that a similar connection exists on the C57BL/6 genetic background, as shown by the fact that mice lacking active FAS due to either a natural (Fas^{lpr/lpr}) or induced (Fas^{Δ/Δ}) mutation possess serum IgE concentrations 50–100 times higher than those in wild-type controls (Figure 2D).

C57BL/6 mice in which FASL binding to FAS was disrupted by a natural mutation (Fas^{gld/gld}) or altered such that the membrane form of the ligand (essential for FAS-mediated apoptosis) can no longer be expressed (Fas^{Δm/Δm}) possessed a similar hyper-IgE phenotype (Figure 2D). Overall, these results indicated that the interaction of membrane-bound FASL with FAS expressed on responding B cells suppresses IgE antibody production in mice. Abnormally high serum IgE is not a recognized clinical feature of ALPS. However, because FAS-FASL function is highly conserved between humans and mice (Fisher et al., 1995; Rieux-Laucat et al., 1995; Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992; Wu et al., 1996), we hypothesized that a subset of ALPS-affected patients would exhibit a hyper-IgE phenotype. Strikingly, retrospective analysis of clinical data from 150 patients and 63 unaffected relatives in the National Institute of Allergy and Infectious Diseases ALPS-FAS study (Price et al., 2014) revealed that 25% of ALPS-affected patients possessed serum IgE concentrations 10–8,000 times higher than the upper limit of the normal range (90 U/ml) and only 3% of healthy relatives fell into this category (Figure 2E). Interestingly, there was no increase in the incidence of allergic diseases among ALPS-affected patients with hyper-IgE (data not shown). Taken together, these data indicate that FAS plays a critical and conserved role in limiting the production of IgE in both humans and mice.

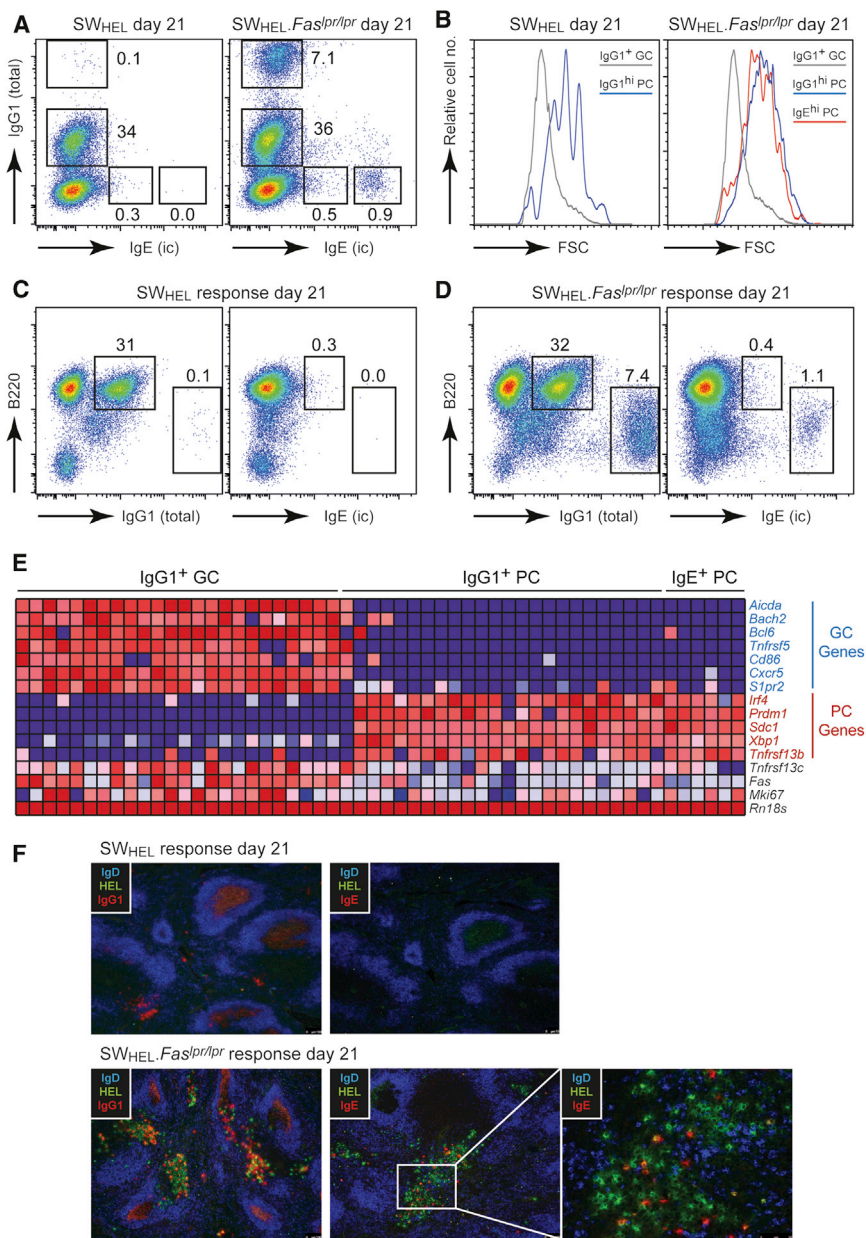


Figure 3. FAS-Deficient B Cells Generate Large Numbers of IgG1⁺ and IgE⁺ PCs

SW_{HEL} or SW_{HEL.Fas^{lpr/lpr}} B cells were transferred into wild-type recipient mice, challenged with HEL^{3X}-SRBCs, and analyzed on day 21.

(A) Flow cytometric analysis of donor-derived splenic GC B cells plus PCs (CD45.1⁺CD45.2⁻CD38^{lo}; see Figure S2A) using staining for total IgG1 and intracellular (i.c.) IgE after cell fixation and permeabilization. IgG1⁺ GC = IgG1^{lo}IgE⁻; IgG1⁺ PC = IgG1^{hi}IgE⁻; IgE⁺ PC = IgG1⁻IgE^{hi}. (B) Histogram overlay of forward light scatter (FSC) of indicated populations of donor-derived cells from (A).

(C and D) Flow cytometric analysis of donor-derived GC B cells plus PCs as for (A) showing surface B220 expression.

Plots in (A)–(D) comprise concatenated data from five mice and are representative of three independent experiments.

(E) Single-cell qPCR analysis of donor-derived subsets (see Figure S2B for gating) from splenic SW_{HEL.Fas^{lpr/lpr}} response. Relative mRNA expression is indicated from high (red) to low (blue).

(F) Immunofluorescence histology of SW_{HEL} and SW_{HEL.Fas^{lpr/lpr}} responses in recipient spleens. Colors representing the various stains are indicated.

staining GC B cell counterparts. To avoid detecting surface CD23-bound IgE, samples were blocked with unconjugated anti-IgE before cell permeabilization and intracellular IgE stores selectively stained with conjugated anti-IgE antibody (Yang et al., 2012). On day 21, wild-type SW_{HEL} B cell responses were characterized by a large IgG1⁺ GC population, small numbers of IgG1^{hi} PCs, and a virtual absence of IgE⁺ cells (Figure 3A). In contrast, the response of SW_{HEL.Fas^{lpr/lpr}} B cells included a 50- to 100-fold expansion of IgG1^{hi} PCs and the emergence of a clearly resolvable population of IgE^{hi} PCs (Figure 3A). As expected, IgE⁺ GC B cells were rare presumably due to their transient nature (Yang et al., 2012).

IgG1^{hi} and IgE^{hi} cells were verified as PCs by their larger size (forward light scatter) relative to IgG1⁺ GC B cells (Figure 3B) and their B220^{lo} phenotype (Figures 3C and 3D). Single-cell analysis (Figure S2B) of cells from the various SW_{HEL.Fas^{lpr/lpr}} B-cell-derived populations revealed gene expression profiles corresponding to those expected for GC B cells and PCs (Figure 3E). Finally, IgE⁺ cells were also located exclusively within the extrafollicular regions of the spleen (Figure 3F), consistent with their identity as antibody-secreting PCs.

IgE⁺ PCs Arise Late in the Response and Derive from GC B Cell Precursors

We next performed a kinetic analysis on the responses of SW_{HEL} B cells lacking functional FAS due to either the *Fas^{lpr}* mutation (SW_{HEL.Fas^{lpr/lpr}}) or targeted ablation of the FAS cytoplasmic

FAS-Deficient B Cells Produce Large Numbers of IgG⁺ and IgE⁺ PCs

To characterize the FAS-deficient cells responsible for IgE production, SW_{HEL} and SW_{HEL.Fas^{lpr/lpr}} B cells were challenged with HEL^{3X}-SRBCs in wild-type recipient mice, identified by flow cytometry, (Figure S2A) and analyzed for IgE expression. IgG1⁺ cells were also examined because these typically form the precursors for IgE-switched cells and IgG1 is the major Ig subclass produced after challenge of SW_{HEL} B cells with HEL^{3X}-SRBCs (Chan et al., 2009; Erazo et al., 2007; Yang et al., 2012). Because previous studies had indicated that IgE-switched B cells exist primarily as plasma cells (PCs) (Erazo et al., 2007; Yang et al., 2012), donor-derived (CD45.1⁺CD45.2⁻) B cells were stained for IgG1 and IgE after fixation and permeabilization to access intracellular antibody stores and thus resolve brightly staining PCs from their more dimly

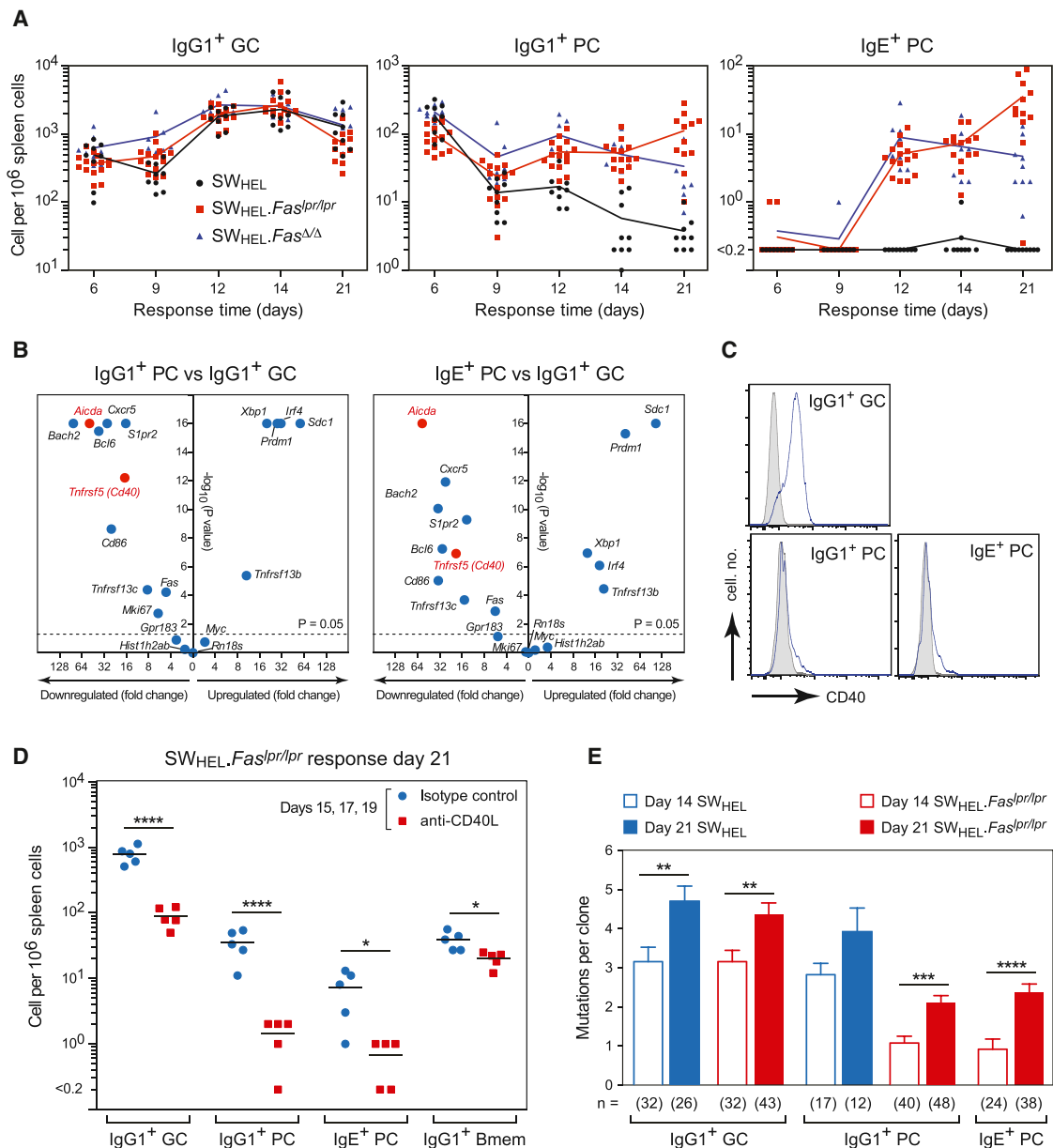


Figure 4. FAS-Deficient $IgG1^+$ and IgE^+ PCs Derive from GC B Cell Precursors

(A) Time course of donor-derived splenic responses of SW_{HEL} , $SW_{HEL}.Fas^{lpr/lpr}$, and $SW_{HEL}.Fas^{\Delta/\Delta}$ B cells. Data pooled from two independent experiments. (B) Volcano plot of qPCR data from Figure 3E ($SW_{HEL}.Fas^{lpr/lpr}$ B cell response to HEL^{3X} -SRBCs) showing fold-change and p values for individual genes between $IgG1^+$ GC B cells and $IgG1^+$ or IgE^+ PCs. (C) Overlay of CD40 staining on cells derived from day 14 splenic response of $SW_{HEL}.Fas^{lpr/lpr}$ B cells to HEL^{3X} -SRBCs. (D) Frequency of splenic donor-derived subsets from $SW_{HEL}.Fas^{lpr/lpr}$ B cells challenged with HEL^{3X} -SRBC on day 21 after 5 days treated with blocking anti-CD40L antibody or isotype control. (E) Mutation rates in the Ig heavy chain variable region gene in donor-derived splenic subsets (see Figure 3B for gating) from SW_{HEL} or $SW_{HEL}.Fas^{lpr/lpr}$ B cells challenged with HEL^{3X} -SRBCs. n = number of clones analyzed. Error bars indicate the SEM.

domain ($SW_{HEL}.Fas^{\Delta/\Delta}$). The early (day 6) response mounted by FAS-deficient and wild-type SW_{HEL} B cells included a burst of $IgG1^+$ PCs but no detectable IgE^+ PCs (Figure 4A). IgE^+ PCs did not emerge until between days 9 and 12 of the response and only when responding B cells lacked functional FAS. The appearance of IgE^+ PCs corresponded with a second wave of $IgG1^+$ PCs from FAS-deficient SW_{HEL} B cells and a ~5-fold

expansion in $IgG1^+$ and total GC B cells (Figures 4A and S3A). This late emergence of IgE^+ PCs in parallel with GC expansion suggested that IgE^+ PCs are derived from GC B cell precursors rather than from persistence of early GC-independent plasmablasts (Chan et al., 2009; William et al., 2002).

To test this proposition, we first examined the effect of ablating the GC B cell response with anti-CD40L mAb (Han et al., 1995a).

Because the PCs produced by $SW_{HEL}.Fas^{lpr/lpr}$ B cells do not express CD40 (*Tnfrsf5*) (Figures 3E, 4B, and 4C), they should be resistant to CD40L blockade if they represent a persisting autonomous population derived from the early plasmablast response. On the other hand, if they are continually replenished from GC precursors, they will be depleted in parallel with GC B cells. Examination of the day 21 $SW_{HEL}.Fas^{lpr/lpr}$ B cell response to HEL^{3X}-SRBCs after 5 days of treatment with anti-CD40L showed that the latter scenario applied, with the numbers of GC B cells as well as IgG1⁺ and IgE⁺ PCs in each case depleted by ~10-fold (Figure 4D). Further confirmation that these PCs are replenished from GC precursors was provided by single-cell sorting (Figure S3B) and SHM analysis. As predicted, both IgG1⁺ and IgE⁺ PCs were somatically mutated and showed increased SHM frequency over the course of the $SW_{HEL}.Fas^{lpr/lpr}$ B cell response (Figure 4E). Because the PCs produced by $SW_{HEL}.Fas^{lpr/lpr}$ B cells do not express AID (*Aicda*) (Figures 3E and 4B), they could not undergo SHM autonomously but must be replenished from AID-expressing, somatically mutating GC B cell precursors.

FAS-Deficient GC B Cells Escape Normal Selection and Produce IgG⁺ and IgE⁺ PCs

The production of PCs from GC B cell precursors is normally tightly regulated such that only those clones with the highest affinity for antigen are selected to undergo PC differentiation (Phan et al., 2006). This is evident in day 21 responses of SW_{HEL} B cells to HEL^{3X}-SRBCs where, similar to IgG1⁺ GC B cells, IgG1^{hi} PCs bind large amounts of HEL^{3X} when stained with limiting concentrations of antigen (Figure 5A). They also invariably carry the high-affinity Ig heavy chain substitution Y53D, often with the additional affinity-enhancing substitutions S31R and/or Y58F (Figure 5C; Chan et al., 2012; Phan et al., 2006). In contrast, very few of the IgG1^{hi} and IgE^{hi} PCs produced during the $SW_{HEL}.Fas^{lpr/lpr}$ B cell response bound HEL^{3X} with high affinity (Figure 5B) or carried mutations normally associated with affinity maturation to HEL^{3X} (Y53D, S31R, Y58F) (Figure 5D). Paradoxically, affinity maturation of $SW_{HEL}.Fas^{lpr/lpr}$ GC B cells progressed relatively normally, with most developing high affinity for HEL^{3X} as indicated by flow cytometry (Figure 5B) and SHM analysis (Figure 5D). Absent FAS expression on responding B cells therefore disrupts the normal regulation of PC differentiation, favoring the production of PCs from “rogue,” non-affinity regulated precursors that must exist at relatively low frequencies within the GC.

To investigate this further, we first bred $SW_{HEL}.Fas^{lpr/lpr}$ mice onto a $Rag1^{\Delta\Delta}$ background to eliminate the possibility that these rare GC B cells might express an alternative BCR. B cells from $SW_{HEL}.Fas^{lpr/lpr}.Rag1^{\Delta\Delta}$ mice responded identically to those from $SW_{HEL}.Fas^{lpr/lpr}$ mice, generating expanded numbers of somatically mutated IgG1⁺ and IgE⁺ PCs that showed little evidence of affinity maturation to HEL^{3X} (Figures S4C and S4D). SHM analysis of donor-derived spleen cells from mice given $SW_{HEL}.Fas^{lpr/lpr}.Rag1^{\Delta\Delta}$ B cells and HEL^{3X}-SRBCs revealed that IgG1⁺ and IgE⁺ PC populations were both clonally restricted and clonally related within individual recipient mice (Figures 5E and S5). IgG1⁺ GC B cells clonally related to these PCs were rare and typically represented only 1%–3% of GC B cells (Figures 5E and S5) but could be enriched by isolation of IgG1⁺ GC B cells with low affinity for HEL^{3X}. By this approach, sufficient numbers of related GC clones were obtained to facilitate a

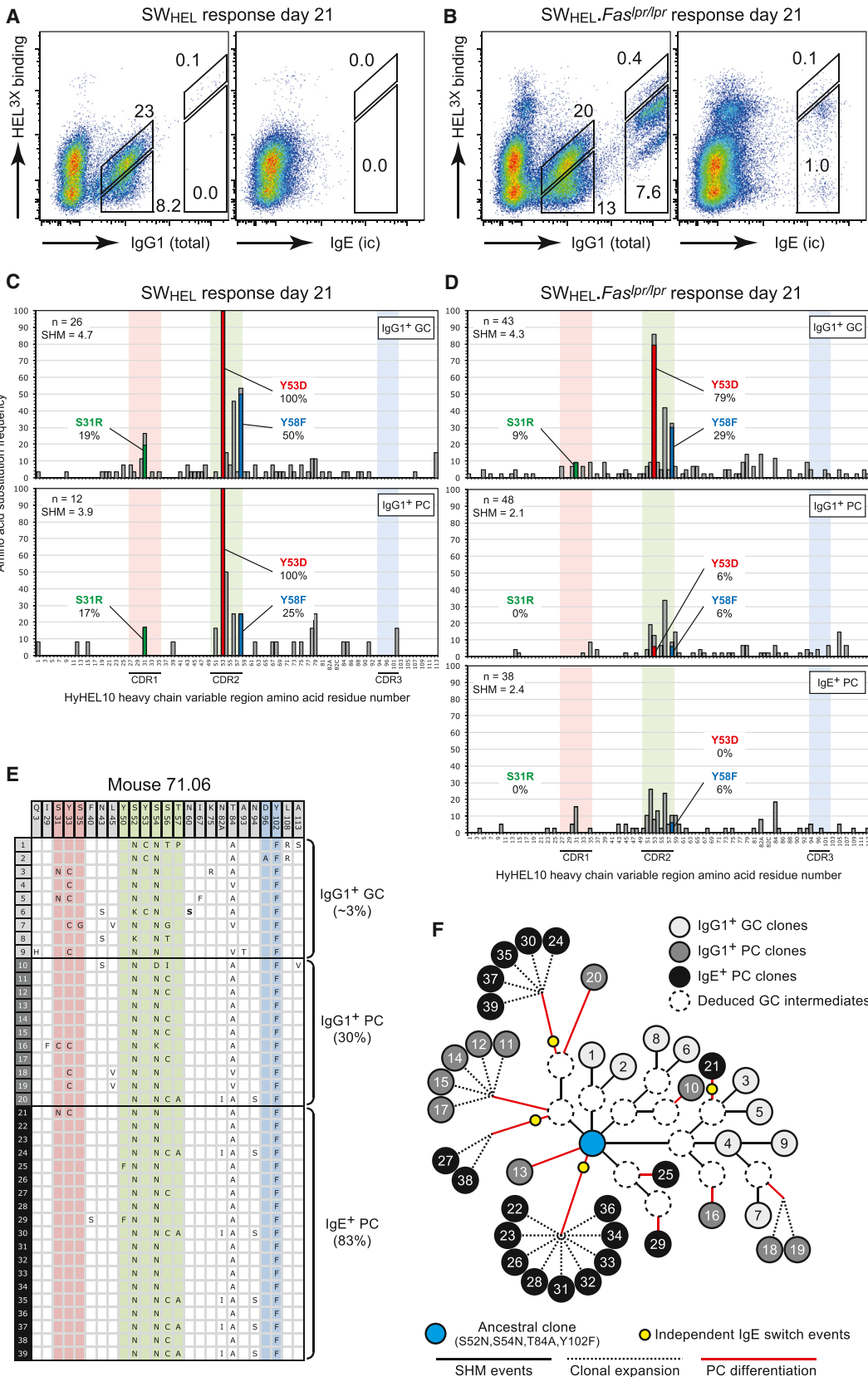
detailed analysis of the clonal evolution of a family of GC B cells and their PC derivatives (PCr). This analysis revealed that the generation of PCr from GC B cells is an ongoing process in which multiple PC differentiation and IgE switching events occur (Figure 5F). Moreover, as predicted by their abnormal SHM patterns and low HEL^{3X}-binding activity, mAbs carrying the variable regions expressed by these clones bound HEL^{3X} antigen with low and often biologically irrelevant ($K_a < 10^5 \text{ M}^{-1}$) affinities (Figure S5A). Unlike the SHM patterns that arise during affinity maturation, the amino acid substitutions observed among rogue FAS-deficient clones were unique to individual mice (Figures 5E and S5). Nevertheless, PCr from individual mice were never observed to be from just a single clonal family (e.g., Figure S5B), indicating that these cells derive from multiple ancestral clones and possibly distinct GCs.

GCr B Cells Accumulate and Generate Late-Response, Autoantibody-Secreting PCs

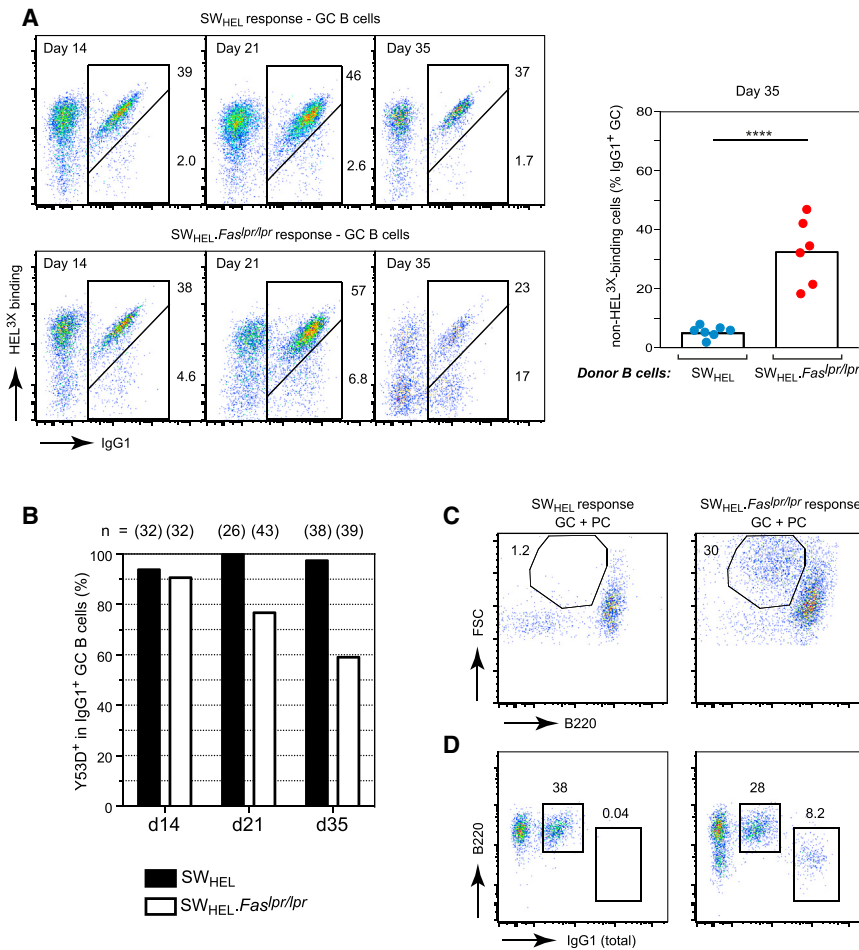
Because GC B cells exhibit ongoing SHM and PC differentiation and are not subject to conventional antigen regulation, we postulated that the emergence and accumulation of such cells might be responsible for the production of autoantibodies that ensues when B cells lack functional FAS. To test this possibility, we first examined the extent to which GC B cells accumulate over the course of FAS-deficient B cell responses. As previously observed (Figures 5C and 5D), early GC B cell affinity maturation occurs regardless of B cell FAS expression, with >90% of both SW_{HEL} and $SW_{HEL}.Fas^{lpr/lpr}$ GC B cells exhibiting high affinity for HEL^{3X} as well as the Y53D substitution on day 14 of the response (Figures 6A and 6B). As expected, high-affinity wild-type SW_{HEL} GC B cell clones remained dominant until at least day 35 of the response. In contrast, low-affinity and Y53D[−] clones progressively accumulated in GCs derived from $SW_{HEL}.Fas^{lpr/lpr}$ GC B cells, resulting in a “heretical” reversal of affinity maturation between days 14 and 35 of the response (Figures 6A and 6B). Correspondingly, sequence analysis of donor-derived cells from single recipient mice at late time points revealed increased clonal restriction within the GC such that clear groups of related (Y53D[−]) GCr clones with corresponding populations of expanded PCr often reached 10%–15% of the GC by days 35–42 (Figure S6) as opposed to 1%–3% on day 21 (Figures 5 and S5). Taken together, these data indicate that GCr B cells become increasingly prevalent over the course of responses mounted by $SW_{HEL}.Fas^{lpr/lpr}$ B cells.

We next determined whether the proclivity of GCr B cells to differentiate into PCs evident on day 21 was maintained at later stages of the response. Analyses performed on day 35 showed that, although wild-type SW_{HEL} PCs were completely absent by this time, a large fraction of both the total and IgG1⁺ splenic response of $SW_{HEL}.Fas^{lpr/lpr}$ donor B cells was still comprised of PCs (Figures 6C and 6D). These cells showed the characteristic clonal restriction (Figure S6) and lack of antigen binding (data not shown) of PCr. We therefore concluded that GCr B cells not only persist and form an increasingly large fraction of the GC response of FAS-deficient B cells but that they continue to produce antibody-secreting PCr long after the conventional PC production has shut down.

Finally, we tested whether the ongoing response by FAS-deficient GCr B cells results in the eventual production of



(legend on next page)



autoantibodies. To do this, we returned to the HEL^{4X} Tg transfer system utilized at the beginning of this study and examined whether self-tolerance, intact during the early response of SW_{HEL}.Fas^{Ipr/Ipr} B cells in HEL^{4X} Tg recipient mice (Figure 1), might break down at later stages when GC and PCr become prominent. Measurement of serum anti-HEL^{4X} autoantibody concentrations revealed that they remained undetectable in HEL^{4X} Tg recipient mice over the course of the response of wild-type SW_{HEL} B cells to HEL^{3X}-SRBCs (Figure 7A). In contrast, anti-HEL^{4X} autoantibodies accumulated in HEL^{4X} Tg recipients of SW_{HEL}.Fas^{Ipr/Ipr} B cells such that, by day 35, the majority of mice exhibited high titers of serum anti-HEL^{4X} IgG1

chain variable regions of these clones revealed the clonal restriction patterns typical of PCr but including substitutions (Y53D, Y53N, Y58F) known to increase the affinity of HyHEL10 for HEL^{4X} (Figure S7). Accordingly, the core mutations present in each of three independent clonal PCr families identified in separate HEL^{4X} Tg recipients were verified to possess affinities (K_a) for HEL^{4X} ranging from $\sim 10^7$ to more than $5 \times 10^8 \text{ M}^{-1}$ (Figure 7D). Overall, these results demonstrate that B cell FAS is not required for the removal of conventional self-reactive GC B cells but is absolutely necessary to prevent the development and persistence of GC B cells that escape normal antigen regulation and initiate IgE and autoantibody production.

Figure 5. FAS-Deficient IgG1⁺ and IgE⁺ PCs Develop from GC B Cells that Escape Normal Antigen Regulation

(A–D) SW_{HEL} or SW_{HEL}.Fas^{Ipr/Ipr} B cells were challenged with HEL^{3X}-SRBCs as for Figure 3.

(A and B) Flow cytometric analysis as for Figures 3C and 3D except counterstained for binding of limiting (50 ng/ml) HEL^{3X} antigen to identify cells expressing high-affinity antibodies. Plots comprise concatenated data from five mice and are representative of three independent experiments.

(C and D) Ig heavy chain variable region replacement mutations in responding cell subsets. HEL^{3X} affinity-increasing mutations Y53D, S31R, and Y58F are highlighted. n, number of clones analyzed; SHM, mean number of mutations per clone.

(E) Ig heavy chain variable region replacement mutations in clonally related cells obtained from a single wild-type recipient mouse (71.06) on day 21 of the response of SW_{HEL}.Fas^{Ipr/Ipr}.Rag1^{Δ/Δ} B cells to HEL^{3X}-SRBCs. Percentages indicate the frequency of the related clones within the respective responder populations. IgG1⁺ GC clones include 2/58 from unfractionated cells and 4/40 from the 20% of cells with the lowest HEL^{3X}-binding activity.

(F) A common ancestral GC clone carrying the S52N, S54N, T84A, and Y102F substitutions diversified into all 39 clones identified in (E) via the indicated SHM, clonal expansion, and PC differentiation steps (SHM was assumed to be limited to GC B cells). Four independent IgE switching events were identified (yellow circles).

Figure 6. GC B Cells Accumulate, Display Abnormal Antigen Selection, and Continue to Generate PCr

SW_{HEL} or SW_{HEL}.Fas^{Ipr/Ipr} B cells were challenged with HEL^{3X}-SRBCs in wild-type recipients as for Figure 5. Splenocytes were harvested at the indicated time points.

(A) Left: Cell surface staining with limiting (50 ng/ml) HEL^{3X} antigen to distinguish high- and low-affinity GC B cells. Right: Summary data from two experiments (combined) indicating the proportion of non-HEL^{3X}-binding cells among IgG1⁺ GC B cells on day 35 using the windows shown on the flow cytometry panels.

(B) SHM analysis of IgG1⁺ GC B cells for clones carrying the high-affinity anti-HEL^{3X} Y53D substitution. n = number of clones analyzed.

Late responses of SW_{HEL}.Fas^{Ipr/Ipr} B cells show anomalous accumulation of low-affinity (A) and Y53D⁺ (B) GC B cells.

(C and D) Identification of B220^{lo} PCs in CD38^{lo} donor-derived cells on day 35 response of SW_{HEL}.Fas^{Ipr/Ipr} but not SW_{HEL} B cells as revealed by high forward light scatter (FSC) (C) and high total IgG1 staining after fixing and permeabilization (D).

(Figure 7A) and IgG2b (data not shown; anti-HEL^{4X} IgE was not detectable). Verification that PCr were responsible for this production of anti-HEL^{4X} autoantibodies was obtained by the identification of donor-derived, HEL^{4X}-binding, IgG1⁺ and IgG1[−] PCs (Figures 7B and 7C). Single-cell sorting and analysis of the heavy

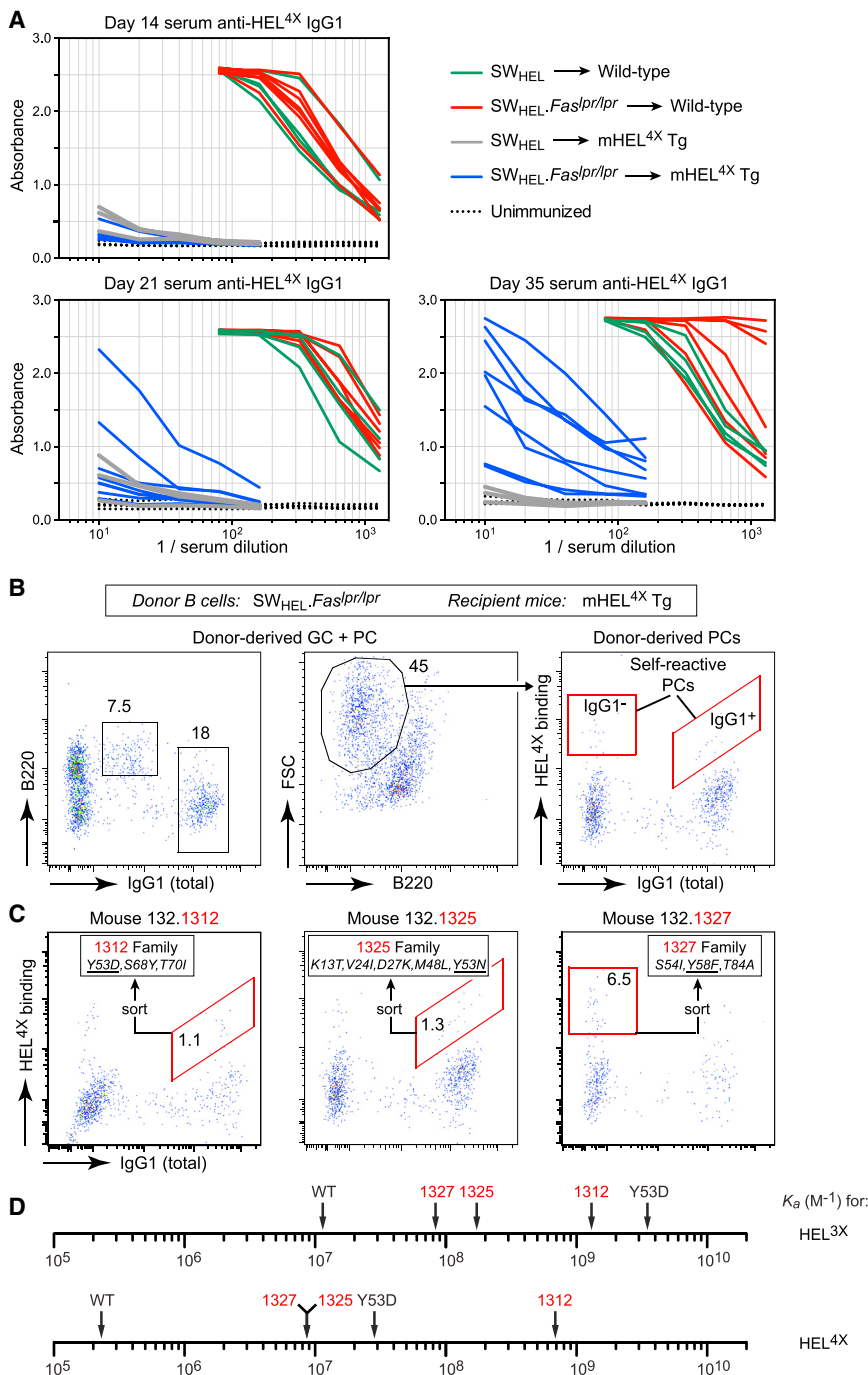


Figure 7. GCr B Cells Produce Autoantibody-Secreting PCr Late in the Response of SW_{HEL}.Fas^{lpr/lpr} B Cells to HEL^{3X}-SRBCs

SW_{HEL} or SW_{HEL}.Fas^{lpr/lpr} B cells were challenged with HEL^{3X}-SRBCs in wild-type or mHEL^{4X} Tg recipients as for Figure 1. Splenocytes and blood were harvested at the indicated time points.

(A) HEL^{4X}-binding IgG1 serum antibodies were measured by ELISA and reveal the absence (day 14), the initial emergence (day 21), and the accumulation (day 35) of autoantibodies in mHEL^{4X} Tg recipients of SW_{HEL}.Fas^{lpr/lpr} but not SW_{HEL} B cells.

(B and C) Detection of self-reactive PCs in mHEL^{4X} Tg recipients of SW_{HEL}.Fas^{lpr/lpr} B cells on day 35 of the response to HEL^{3X}-SRBCs.

(B) Donor-derived CD38^{lo} cells analyzed for HEL^{4X}-binding and total IgG1 after fixing and permeabilization showing IgG1⁺ and IgG1⁻ self-reactive PCs.

(C) Independent mHEL^{4X} Tg recipients showing self-reactive PC populations subjected to single-cell sorting and SHM analyses. Core mutations in each family of clones are shown and substitutions known to increase affinity for HEL^{4X} underlined.

(D) Binding affinities of recombinant HyHEL10 IgG1 carrying the core mutations from the clone families identified in (C) for foreign (HEL^{3X}) and self (HEL^{4X}) antigens. See Figure S7 for further details.

On the other hand, FAS-deficient mice have been found to display aberrant GC B cell selection (Hoch et al., 2000; Takahashi et al., 2001), to develop self-reactive B cells in certain models (Ait Azzouzene et al., 2010), and to produce autoantibodies when FAS inactivation is restricted to B cells (Hao et al., 2008). A major aim of the current study was to gain new perspectives on this unresolved issue by using a high-resolution in vivo mouse model to provide a detailed and comprehensive analysis of how the responses of antigen-specific B cells are affected when they specifically lack FAS. The results not only help reconcile many of these previous findings but also point to a new class of activated lymphocyte, the rogue GC (GCr) B cell, as a potent source of IgE and autoantibody production and the *raison d'être* for FAS expression in the GC.

DISCUSSION

Two decades have passed since the first studies demonstrated that FAS expressed on activated human and mouse B cells can mediate their death (Garrone et al., 1995; Rathmell et al., 1995). Nevertheless, the precise role of FAS in regulating in vivo B cell responses and particularly the GC reaction has been controversial. It has been concluded that FAS has no significant role to play in the GC (Smith et al., 1995) and is not required to remove self-reactive GC B cells (Alabyev et al., 2008; Han et al., 1995b).

The initial result of this study was that FAS was not required to remove self-reactive GC B cells. This is in agreement with the interpretations of some (Alabyev et al., 2008; Han et al., 1995b) but not other (Ait Azzouzene et al., 2010; Hao et al., 2008) earlier investigations. Although it could be argued that different results might be obtained in different systems, the question of whether FAS is required to maintain GC B cell self-tolerance can also be answered differently depending on the readout of tolerance that is employed. In our study, FAS was clearly not required for the removal of conventional GC B cells that acquire self-reactivity

against mHEL^{4X}. However, looking later in the response, it was equally clear that serum anti-HEL^{4X} autoantibodies accumulated to high titers specifically in recipients of FAS-deficient B cells. These results could be reconciled by the fact that a previously unknown population of cells, GC_r B cells, emerged over the course of the response of FAS-deficient B cells. Because these cells do not follow the same rules of selection as their conventional counterparts, SHM ultimately leads to the unchecked production of autoantibody-secreting PC_r and the breakdown of self-tolerance. Thus, although one mechanism of self-tolerance remains intact when GC B cells lack FAS, another fails, which might well explain the previous confusion over the role of FAS in maintaining GC B cell self-tolerance.

A major question to arise from this study is what drives the survival and accumulation of GC_r B cells? These cells are not dependent on recognition of the immunizing antigen and so differ fundamentally from conventional GC B cells. Could they instead be selected on the basis of interactions with certain self-antigens? From our analysis of the specificities encoded by GC_r and PC_r B cells, it seems unlikely that specific self-antigens drive the accumulation of these cells. Thus, although there are strong clonal relationships within individual recipients of SW_{HEL}.Fas^{lpr/lpr} B cells, recurring amino acid substitutions (such as those seen in affinity maturation to HEL^{3X}) are not observed between recipients. An alternative possibility is that GC_r B cells exist because they have acquired mutated antigen receptors that undergo some form of structural change (e.g., auto-aggregation) that results in their “selection” independent of antigen recognition. In this case, the acquisition of self-reactivity by rogue cells might be incidental but (at a population level) highly likely based on their ongoing SHM. Finally, the restricted clonality of GC_r B cells might be explained by an ancestral clone acquiring a mutation in a non-Ig gene (e.g., by off-target SHM) that allows the clone and its daughter cells to be perpetuated without retaining antigen specificity. Here again, self-reactivity might arise as a consequence of ongoing SHM being uncoupled from the need to maintain recognition of the original immunizing antigen. This raises the question of how T cells, presumably GC-localized T_{fh} cells, could be required to maintain GC_r B cells as is indicated by our CD40L-depletion data? One possibility is that they are able to maintain the peptide-MHC complexes as they continue dividing without requiring ongoing antigen contact (Thaumat et al., 2012). Alternatively, they might be able to persist on the basis of “bystander” help that they can access without undergoing cognate interactions with T_{fh} cells.

Whatever the specific mechanism is that drives the production of GC_r B cells, the fact that it does occur as part of normal GC activity explains the need for GC B cells to express FAS. It is unclear at this point whether a specific cell type provides the FASL required to prevent the development of GC_r B cells. Our data show that it is not an autocrine action of activated B cells expressing FASL (Hahne et al., 1996). However, FASL could instead derive from neighboring B cells, follicular dendritic cells (Verbeke et al., 1999), T_{fh}/T_{fr} CD4⁺ cells (Rathmell et al., 1995), or even CD8⁺ T cells (Afshar-Sterle et al., 2014). What feature of GC_r B cells makes them particularly susceptible to the signals delivered through FAS is another question that requires further investigation. Until more functional and phenotypic data have been obtained, we propose that GC_r B cells be defined as a

rare subpopulation of GC B cells that (1) accumulate late in the GC response when FAS expression or function is impaired, (2) do not require ongoing antigen binding to survive, and (3) show a high propensity for PC differentiation including unusually large numbers of IgE⁺ PCs.

Another context in which FAS has been identified to regulate autoantibody production is the accumulation of extrafollicular PCs that undergo chronic stimulation and GC-independent SHM in response to autoantigens such as rheumatoid factor (RF) (William et al., 2002). We determined that the accumulation of SHM in the extrafollicular PCs derived from SW_{HEL}.Fas^{lpr/lpr} B cells does not occur via this mechanism because (1) the PC population we observed did not express AID, (2) unlike the anti-RF PCs (Herlands et al., 2008), the PC population we observed did depend on ongoing T cell help, and (3) clear clonal precursor-product relationships existed between the GC and PC populations in our model.

An unexpected finding was that GC_r B cells act as a source of IgE-secreting PCs that emerge when responding B cells lack functional FAS. The close clonal relationships between IgG1⁺ and IgE⁺ PCs in single recipient mice and evidence of multiple IgE switching events indicate that most IgE⁺ PCs are generated from IgG1⁺ GC B cells, potentially via a transient IgE⁺ GC B cell intermediate (Erazo et al., 2007; Xiong et al., 2012; Yang et al., 2012). Interestingly, human allergic and non-allergic IgE antibodies often exhibit evidence of reduced antigenic selection (Dahlke et al., 2006; Wang et al., 2014), consistent with IgE in these cases being derived at least in part from GC_r B cell clones such as those described here.

The greatly elevated concentrations of serum IgE detected in a major cohort of ALPS patients is consistent with the properties of the GC_r B cells that emerge when FAS is inactivated. The lack of allergic disease among these patients also fits this scenario and points to a link between IgE production and autoimmunity that is independent of allergy. Further evidence for this phenomenon comes from the recent finding that a majority of SLE patients display IgE autoantibodies (Dema et al., 2014b). Similarly, investigations in a mouse model of SLE have demonstrated a role for IgE in disease progression (Dema et al., 2014a) and IgE autoantibodies have a clear pathogenic role in BP (Messingham et al., 2014). Our findings therefore provide a mechanistic basis for the link between IgE antibody production and autoimmune diseases. We propose that the emergence of GC_r B cells, through mutation of FAS or potentially other immunoregulatory disruptions, represents a common phenomenon in autoimmune disease that can not only drive the production of IgG autoantibodies but support the parallel production of pro-inflammatory IgE that might amplify disease. Further investigation of the signals that drive GC_r B cells might therefore reveal new strategies for managing antibody-mediated autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice and Adoptive Transfers

SW_{HEL} mice (Phan et al., 2003) were maintained on a CD45.1 congenic (*Ptprc*^{a/a}) C57BL/6 background and mHEL^{4X} transgenic mice (Chan et al., 2012) on a C57BL/6 background. All other mouse strains were C57BL/6 and are referenced in Supplemental Experimental Procedures. For adoptive transfers, spleen cells from donor SW_{HEL} mice containing 3×10^4 HEL-binding B cells were transferred i.v. into wild-type or mHEL^{4X} Tg recipient mice together with 2×10^8

HEL^{3X}-SRBCs (prepared as previously described) (Paus et al., 2006). Recipient mice were boosted with HEL^{3X}-SRBCs (2×10^8 cells/recipient) 4 days after primary immunization. To abrogate GC B cell responses, 200 μ g of anti-CD40L mAb (MR-1, BioXcell) or isotype control (hamster IgG, BioXcell) was administered intravenously on days 15, 17, and 19 after primary immunization.

HEL Proteins

Purified wild-type hen egg lysozyme (HEL^{WT}) was purchased from Sigma-Aldrich. Recombinant HEL^{3X} and HEL^{4X} proteins (Chan et al., 2012; Paus et al., 2006) were grown as secreted proteins in yeast (*Pichia pastoris*) and purified from culture supernatants as described (Paus et al., 2006).

Flow Cytometric Analysis

Splenocytes were prepared and stained for cell surface HEL^{3X} or HEL^{4X} binding as well as expression of other cell surface markers as described (Chan et al., 2012). In addition, splenic basophils were identified with the following mAbs: anti-Fc ϵ RI (MAR-1, eBioscience), anti-CD49b (DX5, BD Biosciences), and anti-c-Kit (2B8, BD Biosciences). For flow cytometric analysis of fixed and permeabilized cells (Figure S2A), cells were first stained for surface markers and surface IgE (including CD23-bound soluble IgE) blocked with unconjugated anti-IgE (R35-72) antibody (Yang et al., 2012). Thus, for fixed and permeabilized samples, IgG1 stains include surface and intracellular molecules (total) whereas IgE stains are for intracellular (i.c.) IgE only. After surface staining, cells were fixed with 10% formalin (Sigma-Aldrich). Cell permeabilization and subsequent staining steps were performed in 0.5% Saponin/0.1% BSA/PBS. Intracellular staining was performed with 200 ng/ml of HEL^{WT} or 50 ng/ml of HEL^{3X}. After staining for IgG1 (A85-1, BD Biosciences), samples were blocked with 10% normal mouse serum (Jackson ImmunoResearch) and bound HEL^{WT}/HEL^{3X} was revealed with HyHEL9-AlexaFluor647 in 5% normal mouse serum. Subsequent intracellular staining was performed with anti-IgE FITC (R35-72, BD Biosciences) and Streptavidin (SA)-Pacific Blue (Invitrogen). Data were acquired on either the FACS Cantoll or LSR II SORP (BD) and analyzed with FlowJo (TreeStar).

Single-Cell Sorting

To sort single cells for RNA extraction (Figure S2B), cell suspensions from whole spleens were prepared and surface staining performed as detailed earlier for surface flow cytometric analysis. For somatic hypermutation (SHM) analysis (Figure S3B), cell samples were subjected to intracellular staining. Single-cell sorting was performed on the FACS Aria, FACS ARIAII, or Influx (BD Biosciences).

Single-Cell qRT-PCR and Data Analysis

Acquisition and analysis of single-cell qRT-PCR data to determine mRNA expression in sorted cells was carried out according to the methods established by us (Suan et al., 2015) and described in detail in Supplemental Experimental Procedures.

SHM Analysis

The variable region exon of the SW_{HEL} Ig (HyHEL10) heavy chain gene was amplified from sorted single cells by PCR and sequenced as described (Paus et al., 2006).

ELISAs

IgE concentrations in sera were measured by direct ELISA and anti-HEL IgE measured by capturing total serum IgE and detecting with biotinylated HEL. These procedures are described fully in Supplemental Experimental Procedures. Anti-HEL^{4X} IgG1 was measured by direct ELISA with plate-bound HEL^{4X} mice as described (Chan et al., 2012).

ALPS Cohort Description and Human Serum IgE Measurements

The National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIAID, NIH) ALPS program has evaluated patients and family members through mail-in samples and direct evaluation at the NIH Clinical Center (NIH CC) since 1993. 415 individuals from 120 families with 95 unique FAS mutations were investigated (http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=FAS). All participants underwent informed con-

sent process and were enrolled on an IRB-approved NIAID, NIH research protocol (Clinical trials.gov NCT: NCT00001350). Samples analyzed for this study correspond to the 150 ALPS-FAS patients (78 probands and 72 affected relatives) and 63 healthy mutation-positive relatives whose natural histories were recently reported (Price et al., 2014). Serum IgE measurements were performed with IMMULITE 2000 analyzer (Siemens) with a IMMULITE 2000 Total IgE kit for a solid-phase, chemiluminescent immuno-metric assay.

Immunofluorescence Histology

Immunofluorescence histology analysis of splenic sections from recipient mice was carried out as described previously (Chan et al., 2009) and outlined in detail in Supplemental Experimental Procedures.

Recombinant HyHEL10 IgG1 Antibody Production and Binding Analysis

Heavy chain mutants of HyHEL10 were expressed as soluble IgG1 molecules in HEK293F cells from pcDNA3 vectors encoding mutant γ 1 heavy and wild-type κ light chains and purified via Protein G sepharose (AcroBiosystems). Purified IgG1 samples were biotinylated at a 5:1 molar ratio via EZ-Link NHS-PEG4-Biotinylation reagent (Thermo Scientific) and uncoupled biotinylation reagent was removed with a ZebaSpin desalting column (Thermo Scientific). Affinities of HyHEL10 variants were measured via the Blitz system (PALL). SA biosensors were pre-blocked with 1% BSA/PBS for 1 hr. Sensors were then incubated with dilute samples of biotinylated antibody and coupling of antibody quantified with the Blitz software. Coupled sensors were incubated in PBS for 60 s to obtain a stable baseline and then incubated with various dilutions (5 nM–5 μ M) of purified, filtered HEL^{3X}, HEL^{4X}, or HEL^{WT} in PBS. Association and dissociation rates were measured with the Blitz software and global fits of the resulting data were used to determine the K_d , k_a , and k_d of each variant for HEL^{WT} and HEL^{3X}. K_a was taken as $1/K_d$.

Statistical Analysis

A full description of statistical analyses used can be found in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2015.04.010>.

AUTHOR CONTRIBUTIONS

R.B., T.D.C., and D.B. conceptualized and designed the research; D.B., T.D.C., K.B., J.R.H., and A.N. performed experiments; S.P. and V.K.R. collected, analyzed, and interpreted ALPS patient data; P.S. and D.C. produced and analyzed recombinant mAbs; L.A.O'R. and A. Strasser provided *Fas* ^{Δ mi Δ mi} mice and advice; A.N. and A. Statham performed bioinformatic analysis; A.B., C.S.M., S.G.T., and T.G.P. provided specialist advice and analysis; D.B., T.D.C., and R.B. analyzed and interpreted data; D.B., T.D.C., A.N., and R.B. prepared figures; and R.B. prepared text.

ACKNOWLEDGMENTS

We thank R. Salomon, C. Brownlee, and Garvan Institute Flow Facility staff for single-cell sorting and J. Kingham and the staff of Australian BioResources for animal husbandry. We also thank C. Goodnow, A. Collins, and J. Davies for their comments on the manuscript. D.B. is supported by an Australian Postgraduate Award and T.D.C., A. Strasser, D.C., C.S.M., S.G.T., T.G.P., and R.B. by National Health and Medical Research Council (NHMRC) fellowships 1054669, 1020363, 1050146, 1008820, 1042925, 1005097, and 1002280. This work was funded by Asthma Foundation NSW, R.G. Arnott Foundation, NHMRC Project Grant 1044477, and NHMRC Program Grant 1016953. Additional support came from NHMRC Project Grant 1009145, NHMRC Program Grant 1016701, Leukemia and Lymphoma Society (SCOR grants #7413 and #7001-13), and the Intramural Research Program of the National Institute of Allergy and Infectious Diseases and the NIH Clinical Center.

Received: February 8, 2015

Revised: March 9, 2015

Accepted: March 25, 2015

Published: May 12, 2015

REFERENCES

- Afshar-Sterle, S., Zotos, D., Bernard, N.J., Scherger, A.K., Rödling, L., Alsop, A.E., Walker, J., Masson, F., Belz, G.T., Corcoran, L.M., et al. (2014). Fas ligand-mediated immune surveillance by T cells is essential for the control of spontaneous B cell lymphomas. *Nat. Med.* 20, 283–290.
- Ait-Azzouzene, D., Kono, D.H., Gonzalez-Quintial, R., McHeyzer-Williams, L.J., Lim, M., Wickramarachchi, D., Gerdes, T., Gavin, A.L., Skog, P., McHeyzer-Williams, M.G., et al. (2010). Deletion of IgG-switched autoreactive B cells and defects in Fas(lpr) lupus mice. *J. Immunol.* 185, 1015–1027.
- Alabyev, B., Vuyyuru, R., and Manser, T. (2008). Influence of Fas on the regulation of the response of an anti-nuclear antigen B cell clonotype to foreign antigen. *Int. Immunol.* 20, 1279–1287.
- Andrews, B.S., Eisenberg, R.A., Theofilopoulos, A.N., Izui, S., Wilson, C.B., McConahey, P.J., Murphy, E.D., Roths, J.B., and Dixon, F.J. (1978). Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. *J. Exp. Med.* 148, 1198–1215.
- Brink, R. (2014). The imperfect control of self-reactive germinal center B cells. *Curr. Opin. Immunol.* 28, 97–101.
- Chan, T.D., Gatto, D., Wood, K., Camidge, T., Basten, A., and Brink, R. (2009). Antigen affinity controls rapid T-dependent antibody production by driving the expansion rather than the differentiation or extrafollicular migration of early plasmablasts. *J. Immunol.* 183, 3139–3149.
- Chan, T.D., Wood, K., Hermes, J.R., Butt, D., Jolly, C.J., Basten, A., and Brink, R. (2012). Elimination of germinal-center-derived self-reactive B cells is governed by the location and concentration of self-antigen. *Immunity* 37, 893–904.
- Dahlke, I., Nott, D.J., Ruhno, J., Sewell, W.A., and Collins, A.M. (2006). Antigen selection in the IgE response of allergic and nonallergic individuals. *J. Allergy Clin. Immunol.* 117, 1477–1483.
- Dema, B., Charles, N., Pellefigues, C., Ricks, T.K., Suzuki, R., Jiang, C., Scheffell, J., Hasni, S., Hoffman, V., Jablonski, M., et al. (2014a). Immunoglobulin E plays an immunoregulatory role in lupus. *J. Exp. Med.* 211, 2159–2168.
- Dema, B., Pellefigues, C., Hasni, S., Gault, N., Jiang, C., Ricks, T.K., Bonelli, M.M., Scheffell, J., Sacré, K., Jablonski, M., et al. (2014b). Autoreactive IgE is prevalent in systemic lupus erythematosus and is associated with increased disease activity and nephritis. *PLoS ONE* 9, e90424.
- Erazo, A., Kutchukhidze, N., Leung, M., Christ, A.P., Urban, J.F., Jr., Curotto de Lafaille, M.A., and Lafaille, J.J. (2007). Unique maturation program of the IgE response in vivo. *Immunity* 26, 191–203.
- Fisher, G.H., Rosenberg, F.J., Straus, S.E., Dale, J.K., Middleton, L.A., Lin, A.Y., Strober, W., Lenardo, M.J., and Puck, J.M. (1995). Dominant interfering Fas gene mutations inhibit apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell* 81, 935–946.
- Garrone, P., Neidhardt, E.M., Garcia, E., Galibert, L., van Kooten, C., and Banchereau, J. (1995). Fas ligation induces apoptosis of CD40-activated human B lymphocytes. *J. Exp. Med.* 182, 1265–1273.
- Hahne, M., Renno, T., Schroeter, M., Irmeler, M., French, L., Bornard, T., MacDonald, H.R., and Tschopp, J. (1996). Activated B cells express functional Fas ligand. *Eur. J. Immunol.* 26, 721–724.
- Han, S., Hathcock, K., Zheng, B., Kepler, T.B., Hodes, R., and Kelsoe, G. (1995a). Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *J. Immunol.* 155, 556–567.
- Han, S., Zheng, B., Dal Porto, J., and Kelsoe, G. (1995b). In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. IV. Affinity-dependent, antigen-driven B cell apoptosis in germinal centers as a mechanism for maintaining self-tolerance. *J. Exp. Med.* 182, 1635–1644.
- Hao, Z., Duncan, G.S., Seagal, J., Su, Y.W., Hong, C., Haight, J., Chen, N.J., Elia, A., Wakeham, A., Li, W.Y., et al. (2008). Fas receptor expression in germinal-center B cells is essential for T and B lymphocyte homeostasis. *Immunity* 29, 615–627.
- Herlands, R.A., Christensen, S.R., Sweet, R.A., Hershberg, U., and Shlomchik, M.J. (2008). T cell-independent and toll-like receptor-dependent antigen-driven activation of autoreactive B cells. *Immunity* 29, 249–260.
- Hoch, S., Boyd, M., Malone, B., Gonye, G., Schwaber, J., and Schwaber, J. (2000). Fas-mediated apoptosis eliminates B cells that acquire self-reactivity during the germinal center response to NP. *Cell. Immunol.* 203, 103–110.
- Liu, Y.J., Barthélémy, C., de Bouteiller, O., Arpin, C., Durand, I., and Banchereau, J. (1995). Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. *Immunity* 2, 239–248.
- Messingham, K.A., Holahan, H.M., and Fairley, J.A. (2014). Unraveling the significance of IgE autoantibodies in organ-specific autoimmunity: lessons learned from bullous pemphigoid. *Immunol. Res.* 59, 273–278.
- Nossal, G.J. (1988). Somatic mutations in B lymphocytes: new perspectives in tolerance research? *Immunol. Cell Biol.* 66, 105–110.
- O' Reilly, L.A., Tai, L., Lee, L., Kruse, E.A., Grabow, S., Fairlie, W.D., Haynes, N.M., Tarlinton, D.M., Zhang, J.G., Belz, G.T., et al. (2009). Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. *Nature* 461, 659–663.
- Paus, D., Phan, T.G., Chan, T.D., Gardam, S., Basten, A., and Brink, R. (2006). Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *J. Exp. Med.* 203, 1081–1091.
- Permin, H., and Wiik, A. (1978). The prevalence of IgE antinuclear antibodies in rheumatoid arthritis and systemic lupus erythematosus. *Acta Pathol. Microbiol. Scand. [C]* 86C, 245–249.
- Phan, T.G., Amesbury, M., Gardam, S., Crosbie, J., Hasbold, J., Hodgkin, P.D., Basten, A., and Brink, R. (2003). B cell receptor-independent stimuli trigger immunoglobulin (Ig) class switch recombination and production of IgG autoantibodies by anergic self-reactive B cells. *J. Exp. Med.* 197, 845–860.
- Phan, T.G., Paus, D., Chan, T.D., Turner, M.L., Nutt, S.L., Basten, A., and Brink, R. (2006). High affinity germinal center B cells are actively selected into the plasma cell compartment. *J. Exp. Med.* 203, 2419–2424.
- Price, S., Shaw, P.A., Seitz, A., Joshi, G., Davis, J., Niemela, J.E., Perkins, K., Hornung, R.L., Folio, L., Rosenberg, P.S., et al. (2014). Natural history of autoimmune lymphoproliferative syndrome associated with FAS gene mutations. *Blood* 123, 1989–1999.
- Provost, T.T., and Tomasi, T.B., Jr. (1974). Immunopathology of bullous pemphigoid. Basement membrane deposition of IgE, alternate pathway components and fibrin. *Clin. Exp. Immunol.* 18, 193–200.
- Rathmell, J.C., Cooke, M.P., Ho, W.Y., Grein, J., Townsend, S.E., Davis, M.M., and Goodnow, C.C. (1995). CD95 (Fas)-dependent elimination of self-reactive B cells upon interaction with CD4+ T cells. *Nature* 376, 181–184.
- Rieux-Laucat, F., Le Deist, F., Hivroz, C., Roberts, I.A., Debatin, K.M., Fischer, A., and de Villartay, J.P. (1995). Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* 268, 1347–1349.
- Smith, K.G., Nossal, G.J., and Tarlinton, D.M. (1995). FAS is highly expressed in the germinal center but is not required for regulation of the B-cell response to antigen. *Proc. Natl. Acad. Sci. USA* 92, 11628–11632.
- Suan, D., Nguyen, A., Moran, I., Bourne, K., Hermes, J.R., Arshi, M., Hampton, H.R., Tomura, M., Miwa, Y., Kelleher, A.D., et al. (2015). T follicular helper cells have distinct modes of migration and molecular signatures in naive and memory immune responses. *Immunity* 42, 704–718.
- Takahashi, T., Tanaka, M., Brannan, C.I., Jenkins, N.A., Copeland, N.G., Suda, T., and Nagata, S. (1994). Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* 76, 969–976.
- Takahashi, Y., Ohta, H., and Takemori, T. (2001). Fas is required for clonal selection in germinal centers and the subsequent establishment of the memory B cell repertoire. *Immunity* 14, 181–192.
- Takahashi, S., Futatsugi-Yumikura, S., Fukuoka, A., Yoshimoto, T., Nakanishi, K., and Yonehara, S. (2013). Fas deficiency in mice with the Balb/c background

induces blepharitis with allergic inflammation and hyper-IgE production in conjunction with severe autoimmune disease. *Int. Immunol.* 25, 287–293.

Thaunat, O., Granja, A.G., Barral, P., Filby, A., Montaner, B., Collinson, L., Martinez-Martin, N., Harwood, N.E., Bruckbauer, A., and Batista, F.D. (2012). Asymmetric segregation of polarized antigen on B cell division shapes presentation capacity. *Science* 335, 475–479.

Verbeke, C.S., Wenthe, U., and Zentgraf, H. (1999). Fas ligand expression in the germinal centre. *J. Pathol.* 189, 155–160.

Victoria, G.D., and Nussenzweig, M.C. (2012). Germinal centers. *Annu. Rev. Immunol.* 30, 429–457.

Wang, Y., Jackson, K.J., Davies, J., Chen, Z., Gaeta, B.A., Rimmer, J., Sewell, W.A., and Collins, A.M. (2014). IgE-associated IGHV genes from venom and peanut allergic individuals lack mutational evidence of antigen selection. *PLoS ONE* 9, e89730.

Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G., Jenkins, N.A., and Nagata, S. (1992). Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 356, 314–317.

William, J., Euler, C., Christensen, S., and Shlomchik, M.J. (2002). Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science* 297, 2066–2070.

Wu, J., Wilson, J., He, J., Xiang, L., Schur, P.H., and Mountz, J.D. (1996). Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. *J. Clin. Invest.* 98, 1107–1113.

Xiong, H., Dolpady, J., Wabl, M., Curotto de Lafaille, M.A., and Lafaille, J.J. (2012). Sequential class switching is required for the generation of high affinity IgE antibodies. *J. Exp. Med.* 209, 353–364.

Yang, Z., Sullivan, B.M., and Allen, C.D. (2012). Fluorescent in vivo detection reveals that IgE(+) B cells are restrained by an intrinsic cell fate predisposition. *Immunity* 36, 857–872.

Immunity

Supplemental Information

FAS Inactivation Releases Unconventional Germinal Center B Cells that Escape Antigen Control and Drive IgE and Autoantibody Production

Danyal Butt, Tyani D. Chan, Katherine Bourne, Jana R. Hermes, Akira Nguyen, Aaron Statham, Lorraine A. O'Reilly, Andreas Strasser, Susan Price, Peter Schofield, Daniel Christ, Antony Basten, Cindy S. Ma, Stuart G. Tangye, Tri Giang Phan, V. Koneti Rao, and Robert Brink

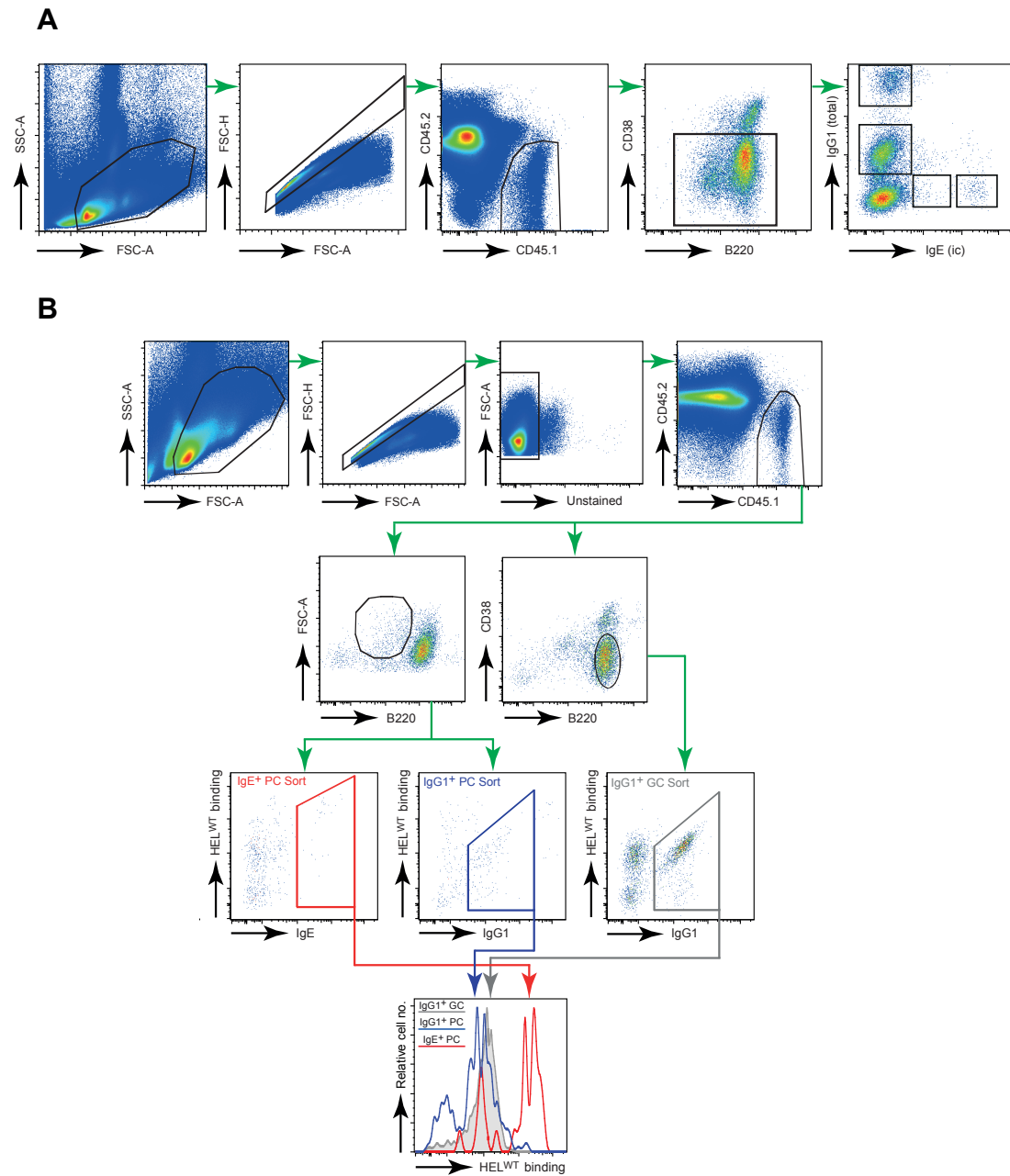


Figure S2, Related to Figure 3. Gating strategies employed in flow cytometric analysis and single cell sorting for qPCR analysis.

Figure S2, Related to Figure 3. Gating strategies employed in flow cytometric analysis and single cell sorting for qPCR analysis.

Recipient spleens were harvested following transfer of CD45.1 donor SW_{HEL} B cells (with or without mutated *Fas* or *Fasl* genes) and challenge with HEL^{3X}-SRBC.

(A) Cells were stained for cell surface markers before being fixed, permeabilized, and stained for IgG1, IgE and HEL^{3X} or HEL^{WT} binding. Following data acquisition and compensation, a lymphocyte light scatter gate was applied (FSC-A vs SSC-A), doublets excluded (FSC-A vs FSC-H), autofluorescent cells excluded (not shown) and a gate for donor-derived cells applied (CD45.1 vs CD45.2). CD38^{hi} cells B cells were then excluded (B220 vs CD38) and class-switched GC B cells identified by low but positive expression of total IgG1 or intracellular (ic) IgE and PCs by high staining for these Ig isotypes.

(B) Single cell sorting for qPCR analysis was performed in a similar fashion but without a fixing and permeabilization step and an additional exclusion of autofluorescent cells (Unstained vs FSC-A). Donor-derived PCs (B220^{lo}, FSC-A^{hi}) and GC B cells (B220^{hi}, CD38^{lo}) were resolved and the IgG1 and IgE switched subsets resolved by surface staining (IgE⁺ GC B cells could not be resolved). Note the high levels of surface Ig expression on IgE⁺ PCs, consistent with previous observations (Yang et al., 2012).

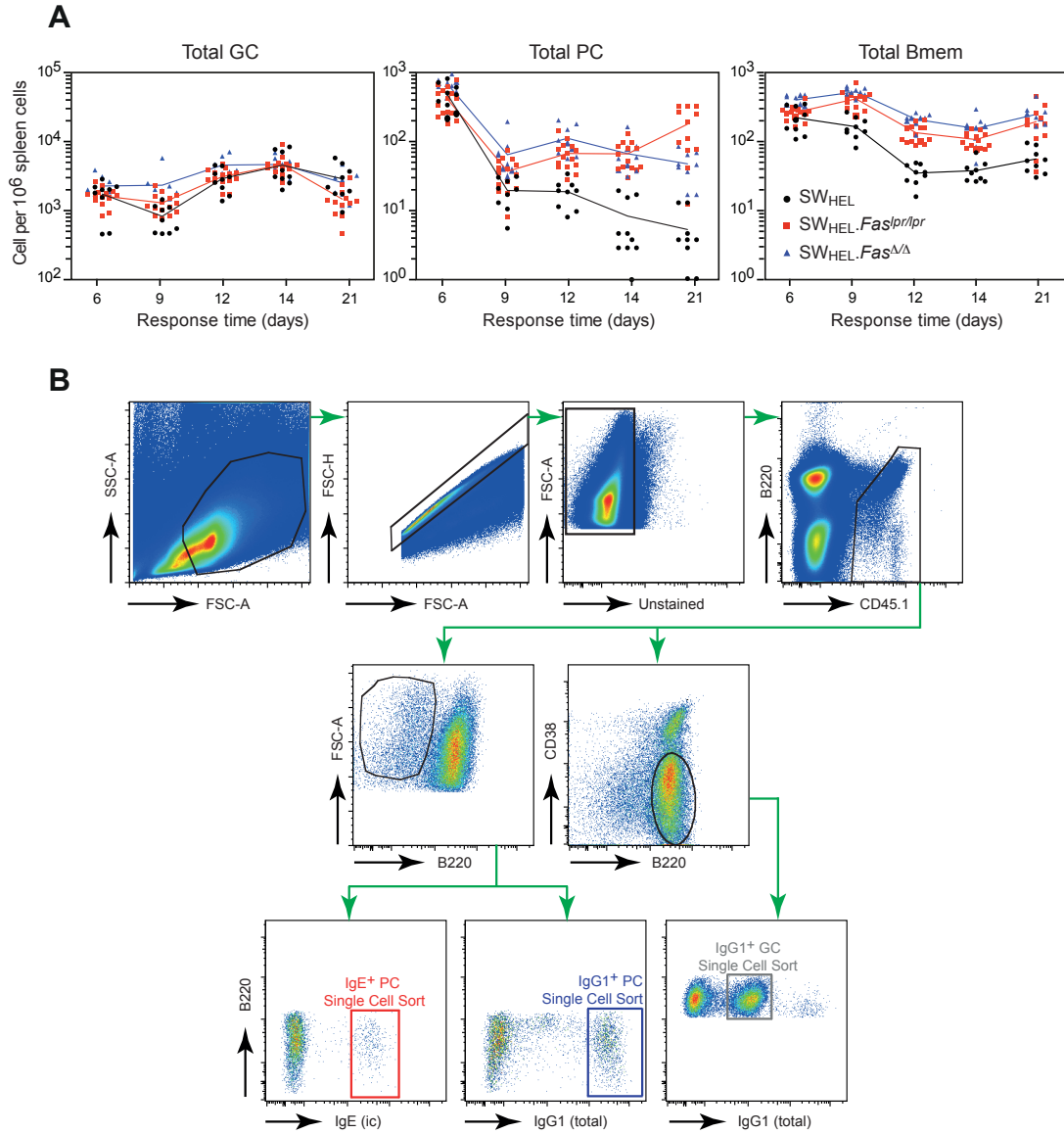


Figure S3, Related to Figure 4. Kinetics of total wild-type vs FAS-deficient SW_{HEL} B cell responses and sort strategy for single cell SHM analysis.

(A) Time course of donor-derived splenic responses of SW_{HEL} , $SW_{HEL}.Fas^{lpr/lpr}$ and $SW_{HEL}.Fas^{\Delta/\Delta}$ B cells challenged with HEL^{3X} -SRBC. Spleen cells were stained as per Figure S2A. Populations reflect all HEL^{WT} -binding donor-derived cells. GC = $B220^{hi}, CD38^{lo}$; PC = $B220^{lo}, CD38^{lo}$, HEL^{WT-hi} ; Bmem = $B220^{hi}, CD38^{hi}$. Data were pooled from two independent experiments.

(B) Sorting strategy for single cell SHM analysis. Cells from recipient spleens were stained as detailed in Figure S2A. Donor-derived cells were identified ($CD45.1^{+}, B220^{lo-hi}$) and PC cell ($B220^{lo}, FSC-A^{hi}$) and GC B cell ($B220^{hi}, CD38^{lo}$) populations resolved. Specific IgG1 and IgE switched populations were then resolved and deposited as single cells in 96 well plates for processing.

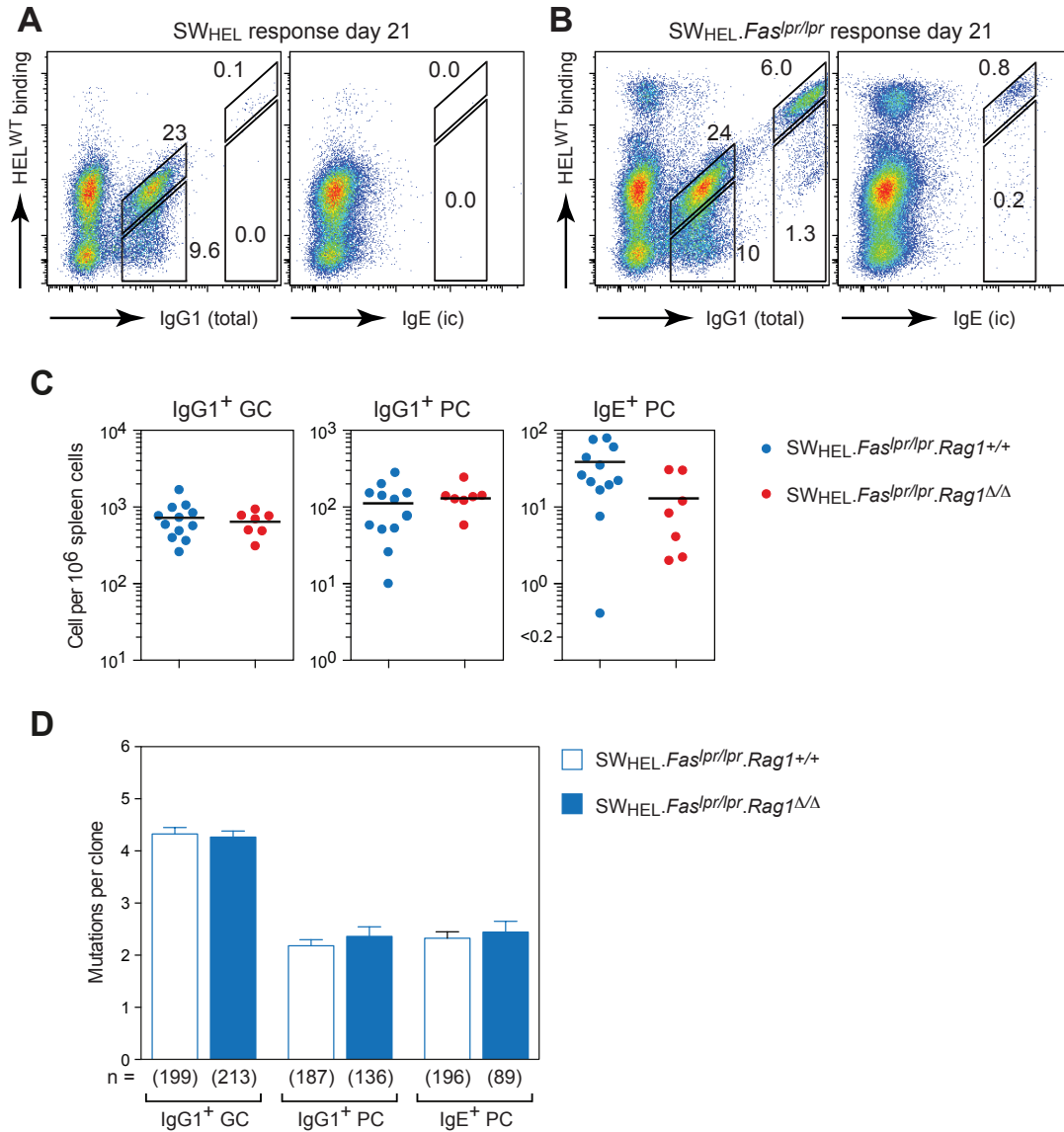


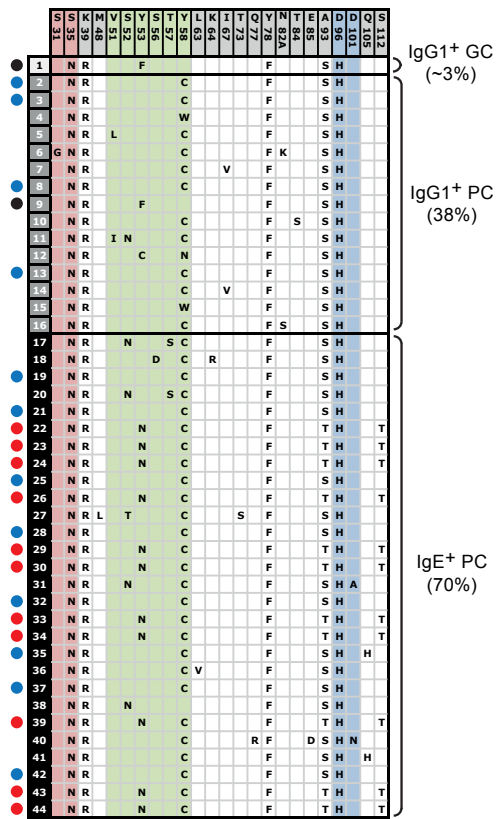
Figure S4, Related to Figure 5. HEL^{WT}-binding of responding SW_{HEL} and SW_{HEL}.Fas^{lpr/lpr} B cells and equivalent responses of RAG1-expressing and RAG1-deficient SW_{HEL}.Fas^{lpr/lpr} B cells.

(A,B) SW_{HEL} or SW_{HEL}.Fas^{lpr/lpr} B cells were challenged with HEL^{3X}-SRBC and recipient spleen cells analyzed on day 21. Data show total IgG1 or intracellular (ic) IgE staining as well as HEL^{WT} binding by donor-derived GC B cells + PCs (CD45.1⁺, CD45.2⁻, CD38^{lo} - see Figure S2A).

(C,D) SW_{HEL}.Fas^{lpr/lpr}.Rag1^{+/+} or SW_{HEL}.Fas^{lpr/lpr}.Rag1^{Δ/Δ} B cells were challenged with HEL^{3X}-SRBC and recipient spleen cells analyzed after 21 days. (C) Enumeration of donor-derived responding subsets. Data are pooled from four independent experiments. (D) Mutation rates in the Ig heavy chain variable region gene in donor-derived splenic subsets (see Figure S3B for gating). n = number of clones analyzed. Errors bars indicate the SEM.

A

Mouse 57.69

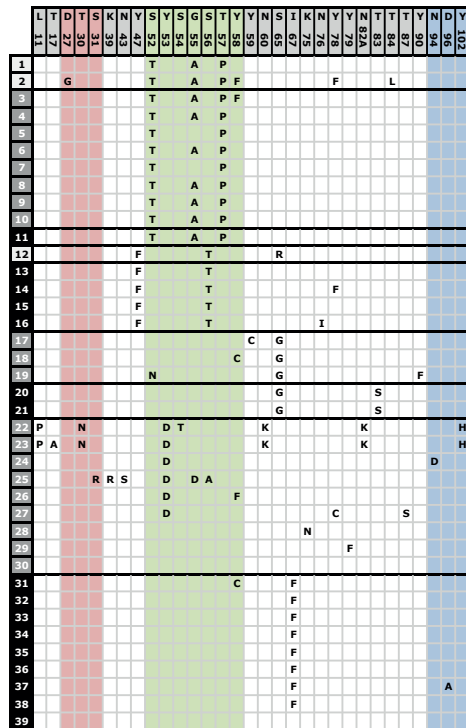


HyHEL10 variant	K_A for HEL ^{3X} (M ⁻¹)	K_A for HEL ^{WT} (M ⁻¹)
Unmutated ("germ-line")	1.3×10^7	$> 10^{10}$
Basic mutant**	$< 10^5$	2.0×10^8
Basic + Y53F	$< 10^5$	1.5×10^8
Basic + Y58C	6.4×10^5	5.5×10^6
Basic + Y53N, Y58C A93T, S112T	$< 10^5$	$< 10^5$

**S35N, K39R, Y58F, A93S, D96H (presumed precursor clone)

B

Mouse 71.02



- IgG1+ GC clones
- IgG1+ PC clones
- IgE+ PC clones

Group 1

Group 2

Group 3

Figure S5, Related to Figure 5. Analysis of clonally-related, donor-derived cells in single recipient mice given SW_{HEL}.*Fas*^{lpr/lpr}.*Rag1*^{Δ/Δ} B cells and challenged with HEL^{3X}-SRBC.

(A) (Left Panel) Ig heavy chain variable region replacement mutations in clonally-related cells obtained from a single recipient mouse (57.69) on day 21 of the response of SW_{HEL}.*Fas*^{lpr/lpr}.*Rag1*^{Δ/Δ} B cells to HEL^{3X}-SRBC. Percentages indicate the frequency of these clones within the respective responder populations and mirror those seen in the independently analyzed mouse 71.06 (Figure 5E). (Right Panel) Affinity for HEL^{3X} and HEL^{WT} of recombinant HyHEL10 soluble IgG1 carrying the indicated heavy chain variable region replacement mutations together with HyHEL10 κ light chain. Clones corresponding exactly to the mAbs analyzed are indicated by the colored dots. The presumed ancestral clone carried the S35N, K39R, Y58F, A93S and D96H substitutions.

(B) All sequenced IgG1⁺ and IgE⁺ PC clones and related IgG1⁺ clones from a single recipient mouse (71.02) obtained on day 21 of the response of SW_{HEL}.*Fas*^{lpr/lpr}.*Rag1*^{Δ/Δ} B cells to HEL^{3X}-SRBC. Three groups of related clones that span two or more cell subsets can be seen with additional related clones apparent within the IgG1⁺ and IgE⁺ PC compartments.

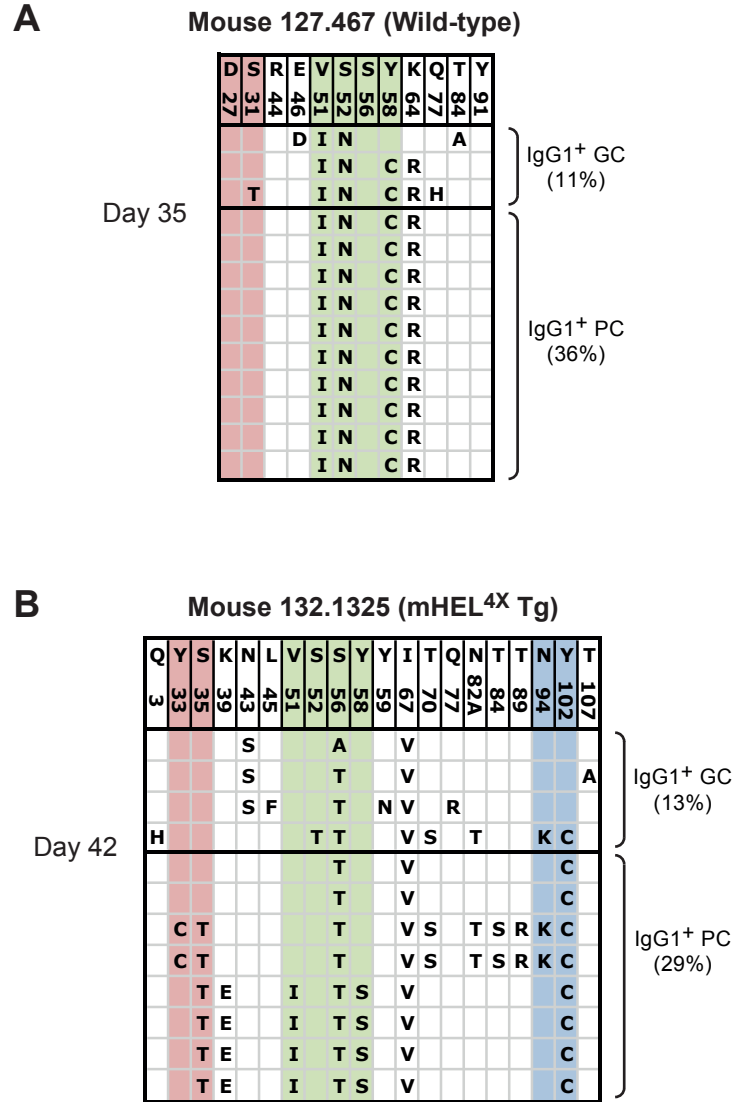


Figure S6, Related to Figure 6. Expanded populations of clonally-related GC_R B cells form large fractions of the late SW_{HEL}.Fas^{lpr/lpr} response.

Ig heavy chain variable region replacement mutations in clonally-related cells obtained from a single recipient (A) wild-type mouse (127.467) on day 35 or (B) mHEL^{4X} Tg mouse on day 42 of the response of SW_{HEL}.Fas^{lpr/lpr} B cells to HEL^{3X}-SRBC. Percentages indicate the frequency of these clonal families within the respective responder populations. These rogue GC (GC_R) B cells show increased representation in the GC at these later time-points compared to day 21 (see Figure S5). IgE⁺ PCs were present but were not sorted at these late time-points.

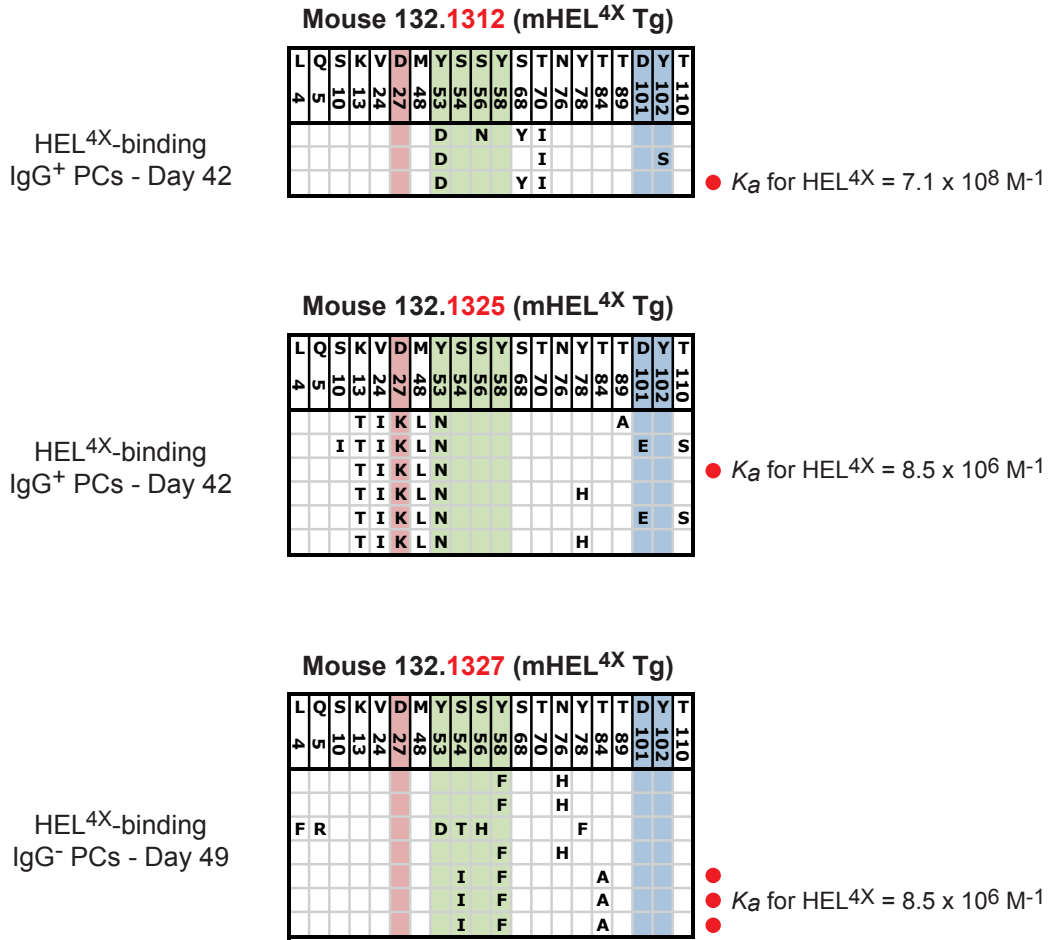


Figure S7, Related to Figure 7. Autoantibodies generated by FAS-deficient PC_R late in the response carry clonally-related Ig heavy chain variable regions.

Single cell sorting and Ig heavy chain variable region gene sequencing was carried out on donor-derived PCs exhibiting high HEL^{4X}-binding from individual mHEL^{4X} Tg recipient mice that had received SW_{HEL}.*Fas*^{lpr/lpr}.*Rag1*^{Δ/Δ} and HEL^{3X}-SRBC. HEL^{4X}-binding gates were applied to either IgG1⁺ PCs at days 42 (mice 132.1312 or 132.1325) or to IgG1⁻ PCs at day 49 (mouse 132.1327) for SHM analysis of the Ig heavy chain variable region gene (see Figure 7C). Self-reactive PCs in individual mice almost exclusively formed clonally-related families. The indicated core clones (red dots) were expressed as soluble IgG1 with the HyHEL10 κ light chain and subjected to binding analyses. Their affinities for HEL^{4X} as measured by Blitz analysis are indicated.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice. SW_{HEL} mice (Phan et al., 2003) were maintained on a CD45.1 congenic (*Ptprc*^{a/a}) C57BL/6 background. *Fas*^{lpr/lpr} (Adachi et al., 1993), *Fas*^{Δ/Δ} (Adachi et al., 1995), *Fasl*^{gld/gld} (Takahashi et al., 1994), *Fasl*^{Δm/Δm} (O' Reilly et al., 2009) and *Rag1*^{Δ/Δ} (Mombaerts et al., 1992) mice were either maintained on a C57BL/6 background or crossed with SW_{HEL} mice on a CD45.1 congenic C57BL/6 background for use in adoptive transfer experiments. mHEL^{4x} transgenic mice (Chan et al., 2012) were maintained on a C57BL/6 background. Wild-type C57BL/6 mice were purchased from either Australian BioResources (Moss Vale, Australia) or Animal Resources Centre (Perth, Australia). All mice were bred and maintained in specific pathogen-free conditions at Australian BioResources and the Garvan Institute of Medical Research Biological Testing Facility. Animal experiments were performed under the approved guidelines of the Garvan Institute of Medical Research/St. Vincent's Hospital Animal Ethics Committee.

Single cell RT-qPCR. RNA was isolated from sorted single cells using Ambion Single Cell to CT kit (Life Technologies) according to manufacturers protocol. Briefly, single IgE⁺ PC, IgG1⁺ PC or IgG1⁺ GC were sorted into 10 μL of single cell lysis/DNAse I solution in 96-well skirted plates chilled to 4°C in the collection chamber of a FACS AriaII (BD Biosciences). Plates were spun and brought to room temperature for 5 min, after which 1 μL of stop solution was added and samples incubated at room temperature for a further 2 min. To perform reverse transcription, 4.5 μL of RT mix was added and samples incubated for 10 min at 25°C, 60 min at 42°C and 5 min at 85°C. For pre-amplification, 11 μL of the PreAmp Mix/Taqman

assays was added to the samples which were then incubated at 95°C for 10 min followed by 14 cycles of 95°C for 15 sec plus 60°C for 4 min, followed by a final incubation at 99°C for 10 min. Incubation steps were performed in a C1000 Thermocycler (BioRad). RT-qPCR on the diluted pre-amplified product was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems).

Pre-processing and analysis of single cell gene expression data. dCt values were obtained by subtracting raw cycle threshold (Ct) values from a cutoff value of 35. These were then expressed as Relative Quantitation (RQ) values ($2^{\Delta Ct}$). Data was then log₂ transformed using the LogTransform module in GenePattern (<https://pwbcr.garvan.org.au/gp>) thereby setting any negative values, where the gene expression levels are below the threshold Ct35, to zero. The NormalizeColumns module in GenePattern allowed for quantile normalization of the single cell expression data sets. For the purpose of normalization, zero values were removed from the dataset and replaced afterwards. Heatmaps were generated using the HeatMapView module in GenePattern. Gene expression analyses were carried out on log-transformed normalized data. LimmaGP, a GenePattern implementation of the Limma (Wettenhall and Smyth, 2004) BioConductor library module in GenePattern, was used to identify differentially expressed genes between the sorted populations, with a false discovery rate (FDR) of <0.05.

ELISAs. Total IgE levels in sera were measured by direct ELISA. 384-well plates (Corning) were coated with purified anti-IgE (R35-72, BD Biosciences) at 5 µg/mL in NPP buffer (35 mM NaHCO₃, 19 mM Na₂CO₃, 3 mM NaN₃, 1 mM MgCl₂) and stored overnight at 4°C. A plate washer (BioTek) was used to wash plates with ELISA

wash buffer (0.05% Tween20/PBS) and all subsequent steps performed at 37°C for 1 h. Wells were blocked with 1% BSA/PBS. Dilutions of serum samples and the standard, IgE isotype control (MEB-38, Biolegend), were prepared in 0.1% BSA/PBS and added in duplicate. Bound IgE antibodies were detected using an anti- κ light chain antibody (187.1, BD Bioscience) diluted in 0.1% BSA/1% skim milk powder/PBS. SA-alkaline phosphatase (Roche) in PBS was added and visualized with the substrate *p*-nitrophenyl phosphate (MP Biomedicals Australia) prepared in NPP buffer. Absorbance at 405 nm was read and a standard curve was constructed to determine the concentration of IgE antibodies in serum. To quantitate anti-HEL IgE serum antibody, 384-well plates were coated with purified anti-IgE (R35-72) at 5 μ g/mL in NPP buffer and stored overnight at 4°C. A plate washer was used to wash plates with ELISA wash buffer and all subsequent steps were incubated at 37°C for 1 h. Wells were blocked with 1% BSA/PBS followed by the addition of serum samples diluted in 0.1% BSA/PBS. Biotinylated HEL^{WT} was prepared using NHS-Biotinylation reagent (Sigma-Aldrich), diluted in 0.1% BSA/1% skim milk powder/PBS and used to detect bound anti-HEL IgE antibodies. SA-alkaline phosphatase was added and then visualized with the substrate *p*-nitrophenyl phosphate as for total IgE ELISA. Absorbance at 405 nm was read and endpoint titers were used to determine serum antibody concentrations (Frey et al., 1998) based on 99.9% confidence levels.

Immunofluorescence histology. Spleens were frozen in Tissue Tek OCT compound (Sakura). 6-7 μ m sections were cut using a CM3050 S cryostat (Leica). Prior to staining for IgE, CD23-bound IgE was removed using an acid wash (Katona et al., 1983). Briefly, sections were incubated for 1 min in a pH 4.0 acetate buffer followed

by neutralization in PBS. Sections were then fixed in ice-cold acetone and blocked with 30% normal horse serum (Invitrogen). Sections were stained with the following antibodies: anti-IgG1 biotin (A85-1, BD Biosciences) or anti-IgE biotin (R35-72, BD Biosciences), anti-IgD AlexaFluor647 (11-26c.2a, Biolegend). Biotinylated antibodies were detected using SA-AlexaFluor555 (Invitrogen). To stain HEL-binding cells, sections were incubated with 200 ng/mL of HEL^{WT} and detected with polyclonal rabbit anti-HEL IgG (Rockland Immunochemicals Inc.) followed by goat anti-rabbit FITC (Southern Biotech). Slides were analyzed with a Leica DM5500 using the x10 or x40 objective and Adobe Photoshop software.

Statistical Analysis. Statistical significance of data was calculated with Prism 6.0 software (Graphpad). For comparisons between two normally distributed groups a two-tailed unpaired *t*-test with Welch's correction was used whilst non-parametric data was analyzed using Mann-Whitney *U* test. For comparison between more than two groups statistical analysis was performed using a one-way ANOVA with the post-hoc Bonferroni test whereas the Kruskal-Wallis test with Dunn's multiple comparison post-test was used in cases where data was non-parametric. Two-way ANOVA with the post-hoc Bonferroni's test was used to compare responses with more than two parameters. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001.

SUPPLEMENTAL REFERENCES

Adachi, M., Suematsu, S., Kondo, T., Ogasawara, J., Tanaka, T., Yoshida, N., and Nagata, S. (1995). Targeted mutation in the Fas gene causes hyperplasia in peripheral lymphoid organs and liver. *Nat Genet* *11*, 294-300.

Adachi, M., Watanabe-Fukunaga, R., and Nagata, S. (1993). Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of lpr mice. *Proc Natl Acad Sci U S A* *90*, 1756-1760.

Frey, A., Di Canzio, J., and Zurakowski, D. (1998). A statistically defined endpoint titer determination method for immunoassays. *J Immunol Methods* *221*, 35-41.

Katona, I.M., Urban, J.F., Jr., Scher, I., Kanellopoulos-Langevin, C., and Finkelman, F.D. (1983). Induction of an IgE response in mice by *Nippostrongylus brasiliensis*: characterization of lymphoid cells with intracytoplasmic or surface IgE. *J Immunol* *130*, 350-356.

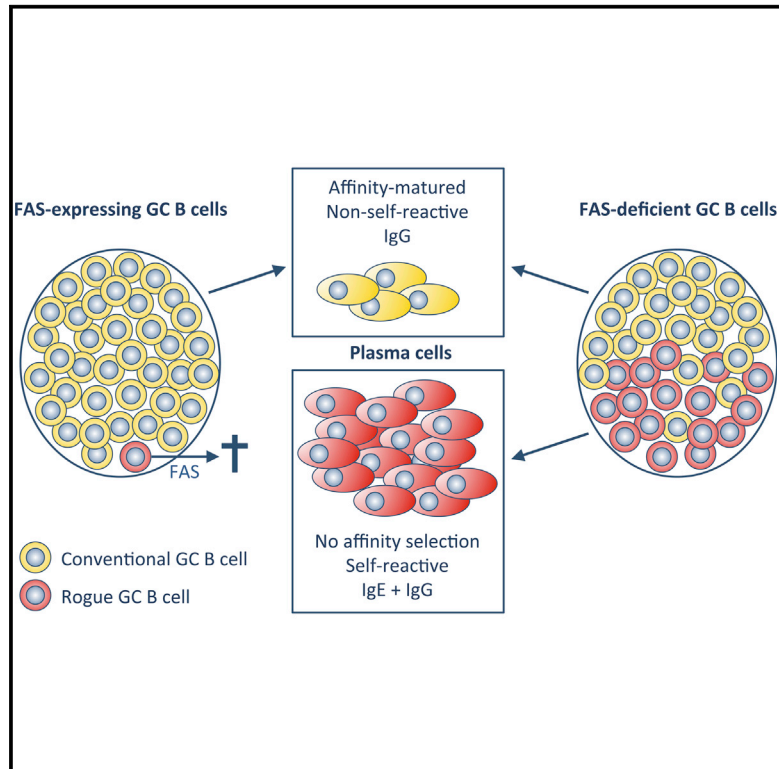
Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., and Papaioannou, V.E. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* *68*, 869-877.

Wettenhall, J.M., and Smyth, G.K. (2004). limmaGUI: a graphical user interface for linear modeling of microarray data. *Bioinformatics* *20*, 3705-3706.

Immunity

FAS Inactivation Releases Unconventional Germinal Center B Cells that Escape Antigen Control and Drive IgE and Autoantibody Production

Graphical Abstract



Authors

Danyal Butt, Tyani D. Chan, ...,
V. Koneti Rao, Robert Brink

Correspondence

r.brink@garvan.org.au

In Brief

FAS deficiency is associated with autoimmunity. Brink and colleagues show that FAS prevents development of “rogue” germinal center (GCr) B cells that escape normal regulatory controls and produce high amounts of IgE and autoantibodies, which might explain the presence of such antibodies in autoimmune disease.

Highlights

- FAS is not required to eliminate conventional self-reactive GC B cells
- FAS is required to prevent development of rogue GC (GCr) B cells
- GCr B cells escape normal selection and show extensive plasma cell differentiation
- GCr B cells might explain the production of IgE and autoantibodies in ALPS and SLE



FAS Inactivation Releases Unconventional Germinal Center B Cells that Escape Antigen Control and Drive IgE and Autoantibody Production

Danyal Butt,^{1,2,6} Tyani D. Chan,^{1,2,6} Katherine Bourne,¹ Jana R. Hermes,¹ Akira Nguyen,¹ Aaron Statham,¹ Lorraine A. O'Reilly,^{3,4} Andreas Strasser,^{3,4} Susan Price,⁵ Peter Schofield,¹ Daniel Christ,^{1,2} Antony Basten,^{1,2} Cindy S. Ma,^{1,2} Stuart G. Tangye,^{1,2} Tri Giang Phan,^{1,2} V. Koneti Rao,⁵ and Robert Brink^{1,2,*}

¹Immunology Division, Garvan Institute of Medical Research, 384 Victoria St., Darlinghurst, NSW 2010, Australia

²St. Vincent's Clinical School, UNSW Australia, Darlinghurst, NSW 2010, Australia

³Molecular Genetics of Cancer Division, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, VIC 3052, Australia

⁴Department of Medical Biology, The University of Melbourne, Parkville, VIC 3010, Australia

⁵Molecular Development Section, Laboratory of Immunology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD 20892, USA

⁶Co-first author

*Correspondence: r.brink@garvan.org.au

<http://dx.doi.org/10.1016/j.immuni.2015.04.010>

SUMMARY

The mechanistic links between genetic variation and autoantibody production in autoimmune disease remain obscure. Autoimmune lymphoproliferative syndrome (ALPS) is caused by inactivating mutations in *FAS* or *FASL*, with autoantibodies thought to arise through failure of FAS-mediated removal of self-reactive germinal center (GC) B cells. Here we show that FAS is in fact not required for this process. Instead, FAS inactivation led to accumulation of a population of unconventional GC B cells that underwent somatic hypermutation, survived despite losing antigen reactivity, and differentiated into a large population of plasma cells that included autoantibody-secreting clones. IgE⁺ plasma cell numbers, in particular, increased after FAS inactivation and a major cohort of ALPS-affected patients were found to have hyper-IgE. We propose that these previously unidentified cells, designated “rogue GC B cells,” are a major driver of autoantibody production and provide a mechanistic explanation for the linked production of IgE and autoantibodies in autoimmune disease.

INTRODUCTION

Production of pathogenic antibodies by B lymphocytes contributes significantly to inflammatory disease. Autoantibodies, typically of the immunoglobulin G (IgG) class, are involved in many human autoimmune diseases including systemic lupus erythematosus (SLE), bullous pemphigoid (BP), and autoimmune lymphoproliferative syndrome (ALPS). Additionally, airborne or ingested proteins can trigger the production of IgE, with resulting induction of allergic diseases such as asthma and food allergies. The processes that regulate autoantibody and allergic IgE production

are often considered separately because of the distinct nature of the antigens targeted (self versus foreign). Nevertheless, the presence of IgE as well as IgG autoantibodies in autoimmune diseases has been recognized since the 1970s (Permin and Wiik, 1978; Provost and Tomasi, 1974) and strong evidence exists that IgE autoantibodies contribute to disease pathogenesis in BP (Messingham et al., 2014) as well as human and murine SLE (Dema et al., 2014a, 2014b). However, the mechanisms underlying the production of IgE together with IgG autoantibodies remain unknown.

A key component of adaptive humoral immunity is the germinal center (GC) reaction. GCs are transient microanatomical structures in which B cells activated by foreign antigen undergo iterative cycles of proliferation and somatic hypermutation (SHM) of their Ig variable region genes to yield the high-affinity antibodies required for long-term immunity (Victora and Nussenzweig, 2012). Because of the random nature of SHM, the development of self-reactive B cells within the GC is inevitable (Nossal, 1988). We showed recently that self-reactive B cells are rapidly removed from the GC and thus prevented from undergoing differentiation into autoantibody-producing plasma cells (PCs) (Chan et al., 2012). However, this process is compromised under some circumstances as indicated by the fact that GCs are a major source of pathogenic IgG autoantibodies (Brink, 2014). In particular, mutations affecting the expression and/or function of molecules involved in the removal of self-reactive GC B cells are likely to make significant contributions to autoimmune diseases such as SLE, BP, and ALPS.

The pro-apoptotic TNF receptor superfamily member FAS is highly expressed on GC B cells (Liu et al., 1995; Smith et al., 1995). Mutations in the genes encoding FAS or its ligand (FASL) result in ALPS in humans (Fisher et al., 1995; Rieux-Laucat et al., 1995; Wu et al., 1996) and model ALPS and SLE in mice (Andrews et al., 1978; Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992). Significantly, the selective removal of FAS from activated B cells results in ALPS-like disease and autoantibody production (Hao et al., 2008). These findings have led to a widely accepted view that the autoimmune predisposition of individuals with *FAS*/*FASL* mutations derives at least in part from a

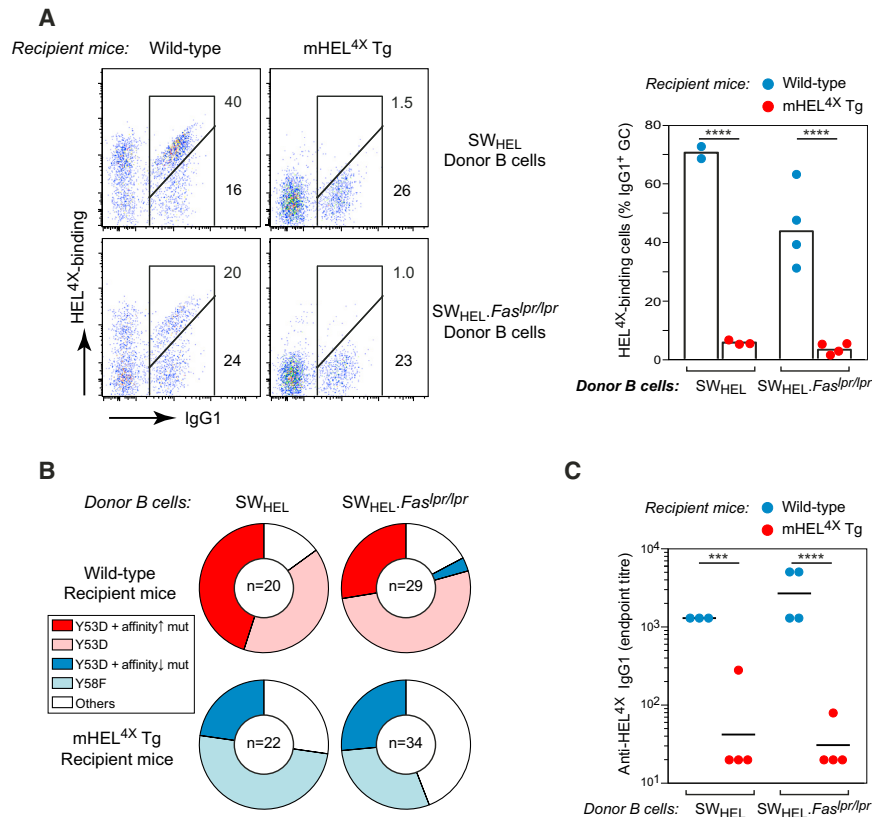


Figure 1. FAS-Mediated Apoptosis Is Not Required to Remove Conventional Self-Reactive GC B Cells

SW_{HEL} or SW_{HEL}.Fas^{Ipr/Ipr} B cells were transferred into wild-type or mHEL^{4X} Tg recipient mice, challenged with HEL^{3X}-SRBCs, and analyzed on day 21. Data are representative of three independent experiments.

(A) Left: Flow cytometric analysis of donor-derived splenic GC B cells (CD45.1⁺CD45.2⁻B220⁺CD38^{lo}). Profiles are concatenated from four mice per group. Right: Summary data from one experiment indicating the proportion of HEL^{4X}-binding cells among IgG1⁺ GC B cells using the windows shown on the flow cytometry panels.

(B) Single-cell SHM analysis of donor-derived splenic GC B cells. Mutations that increase or decrease the affinity of Y53D for HEL^{4X} are S31R/Y58F and L4F/N94K, respectively (see Figure S1). n = number of clones analyzed. Data were obtained from three pooled wild-type or mHEL^{4X} Tg recipient mice. Data from wild-type donor SW_{HEL} B cells have been previously published (Chan et al., 2012).

(C) Endpoint titers of HEL^{4X}-binding IgG1 determined by serum ELISA.

failure to eliminate self-reactive GC B cells (Ait-Azzouzene et al., 2010; Hao et al., 2008). Nevertheless, direct evidence that FAS carries out this function in the GC has remained elusive.

Here we utilize a high-resolution in vivo murine model to unambiguously identify the role of FAS in controlling GC B cells. FAS was found to be dispensable for the removal of conventional self-reactive GC B cells. FAS expression by responding B cells was instead found to be essential for preventing the emergence of a population of unconventional “rogue” GC (GCr) B cells that persist and undergo SHM and PC differentiation independent of normal antigen-dependent regulation. Surprisingly, PCs derived from GCr (PCr) B cells included a large fraction of IgE-producing cells, leading us to identify a large cohort of non-atopic human ALPS patients with hyper-IgE. A proportion of PCr B cells also developed autoreactivity and produced high titers of serum autoantibodies. The identification of GCr B cells explains how B-cell-expressed FAS safeguards against autoantibody responses. It also provides a mechanism for the previously unexplained link between IgG and IgE autoantibody production in antibody-mediated autoimmune diseases.

RESULTS

FAS Expression Is Not Required for the Deletion of Self-Reactive GC B Cells

To test whether FAS is required for the elimination of self-reactive GC B cells, we compared the in vivo responses of antigen-specific B cells that differed only in their ability to express FAS. B cells from wild-type or FAS-deficient (Fas^{Ipr/Ipr}) SW_{HEL} mice (expressing the

hen egg lysozyme [HEL] specificity of the HyHEL10 mAb) (Phan et al., 2003) were transferred into CD45 congenic recipient mice (CD45.1⁺ cells into CD45.2⁺ mice) and challenged with antigen in the form of recombinant HEL^{3X} protein (HEL^{R21Q,R73E,D101R}) coupled to sheep red blood cells (HEL^{3X}-SRBCs) (Paus et al., 2006). SW_{HEL} B cells bind HEL^{3X} with an affinity (K_a) of $\sim 10^7$ M⁻¹ but cannot recognize the closely related HEL^{4X} protein (HEL^{3X,K97R}) (Chan et al., 2012). In wild-type recipient mice, SW_{HEL} GC B cells acquire cross-reactivity with HEL^{4X} as they undergo affinity maturation to HEL^{3X} (Figure 1A). In membrane HEL^{4X} transgenic (mHEL^{4X} Tg) recipients, however, self-tolerance prevents such cells from accumulating (Figure 1A; Chan et al., 2012). When SW_{HEL}.Fas^{Ipr/Ipr} donor B cells were used, self-reactive HEL^{4X}-binding GC B cells still failed to develop in mHEL^{4X} Tg recipients (Figure 1A), and clones carrying the HEL^{4X}-reactive Y53D Ig heavy chain substitution were strongly counter-selected or edited with complementary, deleterious somatic mutations (Figures 1B and S1; Chan et al., 2012). Accordingly, anti-HEL^{4X} serum antibody was absent from mHEL^{4X} Tg recipients on day 21 of the response regardless of whether or not the donor SW_{HEL} B cells expressed FAS (Figure 1C). Thus, contrary to previous assumptions (Ait-Azzouzene et al., 2010; Hao et al., 2008), the expression of FAS by self-reactive B cells generated within the GC is not required for their elimination.

FAS-Deficient Mice and Humans Produce Large Amounts of IgE Antibodies

Although self-reactive GC B cells were removed normally, the response of SW_{HEL}.Fas^{Ipr/Ipr} B cells to HEL^{3X}-SRBCs did exhibit aberrant features. Notably, splenic basophils from recipient mice (CD45.2⁺B220⁻c-Kit⁻CD49b⁺) acquired high HEL-binding activity in recipients of SW_{HEL}.Fas^{Ipr/Ipr} B cells but not in recipients of

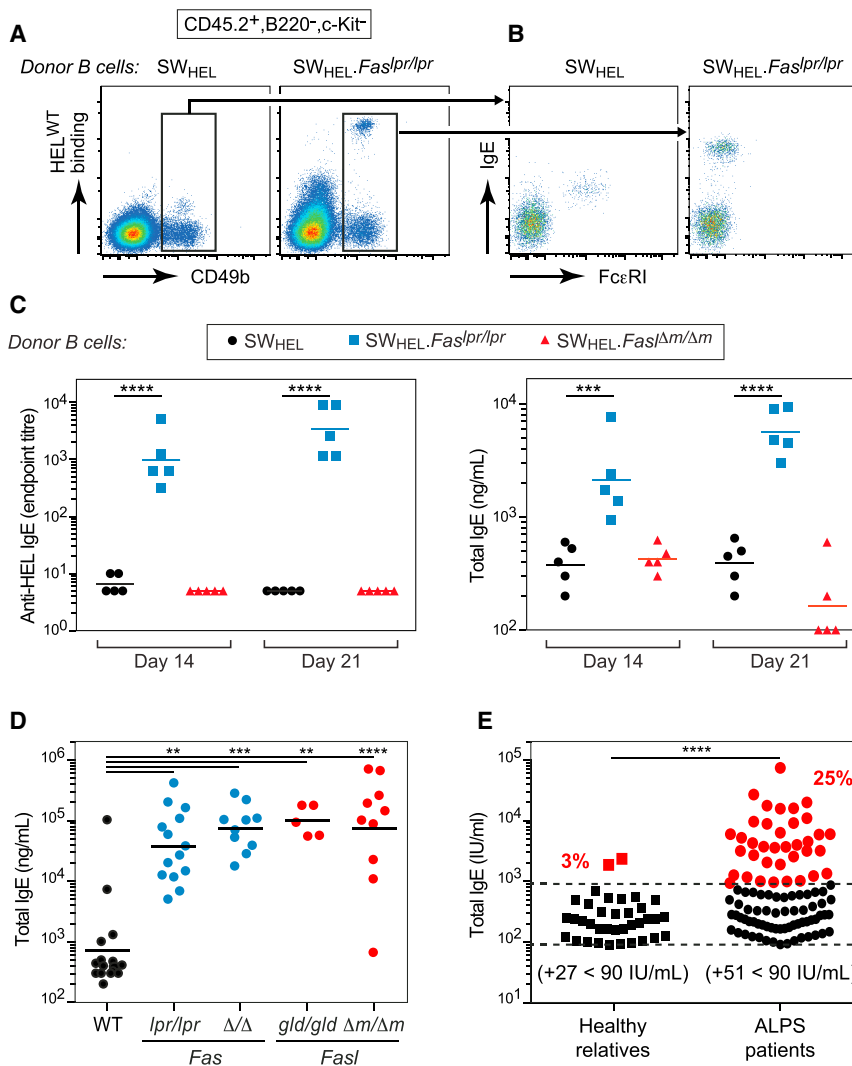


Figure 2. FASL-FAS Suppress IgE Antibody Production in Mice and Humans

(A and B) Flow cytometric analysis of endogenous splenic basophils (CD45.2⁺B220⁺c-Kit⁻CD49b⁺) in wild-type recipients of SW_{HEL} or SW_{HEL}.Fas^{lpr/lpr} B cells. Note that the anti-FcεRI mAb is blocked by receptor-bound IgE. Data are representative of two independent experiments.

(C) SW_{HEL}, SW_{HEL}.Fas^{lpr/lpr}, or SW_{HEL}.Fas^{Δm/Δm} B cells were transferred into wild-type recipient mice and challenged with HEL^{3X}-SRBCs, and sera were taken at days 14 and 21 and analyzed by ELISA. HEL^{WT}-binding IgE (left) was determined by endpoint titer and total IgE was also measured (right). Data are representative of two independent experiments.

(D) Total serum IgE in 16- to 18-week-old wild-type C57BL/6 mice compared to those lacking functional FAS (Fas^{lpr/lpr}, Fas^{Δ/Δ}) or FASL (Fas^{gld/gld}, Fas^{Δm/Δm}).

(E) Total serum IgE in ALPS patients and healthy relatives. Data points < 90 U/ml not shown.

For all figures, significant differences are indicated by asterisk(s). A full explanation of statistical analyses is available in the [Experimental Procedures](#).

wild-type SW_{HEL} B cells (Figure 2A). This occurred regardless of the recipients' expression of mHEL^{4X} (data not shown). Flow cytometric analysis via a mAb that is blocked from binding the high-affinity IgE receptor (FcεRI) by receptor-bound IgE revealed that the FcεRI on these basophils were almost completely saturated with anti-HEL IgE (Figure 2B), suggesting that SW_{HEL}.Fas^{lpr/lpr} B cells were generating large amounts of IgE antibody when challenged with HEL^{3X}-SRBCs.

To test this directly, serum antibody responses in wild-type recipient mice were analyzed on day 21 after transfer and challenge of SW_{HEL} or SW_{HEL}.Fas^{lpr/lpr} B cells. An additional group received SW_{HEL} B cells lacking the membrane form of FASL (SW_{HEL}.Fas^{Δm/Δm} B cells) (O'Reilly et al., 2009). SW_{HEL} and SW_{HEL}.Fas^{Δm/Δm} B cells challenged with HEL^{3X}-SRBCs in wild-type recipient mice failed to mount serum IgE responses (Figure 2C). In contrast, SW_{HEL}.Fas^{lpr/lpr} B cells initiated a strong response, with anti-HEL serum IgE reaching 5–10 μg/ml by day 21 (Figure 2C), verifying that B cells lacking FAS (but not membrane FASL) produce anomalously high IgE antibody responses.

A link between FAS deficiency and IgE production has been suggested from observations in Fas^{lpr/lpr} BALB/c mice (Takaha-

shi et al., 2013). We confirmed that a similar connection exists on the C57BL/6 genetic background, as shown by the fact that mice lacking active FAS due to either a natural (Fas^{lpr/lpr}) or induced (Fas^{Δ/Δ}) mutation possess serum IgE concentrations 50–100 times higher than those in wild-type controls (Figure 2D).

C57BL/6 mice in which FASL binding to FAS was disrupted by a natural mutation (Fas^{gld/gld}) or altered such that the membrane form of the ligand (essential for FAS-mediated apoptosis) can no longer be expressed (Fas^{Δm/Δm}) possessed a similar hyper-IgE phenotype (Figure 2D). Overall, these results indicated that the interaction of membrane-bound FASL with FAS expressed on responding B cells suppresses IgE antibody production in mice. Abnormally high serum IgE is not a recognized clinical feature of ALPS. However, because FAS-FASL function is highly conserved between humans and mice (Fisher et al., 1995; Rieux-Laucat et al., 1995; Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992; Wu et al., 1996), we hypothesized that a subset of ALPS-affected patients would exhibit a hyper-IgE phenotype. Strikingly, retrospective analysis of clinical data from 150 patients and 63 unaffected relatives in the National Institute of Allergy and Infectious Diseases ALPS-FAS study (Price et al., 2014) revealed that 25% of ALPS-affected patients possessed serum IgE concentrations 10–8,000 times higher than the upper limit of the normal range (90 U/ml) and only 3% of healthy relatives fell into this category (Figure 2E). Interestingly, there was no increase in the incidence of allergic diseases among ALPS-affected patients with hyper-IgE (data not shown). Taken together, these data indicate that FAS plays a critical and conserved role in limiting the production of IgE in both humans and mice.

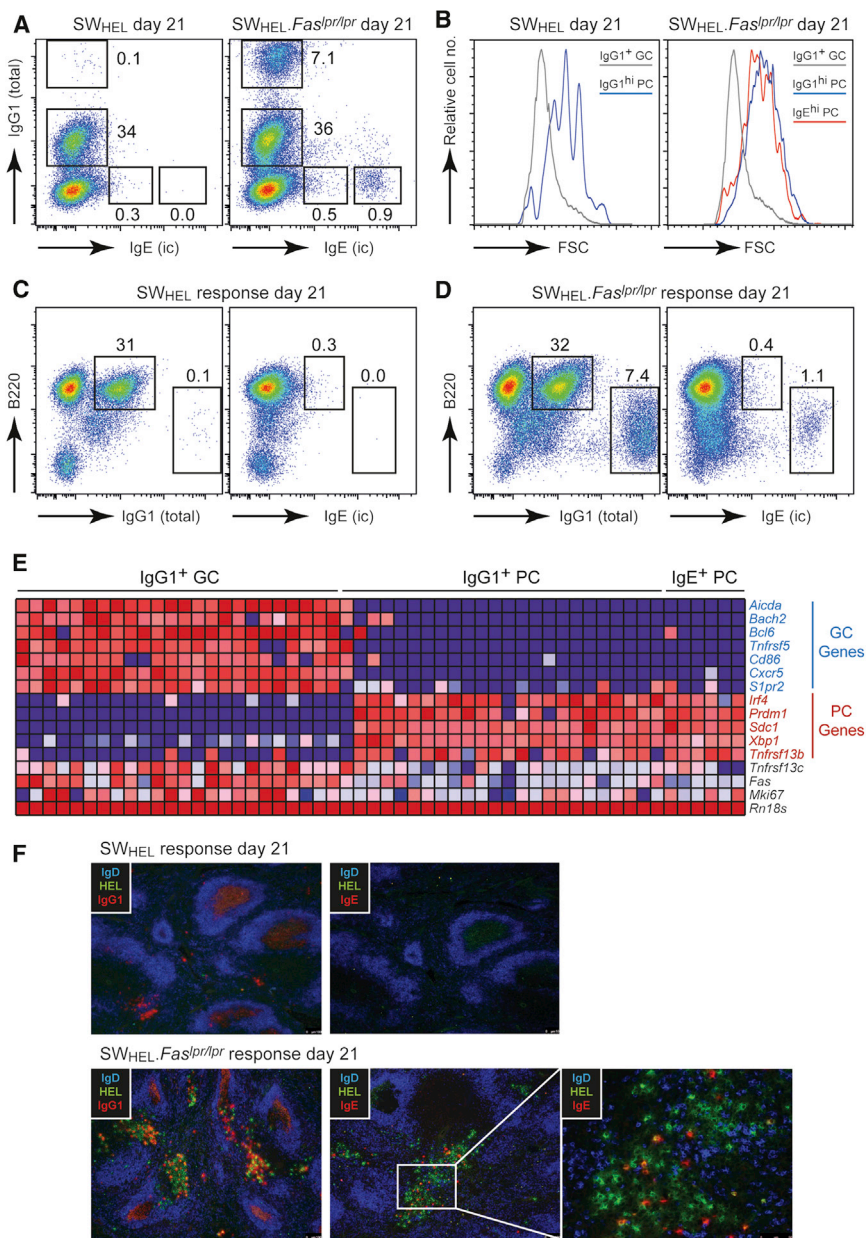


Figure 3. FAS-Deficient B Cells Generate Large Numbers of IgG1⁺ and IgE⁺ PCs

SW_{HEL} or SW_{HEL.Fas^{lpr/lpr}} B cells were transferred into wild-type recipient mice, challenged with HEL^{3X}-SRBCs, and analyzed on day 21.

(A) Flow cytometric analysis of donor-derived splenic GC B cells plus PCs (CD45.1⁺CD45.2⁻CD38^{lo}; see Figure S2A) using staining for total IgG1 and intracellular (i.c.) IgE after cell fixation and permeabilization. IgG1⁺ GC = IgG1^{lo}IgE⁻; IgG1⁺ PC = IgG1^{hi}IgE⁻; IgE⁺ PC = IgG1⁻IgE^{hi}. (B) Histogram overlay of forward light scatter (FSC) of indicated populations of donor-derived cells from (A).

(C and D) Flow cytometric analysis of donor-derived GC B cells plus PCs as for (A) showing surface B220 expression.

Plots in (A)–(D) comprise concatenated data from five mice and are representative of three independent experiments.

(E) Single-cell qPCR analysis of donor-derived subsets (see Figure S2B for gating) from splenic SW_{HEL.Fas^{lpr/lpr}} response. Relative mRNA expression is indicated from high (red) to low (blue).

(F) Immunofluorescence histology of SW_{HEL} and SW_{HEL.Fas^{lpr/lpr}} responses in recipient spleens. Colors representing the various stains are indicated.

staining GC B cell counterparts. To avoid detecting surface CD23-bound IgE, samples were blocked with unconjugated anti-IgE before cell permeabilization and intracellular IgE stores selectively stained with conjugated anti-IgE antibody (Yang et al., 2012). On day 21, wild-type SW_{HEL} B cell responses were characterized by a large IgG1⁺ GC population, small numbers of IgG1^{hi} PCs, and a virtual absence of IgE⁺ cells (Figure 3A). In contrast, the response of SW_{HEL.Fas^{lpr/lpr}} B cells included a 50- to 100-fold expansion of IgG1^{hi} PCs and the emergence of a clearly resolvable population of IgE^{hi} PCs (Figure 3A). As expected, IgE⁺ GC B cells were rare presumably due to their transient nature (Yang et al., 2012).

IgG1^{hi} and IgE^{hi} cells were verified as PCs by their larger size (forward light scatter) relative to IgG1⁺ GC B cells (Figure 3B) and their B220^{lo} phenotype (Figures 3C and 3D). Single-cell analysis (Figure S2B) of cells from the various SW_{HEL.Fas^{lpr/lpr}} B-cell-derived populations revealed gene expression profiles corresponding to those expected for GC B cells and PCs (Figure 3E). Finally, IgE⁺ cells were also located exclusively within the extrafollicular regions of the spleen (Figure 3F), consistent with their identity as antibody-secreting PCs.

IgE⁺ PCs Arise Late in the Response and Derive from GC B Cell Precursors

We next performed a kinetic analysis on the responses of SW_{HEL} B cells lacking functional FAS due to either the *Fas^{lpr}* mutation (SW_{HEL.Fas^{lpr/lpr}}) or targeted ablation of the FAS cytoplasmic

FAS-Deficient B Cells Produce Large Numbers of IgG⁺ and IgE⁺ PCs

To characterize the FAS-deficient cells responsible for IgE production, SW_{HEL} and SW_{HEL.Fas^{lpr/lpr}} B cells were challenged with HEL^{3X}-SRBCs in wild-type recipient mice, identified by flow cytometry, (Figure S2A) and analyzed for IgE expression. IgG1⁺ cells were also examined because these typically form the precursors for IgE-switched cells and IgG1 is the major Ig subclass produced after challenge of SW_{HEL} B cells with HEL^{3X}-SRBCs (Chan et al., 2009; Erazo et al., 2007; Yang et al., 2012). Because previous studies had indicated that IgE-switched B cells exist primarily as plasma cells (PCs) (Erazo et al., 2007; Yang et al., 2012), donor-derived (CD45.1⁺CD45.2⁻) B cells were stained for IgG1 and IgE after fixation and permeabilization to access intracellular antibody stores and thus resolve brightly staining PCs from their more dimly

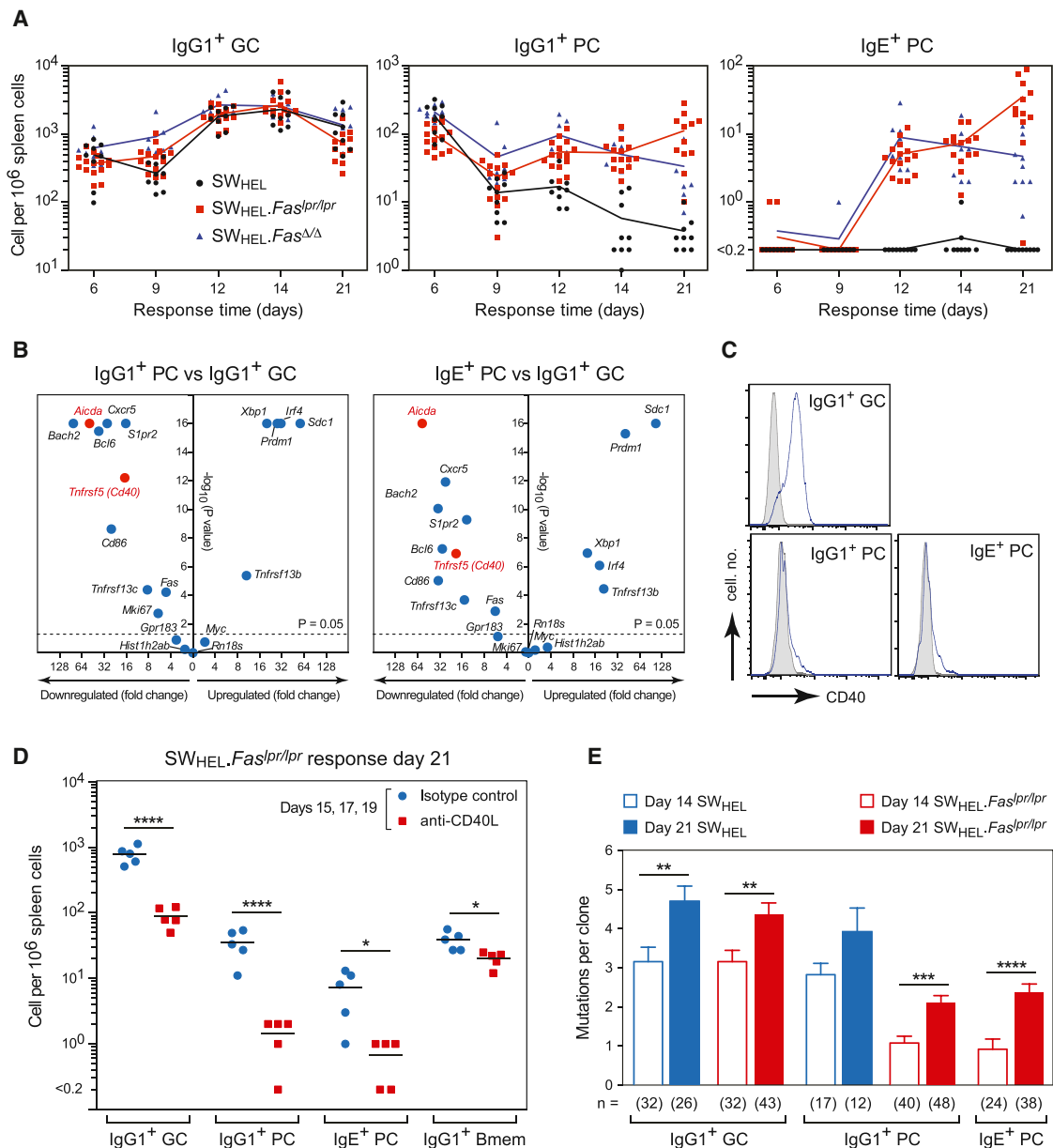


Figure 4. FAS-Deficient IgG1⁺ and IgE⁺ PCs Derive from GC B Cell Precursors

(A) Time course of donor-derived splenic responses of SW_{HEL}, SW_{HEL}.Fas^{lpr/lpr}, and SW_{HEL}.Fas^{Δ/Δ} B cells. Data pooled from two independent experiments. (B) Volcano plot of qPCR data from Figure 3E (SW_{HEL}.Fas^{lpr/lpr} B cell response to HEL^{3X}-SRBCs) showing fold-change and p values for individual genes between IgG1⁺ GC B cells and IgG1⁺ or IgE⁺ PCs. (C) Overlay of CD40 staining on cells derived from day 14 splenic response of SW_{HEL}.Fas^{lpr/lpr} B cells to HEL^{3X}-SRBCs. (D) Frequency of splenic donor-derived subsets from SW_{HEL}.Fas^{lpr/lpr} B cells challenged with HEL^{3X}-SRBC on day 21 after 5 days treated with blocking anti-CD40L antibody or isotype control. (E) Mutation rates in the Ig heavy chain variable region gene in donor-derived splenic subsets (see Figure 3B for gating) from SW_{HEL} or SW_{HEL}.Fas^{lpr/lpr} B cells challenged with HEL^{3X}-SRBCs. n = number of clones analyzed. Error bars indicate the SEM.

domain (SW_{HEL}.Fas^{Δ/Δ}). The early (day 6) response mounted by FAS-deficient and wild-type SW_{HEL} B cells included a burst of IgG1⁺ PCs but no detectable IgE⁺ PCs (Figure 4A). IgE⁺ PCs did not emerge until between days 9 and 12 of the response and only when responding B cells lacked functional FAS. The appearance of IgE⁺ PCs corresponded with a second wave of IgG1⁺ PCs from FAS-deficient SW_{HEL} B cells and a ~5-fold

expansion in IgG1⁺ and total GC B cells (Figures 4A and S3A). This late emergence of IgE⁺ PCs in parallel with GC expansion suggested that IgE⁺ PCs are derived from GC B cell precursors rather than from persistence of early GC-independent plasmablasts (Chan et al., 2009; William et al., 2002).

To test this proposition, we first examined the effect of ablating the GC B cell response with anti-CD40L mAb (Han et al., 1995a).

Because the PCs produced by $SW_{HEL}.Fas^{lpr/lpr}$ B cells do not express CD40 (*Tnfrsf5*) (Figures 3E, 4B, and 4C), they should be resistant to CD40L blockade if they represent a persisting autonomous population derived from the early plasmablast response. On the other hand, if they are continually replenished from GC precursors, they will be depleted in parallel with GC B cells. Examination of the day 21 $SW_{HEL}.Fas^{lpr/lpr}$ B cell response to HEL^{3X}-SRBCs after 5 days of treatment with anti-CD40L showed that the latter scenario applied, with the numbers of GC B cells as well as IgG1⁺ and IgE⁺ PCs in each case depleted by ~10-fold (Figure 4D). Further confirmation that these PCs are replenished from GC precursors was provided by single-cell sorting (Figure S3B) and SHM analysis. As predicted, both IgG1⁺ and IgE⁺ PCs were somatically mutated and showed increased SHM frequency over the course of the $SW_{HEL}.Fas^{lpr/lpr}$ B cell response (Figure 4E). Because the PCs produced by $SW_{HEL}.Fas^{lpr/lpr}$ B cells do not express AID (*Aicda*) (Figures 3E and 4B), they could not undergo SHM autonomously but must be replenished from AID-expressing, somatically mutating GC B cell precursors.

FAS-Deficient GC B Cells Escape Normal Selection and Produce IgG⁺ and IgE⁺ PCs

The production of PCs from GC B cell precursors is normally tightly regulated such that only those clones with the highest affinity for antigen are selected to undergo PC differentiation (Phan et al., 2006). This is evident in day 21 responses of SW_{HEL} B cells to HEL^{3X}-SRBCs where, similar to IgG1⁺ GC B cells, IgG1^{hi} PCs bind large amounts of HEL^{3X} when stained with limiting concentrations of antigen (Figure 5A). They also invariably carry the high-affinity Ig heavy chain substitution Y53D, often with the additional affinity-enhancing substitutions S31R and/or Y58F (Figure 5C; Chan et al., 2012; Phan et al., 2006). In contrast, very few of the IgG1^{hi} and IgE^{hi} PCs produced during the $SW_{HEL}.Fas^{lpr/lpr}$ B cell response bound HEL^{3X} with high affinity (Figure 5B) or carried mutations normally associated with affinity maturation to HEL^{3X} (Y53D, S31R, Y58F) (Figure 5D). Paradoxically, affinity maturation of $SW_{HEL}.Fas^{lpr/lpr}$ GC B cells progressed relatively normally, with most developing high affinity for HEL^{3X} as indicated by flow cytometry (Figure 5B) and SHM analysis (Figure 5D). Absent FAS expression on responding B cells therefore disrupts the normal regulation of PC differentiation, favoring the production of PCs from “rogue,” non-affinity regulated precursors that must exist at relatively low frequencies within the GC.

To investigate this further, we first bred $SW_{HEL}.Fas^{lpr/lpr}$ mice onto a *Rag1*^{ΔΔ} background to eliminate the possibility that these rare GC B cells might express an alternative BCR. B cells from $SW_{HEL}.Fas^{lpr/lpr}.Rag1^{ΔΔ}$ mice responded identically to those from $SW_{HEL}.Fas^{lpr/lpr}$ mice, generating expanded numbers of somatically mutated IgG1⁺ and IgE⁺ PCs that showed little evidence of affinity maturation to HEL^{3X} (Figures S4C and S4D). SHM analysis of donor-derived spleen cells from mice given $SW_{HEL}.Fas^{lpr/lpr}.Rag1^{ΔΔ}$ B cells and HEL^{3X}-SRBCs revealed that IgG1⁺ and IgE⁺ PC populations were both clonally restricted and clonally related within individual recipient mice (Figures 5E and S5). IgG1⁺ GC B cells clonally related to these PCs were rare and typically represented only 1%–3% of GC B cells (Figures 5E and S5) but could be enriched by isolation of IgG1⁺ GC B cells with low affinity for HEL^{3X}. By this approach, sufficient numbers of related GC clones were obtained to facilitate a

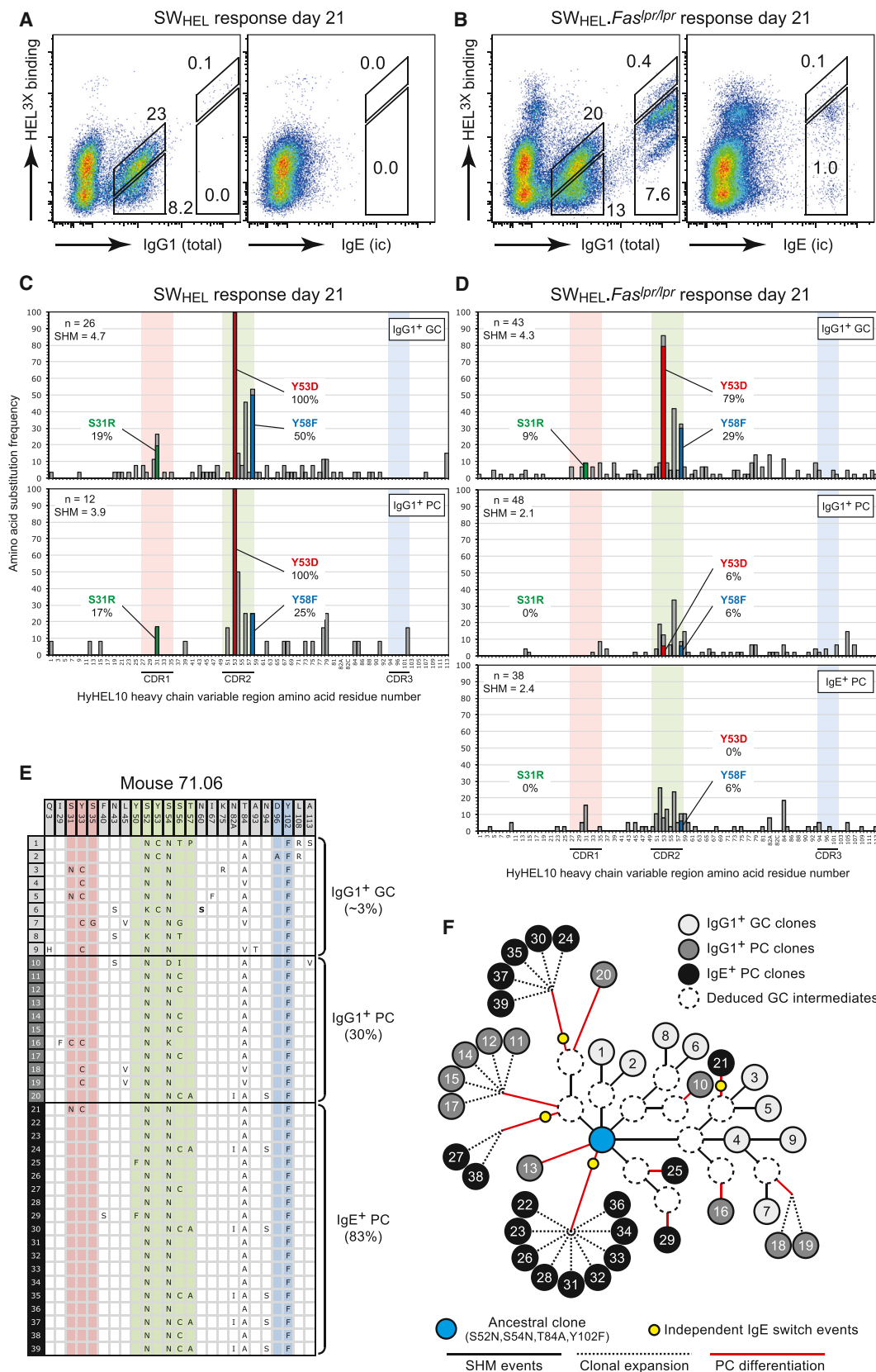
detailed analysis of the clonal evolution of a family of GC B cells and their PC derivatives (PCr). This analysis revealed that the generation of PCr from GC B cells is an ongoing process in which multiple PC differentiation and IgE switching events occur (Figure 5F). Moreover, as predicted by their abnormal SHM patterns and low HEL^{3X}-binding activity, mAbs carrying the variable regions expressed by these clones bound HEL^{3X} antigen with low and often biologically irrelevant ($K_a < 10^5 \text{ M}^{-1}$) affinities (Figure S5A). Unlike the SHM patterns that arise during affinity maturation, the amino acid substitutions observed among rogue FAS-deficient clones were unique to individual mice (Figures 5E and S5). Nevertheless, PCr from individual mice were never observed to be from just a single clonal family (e.g., Figure S5B), indicating that these cells derive from multiple ancestral clones and possibly distinct GCs.

GCr B Cells Accumulate and Generate Late-Response, Autoantibody-Secreting PCs

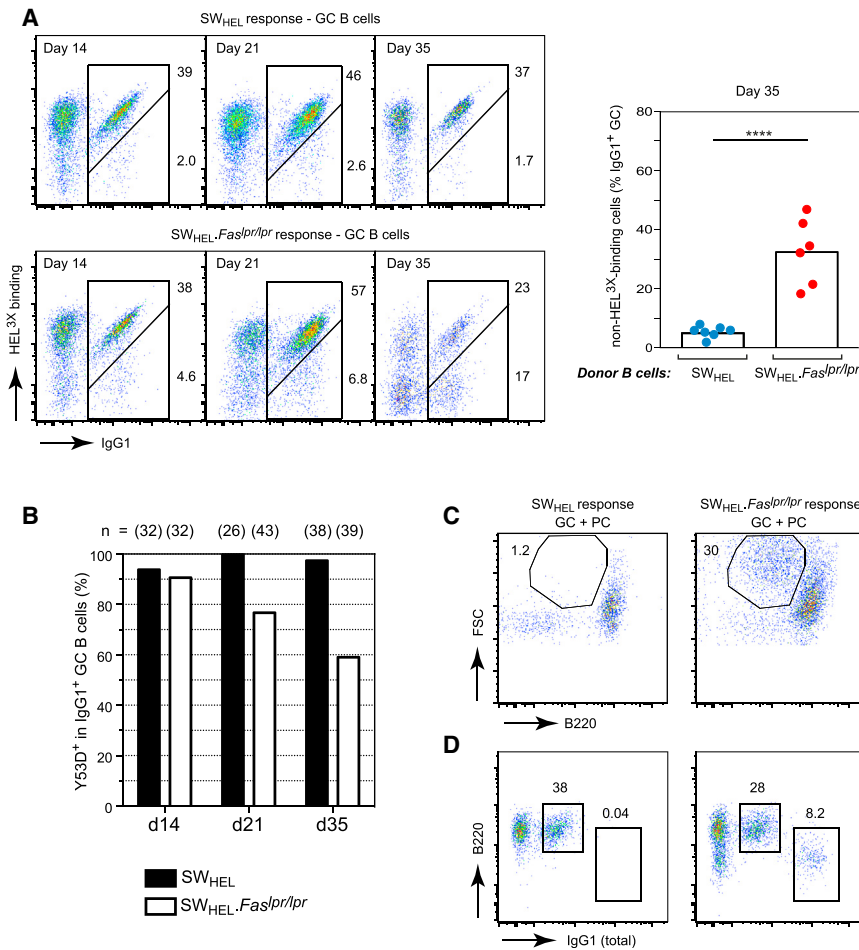
Because GC B cells exhibit ongoing SHM and PC differentiation and are not subject to conventional antigen regulation, we postulated that the emergence and accumulation of such cells might be responsible for the production of autoantibodies that ensues when B cells lack functional FAS. To test this possibility, we first examined the extent to which GCr B cells accumulate over the course of FAS-deficient B cell responses. As previously observed (Figures 5C and 5D), early GC B cell affinity maturation occurs regardless of B cell FAS expression, with >90% of both SW_{HEL} and $SW_{HEL}.Fas^{lpr/lpr}$ GC B cells exhibiting high affinity for HEL^{3X} as well as the Y53D substitution on day 14 of the response (Figures 6A and 6B). As expected, high-affinity wild-type SW_{HEL} GC B cell clones remained dominant until at least day 35 of the response. In contrast, low-affinity and Y53D[−] clones progressively accumulated in GCs derived from $SW_{HEL}.Fas^{lpr/lpr}$ GC B cells, resulting in a “heretical” reversal of affinity maturation between days 14 and 35 of the response (Figures 6A and 6B). Correspondingly, sequence analysis of donor-derived cells from single recipient mice at late time points revealed increased clonal restriction within the GC such that clear groups of related (Y53D[−]) GCr clones with corresponding populations of expanded PCr often reached 10%–15% of the GC by days 35–42 (Figure S6) as opposed to 1%–3% on day 21 (Figures 5 and S5). Taken together, these data indicate that GCr B cells become increasingly prevalent over the course of responses mounted by $SW_{HEL}.Fas^{lpr/lpr}$ B cells.

We next determined whether the proclivity of GCr B cells to differentiate into PCs evident on day 21 was maintained at later stages of the response. Analyses performed on day 35 showed that, although wild-type SW_{HEL} PCs were completely absent by this time, a large fraction of both the total and IgG1⁺ splenic response of $SW_{HEL}.Fas^{lpr/lpr}$ donor B cells was still comprised of PCs (Figures 6C and 6D). These cells showed the characteristic clonal restriction (Figure S6) and lack of antigen binding (data not shown) of PCr. We therefore concluded that GCr B cells not only persist and form an increasingly large fraction of the GC response of FAS-deficient B cells but that they continue to produce antibody-secreting PCr long after the conventional PC production has shut down.

Finally, we tested whether the ongoing response by FAS-deficient GCr B cells results in the eventual production of



(legend on next page)



autoantibodies. To do this, we returned to the HEL^{4X} Tg transfer system utilized at the beginning of this study and examined whether self-tolerance, intact during the early response of SW_{HEL}.Fas^{Ipr/Ipr} B cells in HEL^{4X} Tg recipient mice (Figure 1), might break down at later stages when GCr and PCr become prominent. Measurement of serum anti-HEL^{4X} autoantibody concentrations revealed that they remained undetectable in HEL^{4X} Tg recipient mice over the course of the response of wild-type SW_{HEL} B cells to HEL^{3X}-SRBCs (Figure 7A). In contrast, anti-HEL^{4X} autoantibodies accumulated in HEL^{4X} Tg recipients of SW_{HEL}.Fas^{Ipr/Ipr} B cells such that, by day 35, the majority of mice exhibited high titers of serum anti-HEL^{4X} IgG1

chain variable regions of these clones revealed the clonal restriction patterns typical of PCr but including substitutions (Y53D, Y53N, Y58F) known to increase the affinity of HyHEL10 for HEL^{4X} (Figure S7). Accordingly, the core mutations present in each of three independent clonal PCr families identified in separate HEL^{4X} Tg recipients were verified to possess affinities (K_a) for HEL^{4X} ranging from $\sim 10^7$ to more than $5 \times 10^8 \text{ M}^{-1}$ (Figure 7D). Overall, these results demonstrate that B cell FAS is not required for the removal of conventional self-reactive GC B cells but is absolutely necessary to prevent the development and persistence of GCr B cells that escape normal antigen regulation and initiate IgE and autoantibody production.

Figure 5. FAS-Deficient IgG1⁺ and IgE⁺ PCs Develop from GCr B Cells that Escape Normal Antigen Regulation

(A–D) SW_{HEL} or SW_{HEL}.Fas^{Ipr/Ipr} B cells were challenged with HEL^{3X}-SRBCs as for Figure 3.

(A and B) Flow cytometric analysis as for Figures 3C and 3D except counterstained for binding of limiting (50 ng/ml) HEL^{3X} antigen to identify cells expressing high-affinity antibodies. Plots comprise concatenated data from five mice and are representative of three independent experiments.

(C and D) Ig heavy chain variable region replacement mutations in responding cell subsets. HEL^{3X} affinity-increasing mutations Y53D, S31R, and Y58F are highlighted. n, number of clones analyzed; SHM, mean number of mutations per clone.

(E) Ig heavy chain variable region replacement mutations in clonally related cells obtained from a single wild-type recipient mouse (71.06) on day 21 of the response of SW_{HEL}.Fas^{Ipr/Ipr}.Rag1^{Δ/Δ} B cells to HEL^{3X}-SRBCs. Percentages indicate the frequency of the related clones within the respective responder populations. IgG1⁺ GC clones include 2/58 from unfractionated cells and 4/40 from the 20% of cells with the lowest HEL^{3X}-binding activity.

(F) A common ancestral GC clone carrying the S52N, S54N, T84A, and Y102F substitutions diversified into all 39 clones identified in (E) via the indicated SHM, clonal expansion, and PC differentiation steps (SHM was assumed to be limited to GC B cells). Four independent IgE switching events were identified (yellow circles).

Figure 6. GCr B Cells Accumulate, Display Abnormal Antigen Selection, and Continue to Generate PCr

SW_{HEL} or SW_{HEL}.Fas^{Ipr/Ipr} B cells were challenged with HEL^{3X}-SRBCs in wild-type recipients as for Figure 5. Splenocytes were harvested at the indicated time points.

(A) Left: Cell surface staining with limiting (50 ng/ml) HEL^{3X} antigen to distinguish high- and low-affinity GC B cells. Right: Summary data from two experiments (combined) indicating the proportion of non-HEL^{3X}-binding cells among IgG1⁺ GC B cells on day 35 using the windows shown on the flow cytometry panels.

(B) SHM analysis of IgG1⁺ GC B cells for clones carrying the high-affinity anti-HEL^{3X} Y53D substitution. n = number of clones analyzed.

Late responses of SW_{HEL}.Fas^{Ipr/Ipr} B cells show anomalous accumulation of low-affinity (A) and Y53D⁺ (B) GC B cells.

(C and D) Identification of B220^{lo} PCs in CD38^{lo} donor-derived cells on day 35 response of SW_{HEL}.Fas^{Ipr/Ipr} but not SW_{HEL} B cells as revealed by high forward light scatter (FSC) (C) and high total IgG1 staining after fixing and permeabilization (D).

(Figure 7A) and IgG2b (data not shown; anti-HEL^{4X} IgE was not detectable). Verification that PCr were responsible for this production of anti-HEL^{4X} autoantibodies was obtained by the identification of donor-derived, HEL^{4X}-binding, IgG1⁺ and IgG1[−] PCs (Figures 7B and 7C). Single-cell sorting and analysis of the heavy

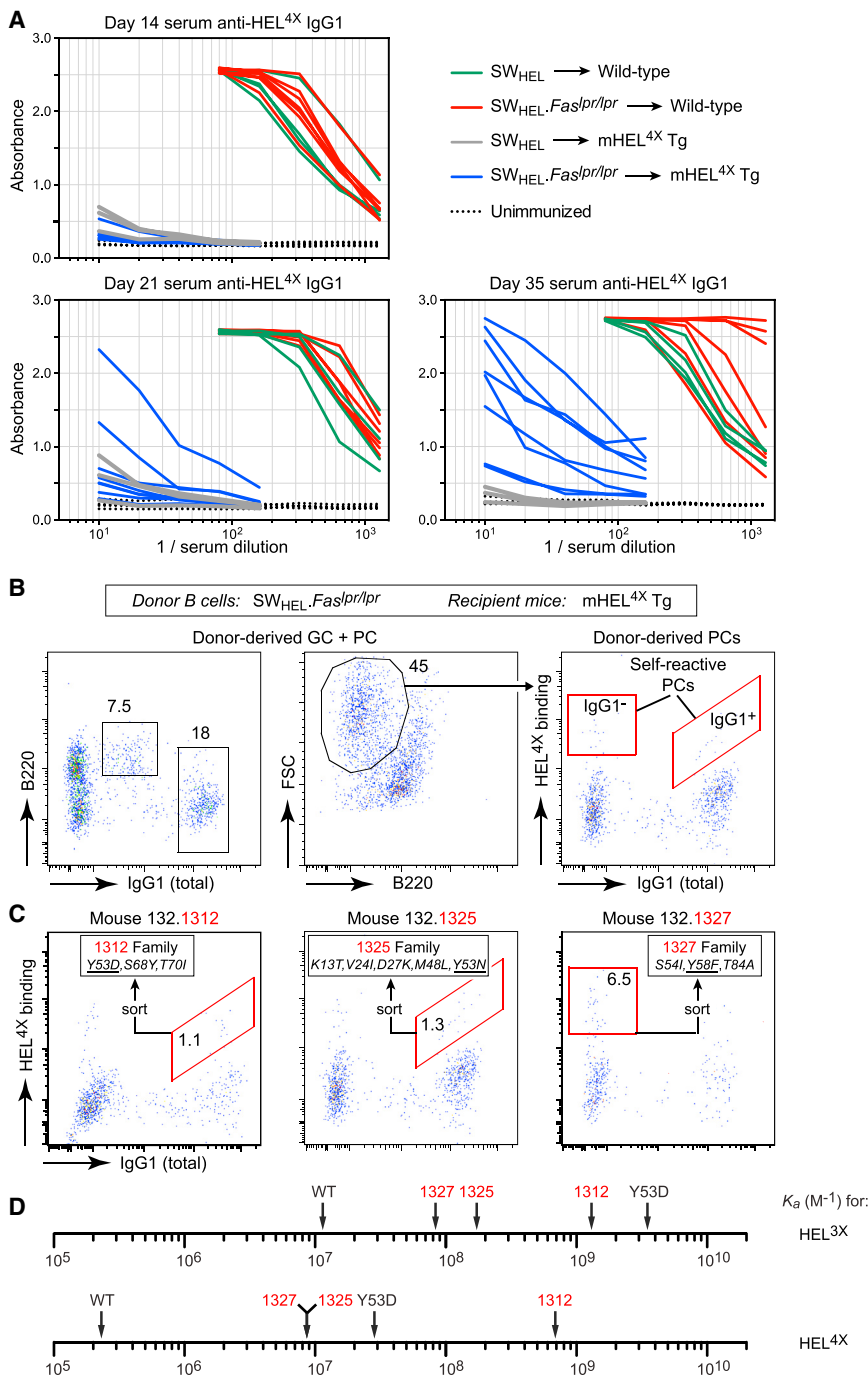


Figure 7. GCr B Cells Produce Autoantibody-Secreting PCr Late in the Response of SW_{HEL}.Fas^{lpr/lpr} B Cells to HEL^{3X}-SRBCs

SW_{HEL} or SW_{HEL}.Fas^{lpr/lpr} B cells were challenged with HEL^{3X}-SRBCs in wild-type or mHEL^{4X} Tg recipients as for Figure 1. Splenocytes and blood were harvested at the indicated time points.

(A) HEL^{4X}-binding IgG1 serum antibodies were measured by ELISA and reveal the absence (day 14), the initial emergence (day 21), and the accumulation (day 35) of autoantibodies in mHEL^{4X} Tg recipients of SW_{HEL}.Fas^{lpr/lpr} but not SW_{HEL} B cells.

(B and C) Detection of self-reactive PCs in mHEL^{4X} Tg recipients of SW_{HEL}.Fas^{lpr/lpr} B cells on day 35 of the response to HEL^{3X}-SRBCs.

(B) Donor-derived CD38^{lo} cells analyzed for HEL^{4X}-binding and total IgG1 after fixing and permeabilization showing IgG1⁺ and IgG1⁻ self-reactive PCs.

(C) Independent mHEL^{4X} Tg recipients showing self-reactive PC populations subjected to single-cell sorting and SHM analyses. Core mutations in each family of clones are shown and substitutions known to increase affinity for HEL^{4X} underlined.

(D) Binding affinities of recombinant HyHEL10 IgG1 carrying the core mutations from the clone families identified in (C) for foreign (HEL^{3X}) and self (HEL^{4X}) antigens. See Figure S7 for further details.

On the other hand, FAS-deficient mice have been found to display aberrant GC B cell selection (Hoch et al., 2000; Takahashi et al., 2001), to develop self-reactive B cells in certain models (Ait Azzouzene et al., 2010), and to produce autoantibodies when FAS inactivation is restricted to B cells (Hao et al., 2008). A major aim of the current study was to gain new perspectives on this unresolved issue by using a high-resolution in vivo mouse model to provide a detailed and comprehensive analysis of how the responses of antigen-specific B cells are affected when they specifically lack FAS. The results not only help reconcile many of these previous findings but also point to a new class of activated lymphocyte, the rogue GC (GCr) B cell, as a potent source of IgE and autoantibody production and the *raison d'être* for FAS expression in the GC.

DISCUSSION

Two decades have passed since the first studies demonstrated that FAS expressed on activated human and mouse B cells can mediate their death (Garrone et al., 1995; Rathmell et al., 1995). Nevertheless, the precise role of FAS in regulating in vivo B cell responses and particularly the GC reaction has been controversial. It has been concluded that FAS has no significant role to play in the GC (Smith et al., 1995) and is not required to remove self-reactive GC B cells (Alabyev et al., 2008; Han et al., 1995b).

The initial result of this study was that FAS was not required to remove self-reactive GC B cells. This is in agreement with the interpretations of some (Alabyev et al., 2008; Han et al., 1995b) but not other (Ait Azzouzene et al., 2010; Hao et al., 2008) earlier investigations. Although it could be argued that different results might be obtained in different systems, the question of whether FAS is required to maintain GC B cell self-tolerance can also be answered differently depending on the readout of tolerance that is employed. In our study, FAS was clearly not required for the removal of conventional GC B cells that acquire self-reactivity

against mHEL^{4X}. However, looking later in the response, it was equally clear that serum anti-HEL^{4X} autoantibodies accumulated to high titers specifically in recipients of FAS-deficient B cells. These results could be reconciled by the fact that a previously unknown population of cells, GC_r B cells, emerged over the course of the response of FAS-deficient B cells. Because these cells do not follow the same rules of selection as their conventional counterparts, SHM ultimately leads to the unchecked production of autoantibody-secreting PC_r and the breakdown of self-tolerance. Thus, although one mechanism of self-tolerance remains intact when GC B cells lack FAS, another fails, which might well explain the previous confusion over the role of FAS in maintaining GC B cell self-tolerance.

A major question to arise from this study is what drives the survival and accumulation of GC_r B cells? These cells are not dependent on recognition of the immunizing antigen and so differ fundamentally from conventional GC B cells. Could they instead be selected on the basis of interactions with certain self-antigens? From our analysis of the specificities encoded by GC_r and PC_r B cells, it seems unlikely that specific self-antigens drive the accumulation of these cells. Thus, although there are strong clonal relationships within individual recipients of SW_{HEL}.Fas^{lpr/lpr} B cells, recurring amino acid substitutions (such as those seen in affinity maturation to HEL^{3X}) are not observed between recipients. An alternative possibility is that GC_r B cells exist because they have acquired mutated antigen receptors that undergo some form of structural change (e.g., auto-aggregation) that results in their “selection” independent of antigen recognition. In this case, the acquisition of self-reactivity by rogue cells might be incidental but (at a population level) highly likely based on their ongoing SHM. Finally, the restricted clonality of GC_r B cells might be explained by an ancestral clone acquiring a mutation in a non-Ig gene (e.g., by off-target SHM) that allows the clone and its daughter cells to be perpetuated without retaining antigen specificity. Here again, self-reactivity might arise as a consequence of ongoing SHM being uncoupled from the need to maintain recognition of the original immunizing antigen. This raises the question of how T cells, presumably GC-localized T_{fh} cells, could be required to maintain GC_r B cells as is indicated by our CD40L-depletion data? One possibility is that they are able to maintain the peptide-MHC complexes as they continue dividing without requiring ongoing antigen contact (Thaumat et al., 2012). Alternatively, they might be able to persist on the basis of “bystander” help that they can access without undergoing cognate interactions with T_{fh} cells.

Whatever the specific mechanism is that drives the production of GC_r B cells, the fact that it does occur as part of normal GC activity explains the need for GC B cells to express FAS. It is unclear at this point whether a specific cell type provides the FASL required to prevent the development of GC_r B cells. Our data show that it is not an autocrine action of activated B cells expressing FASL (Hahne et al., 1996). However, FASL could instead derive from neighboring B cells, follicular dendritic cells (Verbeke et al., 1999), T_{fh}/T_{fr} CD4⁺ cells (Rathmell et al., 1995), or even CD8⁺ T cells (Afshar-Sterle et al., 2014). What feature of GC_r B cells makes them particularly susceptible to the signals delivered through FAS is another question that requires further investigation. Until more functional and phenotypic data have been obtained, we propose that GC_r B cells be defined as a

rare subpopulation of GC B cells that (1) accumulate late in the GC response when FAS expression or function is impaired, (2) do not require ongoing antigen binding to survive, and (3) show a high propensity for PC differentiation including unusually large numbers of IgE⁺ PCs.

Another context in which FAS has been identified to regulate autoantibody production is the accumulation of extrafollicular PCs that undergo chronic stimulation and GC-independent SHM in response to autoantigens such as rheumatoid factor (RF) (William et al., 2002). We determined that the accumulation of SHM in the extrafollicular PCs derived from SW_{HEL}.Fas^{lpr/lpr} B cells does not occur via this mechanism because (1) the PC population we observed did not express AID, (2) unlike the anti-RF PCs (Herlands et al., 2008), the PC population we observed did depend on ongoing T cell help, and (3) clear clonal precursor-product relationships existed between the GC and PC populations in our model.

An unexpected finding was that GC_r B cells act as a source of IgE-secreting PCs that emerge when responding B cells lack functional FAS. The close clonal relationships between IgG1⁺ and IgE⁺ PCs in single recipient mice and evidence of multiple IgE switching events indicate that most IgE⁺ PCs are generated from IgG1⁺ GC B cells, potentially via a transient IgE⁺ GC B cell intermediate (Erazo et al., 2007; Xiong et al., 2012; Yang et al., 2012). Interestingly, human allergic and non-allergic IgE antibodies often exhibit evidence of reduced antigenic selection (Dahlke et al., 2006; Wang et al., 2014), consistent with IgE in these cases being derived at least in part from GC_r B cell clones such as those described here.

The greatly elevated concentrations of serum IgE detected in a major cohort of ALPS patients is consistent with the properties of the GC_r B cells that emerge when FAS is inactivated. The lack of allergic disease among these patients also fits this scenario and points to a link between IgE production and autoimmunity that is independent of allergy. Further evidence for this phenomenon comes from the recent finding that a majority of SLE patients display IgE autoantibodies (Dema et al., 2014b). Similarly, investigations in a mouse model of SLE have demonstrated a role for IgE in disease progression (Dema et al., 2014a) and IgE autoantibodies have a clear pathogenic role in BP (Messingham et al., 2014). Our findings therefore provide a mechanistic basis for the link between IgE antibody production and autoimmune diseases. We propose that the emergence of GC_r B cells, through mutation of FAS or potentially other immunoregulatory disruptions, represents a common phenomenon in autoimmune disease that can not only drive the production of IgG autoantibodies but support the parallel production of pro-inflammatory IgE that might amplify disease. Further investigation of the signals that drive GC_r B cells might therefore reveal new strategies for managing antibody-mediated autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice and Adoptive Transfers

SW_{HEL} mice (Phan et al., 2003) were maintained on a CD45.1 congenic (*Ptprc*^{a/a}) C57BL/6 background and mHEL^{4X} transgenic mice (Chan et al., 2012) on a C57BL/6 background. All other mouse strains were C57BL/6 and are referenced in Supplemental Experimental Procedures. For adoptive transfers, spleen cells from donor SW_{HEL} mice containing 3×10^4 HEL-binding B cells were transferred i.v. into wild-type or mHEL^{4X} Tg recipient mice together with 2×10^8

HEL^{3X}-SRBCs (prepared as previously described) (Paus et al., 2006). Recipient mice were boosted with HEL^{3X}-SRBCs (2×10^8 cells/recipient) 4 days after primary immunization. To abrogate GC B cell responses, 200 μ g of anti-CD40L mAb (MR-1, BioXcell) or isotype control (hamster IgG, BioXcell) was administered intravenously on days 15, 17, and 19 after primary immunization.

HEL Proteins

Purified wild-type hen egg lysozyme (HEL^{WT}) was purchased from Sigma-Aldrich. Recombinant HEL^{3X} and HEL^{4X} proteins (Chan et al., 2012; Paus et al., 2006) were grown as secreted proteins in yeast (*Pichia pastoris*) and purified from culture supernatants as described (Paus et al., 2006).

Flow Cytometric Analysis

Splenocytes were prepared and stained for cell surface HEL^{3X} or HEL^{4X} binding as well as expression of other cell surface markers as described (Chan et al., 2012). In addition, splenic basophils were identified with the following mAbs: anti-Fc ϵ RI (MAR-1, eBioscience), anti-CD49b (DX5, BD Biosciences), and anti-c-Kit (2B8, BD Biosciences). For flow cytometric analysis of fixed and permeabilized cells (Figure S2A), cells were first stained for surface markers and surface IgE (including CD23-bound soluble IgE) blocked with unconjugated anti-IgE (R35-72) antibody (Yang et al., 2012). Thus, for fixed and permeabilized samples, IgG1 stains include surface and intracellular molecules (total) whereas IgE stains are for intracellular (i.c.) IgE only. After surface staining, cells were fixed with 10% formalin (Sigma-Aldrich). Cell permeabilization and subsequent staining steps were performed in 0.5% Saponin/0.1% BSA/PBS. Intracellular staining was performed with 200 ng/ml of HEL^{WT} or 50 ng/ml of HEL^{3X}. After staining for IgG1 (A85-1, BD Biosciences), samples were blocked with 10% normal mouse serum (Jackson ImmunoResearch) and bound HEL^{WT}/HEL^{3X} was revealed with HyHEL9-AlexaFluor647 in 5% normal mouse serum. Subsequent intracellular staining was performed with anti-IgE FITC (R35-72, BD Biosciences) and Streptavidin (SA)-Pacific Blue (Invitrogen). Data were acquired on either the FACS Cantoll or LSR II SORP (BD) and analyzed with FlowJo (TreeStar).

Single-Cell Sorting

To sort single cells for RNA extraction (Figure S2B), cell suspensions from whole spleens were prepared and surface staining performed as detailed earlier for surface flow cytometric analysis. For somatic hypermutation (SHM) analysis (Figure S3B), cell samples were subjected to intracellular staining. Single-cell sorting was performed on the FACS Aria, FACS ARIAII, or Influx (BD Biosciences).

Single-Cell qRT-PCR and Data Analysis

Acquisition and analysis of single-cell qRT-PCR data to determine mRNA expression in sorted cells was carried out according to the methods established by us (Suan et al., 2015) and described in detail in Supplemental Experimental Procedures.

SHM Analysis

The variable region exon of the SW_{HEL} Ig (HyHEL10) heavy chain gene was amplified from sorted single cells by PCR and sequenced as described (Paus et al., 2006).

ELISAs

IgE concentrations in sera were measured by direct ELISA and anti-HEL IgE measured by capturing total serum IgE and detecting with biotinylated HEL. These procedures are described fully in Supplemental Experimental Procedures. Anti-HEL^{4X} IgG1 was measured by direct ELISA with plate-bound HEL^{4X} mice as described (Chan et al., 2012).

ALPS Cohort Description and Human Serum IgE Measurements

The National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIAID, NIH) ALPS program has evaluated patients and family members through mail-in samples and direct evaluation at the NIH Clinical Center (NIH CC) since 1993. 415 individuals from 120 families with 95 unique FAS mutations were investigated (http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=FAS). All participants underwent informed con-

sent process and were enrolled on an IRB-approved NIAID, NIH research protocol (Clinical [trials.gov](https://clinicaltrials.gov) NCT: NCT00001350). Samples analyzed for this study correspond to the 150 ALPS-FAS patients (78 probands and 72 affected relatives) and 63 healthy mutation-positive relatives whose natural histories were recently reported (Price et al., 2014). Serum IgE measurements were performed with IMMULITE 2000 analyzer (Siemens) with a IMMULITE 2000 Total IgE kit for a solid-phase, chemiluminescent immuno-metric assay.

Immunofluorescence Histology

Immunofluorescence histology analysis of splenic sections from recipient mice was carried out as described previously (Chan et al., 2009) and outlined in detail in Supplemental Experimental Procedures.

Recombinant HyHEL10 IgG1 Antibody Production and Binding Analysis

Heavy chain mutants of HyHEL10 were expressed as soluble IgG1 molecules in HEK293F cells from pcDNA3 vectors encoding mutant γ 1 heavy and wild-type κ light chains and purified via Protein G sepharose (AcroBiosystems). Purified IgG1 samples were biotinylated at a 5:1 molar ratio via EZ-Link NHS-PEG4-Biotinylation reagent (Thermo Scientific) and uncoupled biotinylation reagent was removed with a ZebaSpin desalting column (Thermo Scientific). Affinities of HyHEL10 variants were measured via the Blitz system (PALL). SA biosensors were pre-blocked with 1% BSA/PBS for 1 hr. Sensors were then incubated with dilute samples of biotinylated antibody and coupling of antibody quantified with the Blitz software. Coupled sensors were incubated in PBS for 60 s to obtain a stable baseline and then incubated with various dilutions (5 nM–5 μ M) of purified, filtered HEL^{3X}, HEL^{4X}, or HEL^{WT} in PBS. Association and dissociation rates were measured with the Blitz software and global fits of the resulting data were used to determine the K_d , k_a , and k_d of each variant for HEL^{WT} and HEL^{3X}. K_a was taken as $1/K_d$.

Statistical Analysis

A full description of statistical analyses used can be found in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2015.04.010>.

AUTHOR CONTRIBUTIONS

R.B., T.D.C., and D.B. conceptualized and designed the research; D.B., T.D.C., K.B., J.R.H., and A.N. performed experiments; S.P. and V.K.R. collected, analyzed, and interpreted ALPS patient data; P.S. and D.C. produced and analyzed recombinant mAbs; L.A.O'R. and A. Strasser provided *Fas* ^{Δ m/ Δ m} mice and advice; A.N. and A. Statham performed bioinformatic analysis; A.B., C.S.M., S.G.T., and T.G.P. provided specialist advice and analysis; D.B., T.D.C., and R.B. analyzed and interpreted data; D.B., T.D.C., A.N., and R.B. prepared figures; and R.B. prepared text.

ACKNOWLEDGMENTS

We thank R. Salomon, C. Brownlee, and Garvan Institute Flow Facility staff for single-cell sorting and J. Kingham and the staff of Australian BioResources for animal husbandry. We also thank C. Goodnow, A. Collins, and J. Davies for their comments on the manuscript. D.B. is supported by an Australian Postgraduate Award and T.D.C., A. Strasser, D.C., C.S.M., S.G.T., T.G.P., and R.B. by National Health and Medical Research Council (NHMRC) fellowships 1054669, 1020363, 1050146, 1008820, 1042925, 1005097, and 1002280. This work was funded by Asthma Foundation NSW, R.G. Arnott Foundation, NHMRC Project Grant 1044477, and NHMRC Program Grant 1016953. Additional support came from NHMRC Project Grant 1009145, NHMRC Program Grant 1016701, Leukemia and Lymphoma Society (SCOR grants #7413 and #7001-13), and the Intramural Research Program of the National Institute of Allergy and Infectious Diseases and the NIH Clinical Center.

Received: February 8, 2015

Revised: March 9, 2015

Accepted: March 25, 2015

Published: May 12, 2015

REFERENCES

- Afshar-Sterle, S., Zotos, D., Bernard, N.J., Scherger, A.K., Rödling, L., Alsop, A.E., Walker, J., Masson, F., Belz, G.T., Corcoran, L.M., et al. (2014). Fas ligand-mediated immune surveillance by T cells is essential for the control of spontaneous B cell lymphomas. *Nat. Med.* 20, 283–290.
- Ait-Azzouzene, D., Kono, D.H., Gonzalez-Quintial, R., McHeyzer-Williams, L.J., Lim, M., Wickramarachchi, D., Gerdes, T., Gavin, A.L., Skog, P., McHeyzer-Williams, M.G., et al. (2010). Deletion of IgG-switched autoreactive B cells and defects in Fas(lpr) lupus mice. *J. Immunol.* 185, 1015–1027.
- Alabyev, B., Vuyyuru, R., and Manser, T. (2008). Influence of Fas on the regulation of the response of an anti-nuclear antigen B cell clonotype to foreign antigen. *Int. Immunol.* 20, 1279–1287.
- Andrews, B.S., Eisenberg, R.A., Theofilopoulos, A.N., Izui, S., Wilson, C.B., McConahey, P.J., Murphy, E.D., Roths, J.B., and Dixon, F.J. (1978). Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. *J. Exp. Med.* 148, 1198–1215.
- Brink, R. (2014). The imperfect control of self-reactive germinal center B cells. *Curr. Opin. Immunol.* 28, 97–101.
- Chan, T.D., Gatto, D., Wood, K., Camidge, T., Basten, A., and Brink, R. (2009). Antigen affinity controls rapid T-dependent antibody production by driving the expansion rather than the differentiation or extrafollicular migration of early plasmablasts. *J. Immunol.* 183, 3139–3149.
- Chan, T.D., Wood, K., Hermes, J.R., Butt, D., Jolly, C.J., Basten, A., and Brink, R. (2012). Elimination of germinal-center-derived self-reactive B cells is governed by the location and concentration of self-antigen. *Immunity* 37, 893–904.
- Dahlke, I., Nott, D.J., Ruhno, J., Sewell, W.A., and Collins, A.M. (2006). Antigen selection in the IgE response of allergic and nonallergic individuals. *J. Allergy Clin. Immunol.* 117, 1477–1483.
- Dema, B., Charles, N., Pellefigues, C., Ricks, T.K., Suzuki, R., Jiang, C., Scheffell, J., Hasni, S., Hoffman, V., Jablonski, M., et al. (2014a). Immunoglobulin E plays an immunoregulatory role in lupus. *J. Exp. Med.* 211, 2159–2168.
- Dema, B., Pellefigues, C., Hasni, S., Gault, N., Jiang, C., Ricks, T.K., Bonelli, M.M., Scheffell, J., Sacré, K., Jablonski, M., et al. (2014b). Autoreactive IgE is prevalent in systemic lupus erythematosus and is associated with increased disease activity and nephritis. *PLoS ONE* 9, e90424.
- Erazo, A., Kutchukhidze, N., Leung, M., Christ, A.P., Urban, J.F., Jr., Curotto de Lafaille, M.A., and Lafaille, J.J. (2007). Unique maturation program of the IgE response in vivo. *Immunity* 26, 191–203.
- Fisher, G.H., Rosenberg, F.J., Straus, S.E., Dale, J.K., Middleton, L.A., Lin, A.Y., Strober, W., Lenardo, M.J., and Puck, J.M. (1995). Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell* 81, 935–946.
- Garrone, P., Neidhardt, E.M., Garcia, E., Galibert, L., van Kooten, C., and Banchereau, J. (1995). Fas ligation induces apoptosis of CD40-activated human B lymphocytes. *J. Exp. Med.* 182, 1265–1273.
- Hahne, M., Renno, T., Schroeter, M., Irmeler, M., French, L., Bornard, T., MacDonald, H.R., and Tschopp, J. (1996). Activated B cells express functional Fas ligand. *Eur. J. Immunol.* 26, 721–724.
- Han, S., Hathcock, K., Zheng, B., Kepler, T.B., Hodes, R., and Kelsoe, G. (1995a). Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *J. Immunol.* 155, 556–567.
- Han, S., Zheng, B., Dal Porto, J., and Kelsoe, G. (1995b). In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. IV. Affinity-dependent, antigen-driven B cell apoptosis in germinal centers as a mechanism for maintaining self-tolerance. *J. Exp. Med.* 182, 1635–1644.
- Hao, Z., Duncan, G.S., Seagal, J., Su, Y.W., Hong, C., Haight, J., Chen, N.J., Elia, A., Wakeham, A., Li, W.Y., et al. (2008). Fas receptor expression in germinal-center B cells is essential for T and B lymphocyte homeostasis. *Immunity* 29, 615–627.
- Herlands, R.A., Christensen, S.R., Sweet, R.A., Hershberg, U., and Shlomchik, M.J. (2008). T cell-independent and toll-like receptor-dependent antigen-driven activation of autoreactive B cells. *Immunity* 29, 249–260.
- Hoch, S., Boyd, M., Malone, B., Gonye, G., Schwaber, J., and Schwaber, J. (2000). Fas-mediated apoptosis eliminates B cells that acquire self-reactivity during the germinal center response to NP. *Cell. Immunol.* 203, 103–110.
- Liu, Y.J., Barthélémy, C., de Bouteiller, O., Arpin, C., Durand, I., and Banchereau, J. (1995). Memory B cells from human tonsils colonize mucosal epithelium and directly present antigen to T cells by rapid up-regulation of B7-1 and B7-2. *Immunity* 2, 239–248.
- Messingham, K.A., Holahan, H.M., and Fairley, J.A. (2014). Unraveling the significance of IgE autoantibodies in organ-specific autoimmunity: lessons learned from bullous pemphigoid. *Immunol. Res.* 59, 273–278.
- Nossal, G.J. (1988). Somatic mutations in B lymphocytes: new perspectives in tolerance research? *Immunol. Cell Biol.* 66, 105–110.
- O’Reilly, L.A., Tai, L., Lee, L., Kruse, E.A., Grabow, S., Fairlie, W.D., Haynes, N.M., Tarlinton, D.M., Zhang, J.G., Belz, G.T., et al. (2009). Membrane-bound Fas ligand only is essential for Fas-induced apoptosis. *Nature* 461, 659–663.
- Paus, D., Phan, T.G., Chan, T.D., Gardam, S., Basten, A., and Brink, R. (2006). Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation. *J. Exp. Med.* 203, 1081–1091.
- Permin, H., and Wiik, A. (1978). The prevalence of IgE antinuclear antibodies in rheumatoid arthritis and systemic lupus erythematosus. *Acta Pathol. Microbiol. Scand. [C]* 86C, 245–249.
- Phan, T.G., Amesbury, M., Gardam, S., Crosbie, J., Hasbold, J., Hodgkin, P.D., Basten, A., and Brink, R. (2003). B cell receptor-independent stimuli trigger immunoglobulin (Ig) class switch recombination and production of IgG autoantibodies by anergic self-reactive B cells. *J. Exp. Med.* 197, 845–860.
- Phan, T.G., Paus, D., Chan, T.D., Turner, M.L., Nutt, S.L., Basten, A., and Brink, R. (2006). High affinity germinal center B cells are actively selected into the plasma cell compartment. *J. Exp. Med.* 203, 2419–2424.
- Price, S., Shaw, P.A., Seitz, A., Joshi, G., Davis, J., Niemela, J.E., Perkins, K., Hornung, R.L., Folio, L., Rosenberg, P.S., et al. (2014). Natural history of autoimmune lymphoproliferative syndrome associated with FAS gene mutations. *Blood* 123, 1989–1999.
- Provost, T.T., and Tomasi, T.B., Jr. (1974). Immunopathology of bullous pemphigoid. Basement membrane deposition of IgE, alternate pathway components and fibrin. *Clin. Exp. Immunol.* 18, 193–200.
- Rathmell, J.C., Cooke, M.P., Ho, W.Y., Grein, J., Townsend, S.E., Davis, M.M., and Goodnow, C.C. (1995). CD95 (Fas)-dependent elimination of self-reactive B cells upon interaction with CD4+ T cells. *Nature* 376, 181–184.
- Rieux-Laucat, F., Le Deist, F., Hivroz, C., Roberts, I.A., Debatin, K.M., Fischer, A., and de Villartay, J.P. (1995). Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* 268, 1347–1349.
- Smith, K.G., Nossal, G.J., and Tarlinton, D.M. (1995). FAS is highly expressed in the germinal center but is not required for regulation of the B-cell response to antigen. *Proc. Natl. Acad. Sci. USA* 92, 11628–11632.
- Suan, D., Nguyen, A., Moran, I., Bourne, K., Hermes, J.R., Arshi, M., Hampton, H.R., Tomura, M., Miwa, Y., Kelleher, A.D., et al. (2015). T follicular helper cells have distinct modes of migration and molecular signatures in naive and memory immune responses. *Immunity* 42, 704–718.
- Takahashi, T., Tanaka, M., Brannan, C.I., Jenkins, N.A., Copeland, N.G., Suda, T., and Nagata, S. (1994). Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* 76, 969–976.
- Takahashi, Y., Ohta, H., and Takemori, T. (2001). Fas is required for clonal selection in germinal centers and the subsequent establishment of the memory B cell repertoire. *Immunity* 14, 181–192.
- Takahashi, S., Futatsugi-Yumikura, S., Fukuoka, A., Yoshimoto, T., Nakanishi, K., and Yonehara, S. (2013). Fas deficiency in mice with the Balb/c background

induces blepharitis with allergic inflammation and hyper-IgE production in conjunction with severe autoimmune disease. *Int. Immunol.* 25, 287–293.

Thaunat, O., Granja, A.G., Barral, P., Filby, A., Montaner, B., Collinson, L., Martinez-Martin, N., Harwood, N.E., Bruckbauer, A., and Batista, F.D. (2012). Asymmetric segregation of polarized antigen on B cell division shapes presentation capacity. *Science* 335, 475–479.

Verbeke, C.S., Wenthe, U., and Zentgraf, H. (1999). Fas ligand expression in the germinal centre. *J. Pathol.* 189, 155–160.

Victoria, G.D., and Nussenzweig, M.C. (2012). Germinal centers. *Annu. Rev. Immunol.* 30, 429–457.

Wang, Y., Jackson, K.J., Davies, J., Chen, Z., Gaeta, B.A., Rimmer, J., Sewell, W.A., and Collins, A.M. (2014). IgE-associated IGHV genes from venom and peanut allergic individuals lack mutational evidence of antigen selection. *PLoS ONE* 9, e89730.

Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G., Jenkins, N.A., and Nagata, S. (1992). Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 356, 314–317.

William, J., Euler, C., Christensen, S., and Shlomchik, M.J. (2002). Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science* 297, 2066–2070.

Wu, J., Wilson, J., He, J., Xiang, L., Schur, P.H., and Mountz, J.D. (1996). Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. *J. Clin. Invest.* 98, 1107–1113.

Xiong, H., Dolpady, J., Wabl, M., Curotto de Lafaille, M.A., and Lafaille, J.J. (2012). Sequential class switching is required for the generation of high affinity IgE antibodies. *J. Exp. Med.* 209, 353–364.

Yang, Z., Sullivan, B.M., and Allen, C.D. (2012). Fluorescent in vivo detection reveals that IgE(+) B cells are restrained by an intrinsic cell fate predisposition. *Immunity* 36, 857–872.

Immunity

Supplemental Information

FAS Inactivation Releases Unconventional Germinal Center B Cells that Escape Antigen Control and Drive IgE and Autoantibody Production

Danyal Butt, Tyani D. Chan, Katherine Bourne, Jana R. Hermes, Akira Nguyen, Aaron Statham, Lorraine A. O'Reilly, Andreas Strasser, Susan Price, Peter Schofield, Daniel Christ, Antony Basten, Cindy S. Ma, Stuart G. Tangye, Tri Giang Phan, V. Koneti Rao, and Robert Brink

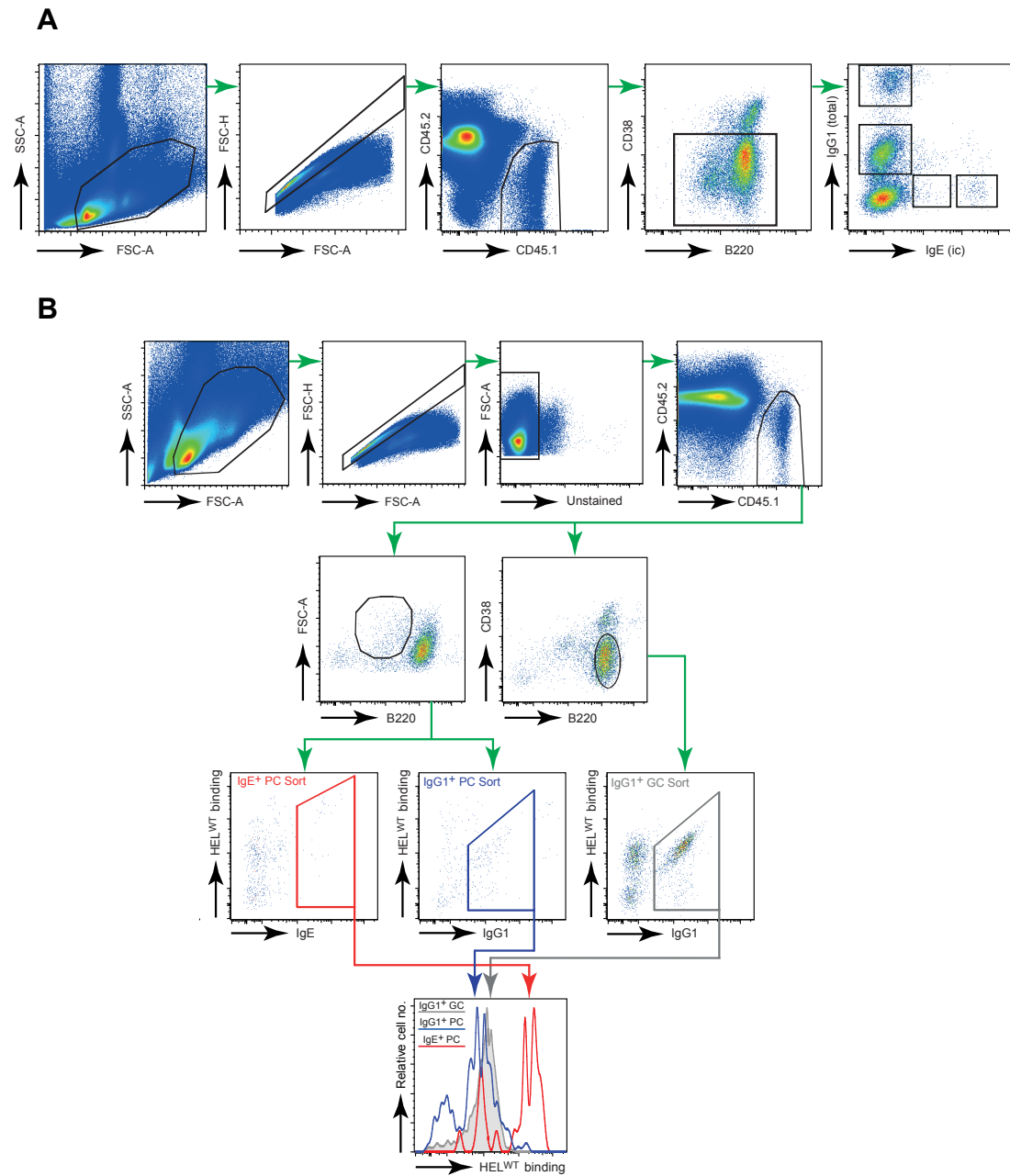


Figure S2, Related to Figure 3. Gating strategies employed in flow cytometric analysis and single cell sorting for qPCR analysis.

Figure S2, Related to Figure 3. Gating strategies employed in flow cytometric analysis and single cell sorting for qPCR analysis.

Recipient spleens were harvested following transfer of CD45.1 donor SW_{HEL} B cells (with or without mutated *Fas* or *Fasl* genes) and challenge with HEL^{3X}-SRBC.

(A) Cells were stained for cell surface markers before being fixed, permeabilized, and stained for IgG1, IgE and HEL^{3X} or HEL^{WT} binding. Following data acquisition and compensation, a lymphocyte light scatter gate was applied (FSC-A vs SSC-A), doublets excluded (FSC-A vs FSC-H), autofluorescent cells excluded (not shown) and a gate for donor-derived cells applied (CD45.1 vs CD45.2). CD38^{hi} cells B cells were then excluded (B220 vs CD38) and class-switched GC B cells identified by low but positive expression of total IgG1 or intracellular (ic) IgE and PCs by high staining for these Ig isotypes.

(B) Single cell sorting for qPCR analysis was performed in a similar fashion but without a fixing and permeabilization step and an additional exclusion of autofluorescent cells (Unstained vs FSC-A). Donor-derived PCs (B220^{lo}, FSC-A^{hi}) and GC B cells (B220^{hi}, CD38^{lo}) were resolved and the IgG1 and IgE switched subsets resolved by surface staining (IgE⁺ GC B cells could not be resolved). Note the high levels of surface Ig expression on IgE⁺ PCs, consistent with previous observations (Yang et al., 2012).

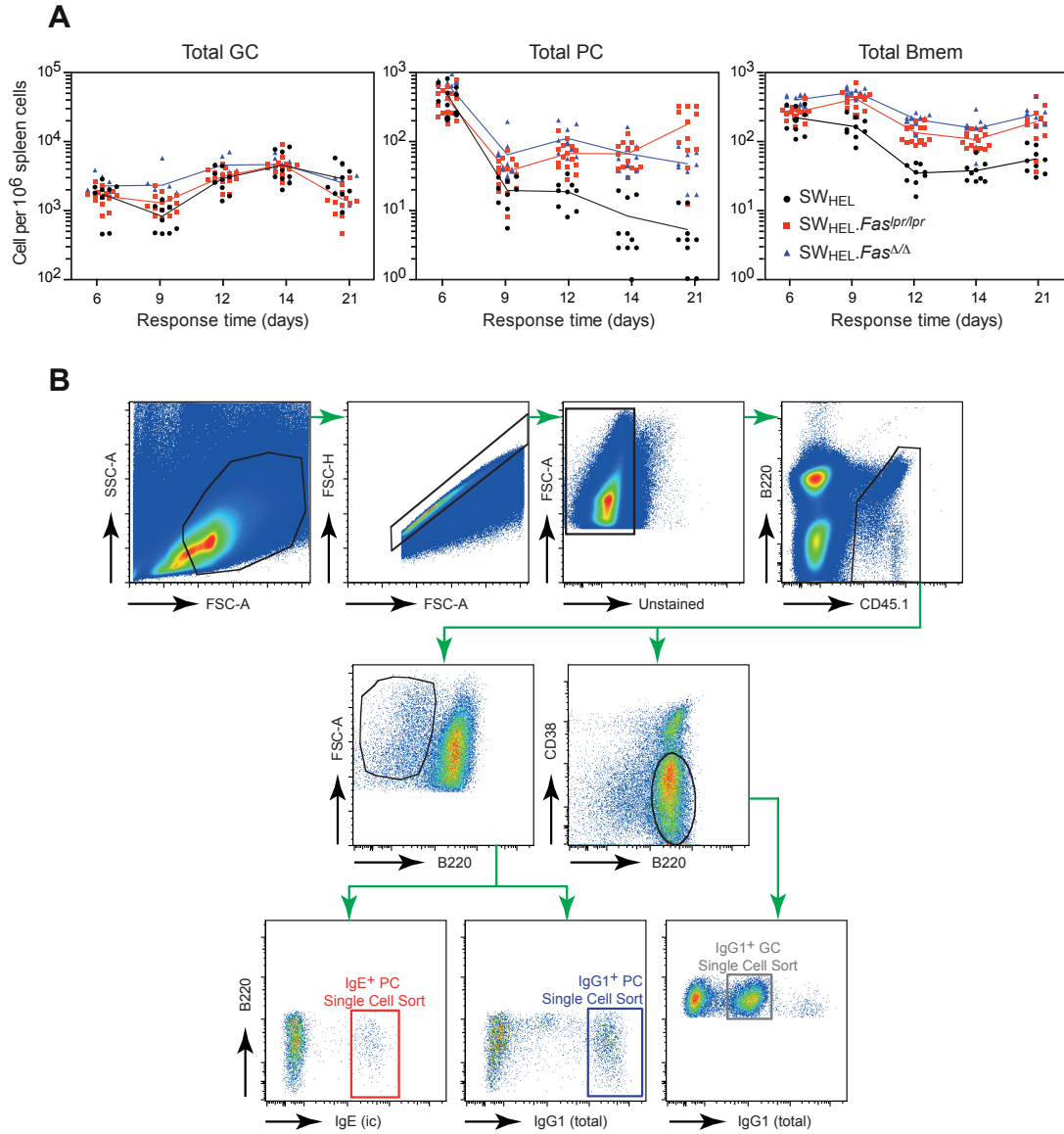


Figure S3, Related to Figure 4. Kinetics of total wild-type vs FAS-deficient SW_{HEL} B cell responses and sort strategy for single cell SHM analysis.

(A) Time course of donor-derived splenic responses of SW_{HEL} , $SW_{HEL}.Fas^{lpr/lpr}$ and $SW_{HEL}.Fas^{\Delta/\Delta}$ B cells challenged with HEL^{3X} -SRBC. Spleen cells were stained as per Figure S2A. Populations reflect all HEL^{WT} -binding donor-derived cells. GC = $B220^{hi}, CD38^{lo}$; PC = $B220^{lo}, CD38^{lo}$, HEL^{WT-hi} ; Bmem = $B220^{hi}, CD38^{hi}$. Data were pooled from two independent experiments.

(B) Sorting strategy for single cell SHM analysis. Cells from recipient spleens were stained as detailed in Figure S2A. Donor-derived cells were identified ($CD45.1^{+}, B220^{lo-hi}$) and PC cell ($B220^{lo}, FSC-A^{hi}$) and GC B cell ($B220^{hi}, CD38^{lo}$) populations resolved. Specific IgG1 and IgE switched populations were then resolved and deposited as single cells in 96 well plates for processing.

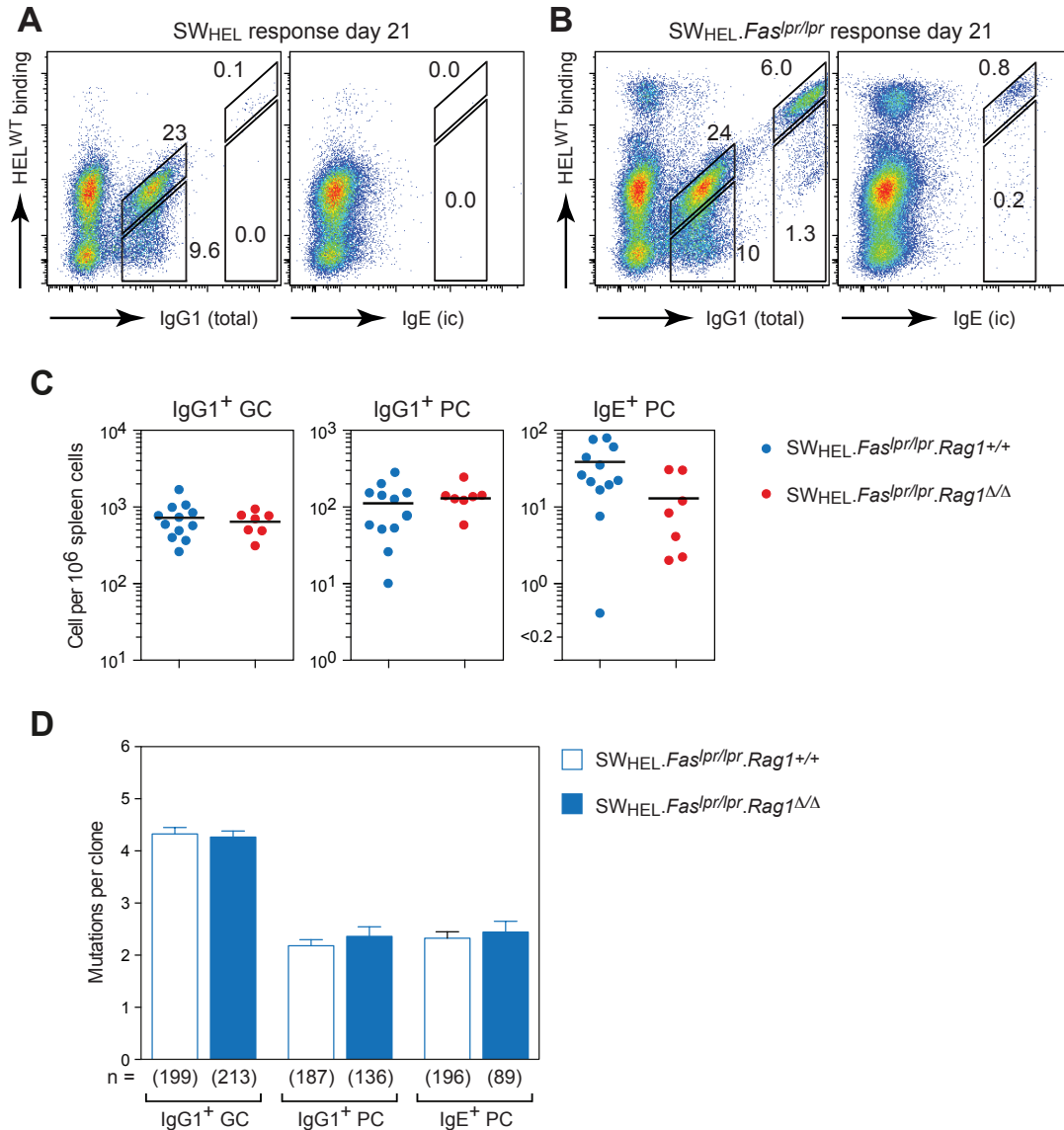


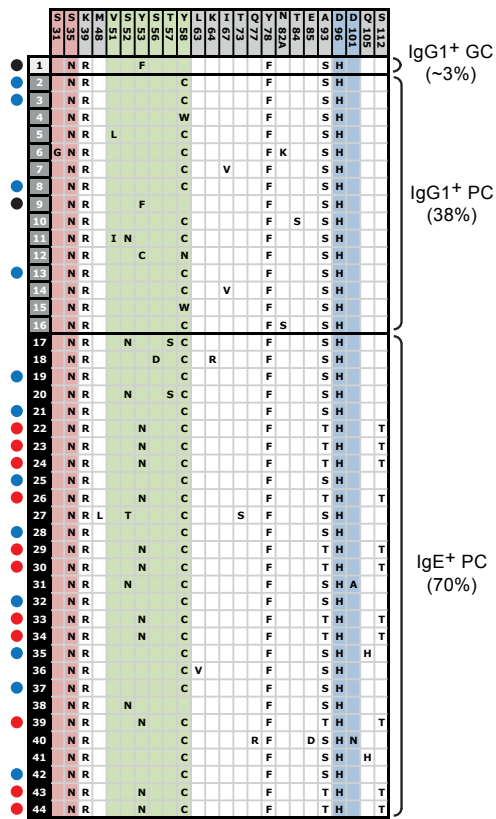
Figure S4, Related to Figure 5. HEL^{WT}-binding of responding SW_{HEL} and SW_{HEL}.Fas^{lpr/lpr} B cells and equivalent responses of RAG1-expressing and RAG1-deficient SW_{HEL}.Fas^{lpr/lpr} B cells.

(A,B) SW_{HEL} or SW_{HEL}.Fas^{lpr/lpr} B cells were challenged with HEL^{3X}-SRBC and recipient spleen cells analyzed on day 21. Data show total IgG1 or intracellular (ic) IgE staining as well as HEL^{WT} binding by donor-derived GC B cells + PCs (CD45.1⁺, CD45.2⁻, CD38^{lo} - see Figure S2A).

(C,D) SW_{HEL}.Fas^{lpr/lpr}.Rag1^{+/+} or SW_{HEL}.Fas^{lpr/lpr}.Rag1^{Δ/Δ} B cells were challenged with HEL^{3X}-SRBC and recipient spleen cells analyzed after 21 days. (C) Enumeration of donor-derived responding subsets. Data are pooled from four independent experiments. (D) Mutation rates in the Ig heavy chain variable region gene in donor-derived splenic subsets (see Figure S3B for gating). n = number of clones analyzed. Errors bars indicate the SEM.

A

Mouse 57.69

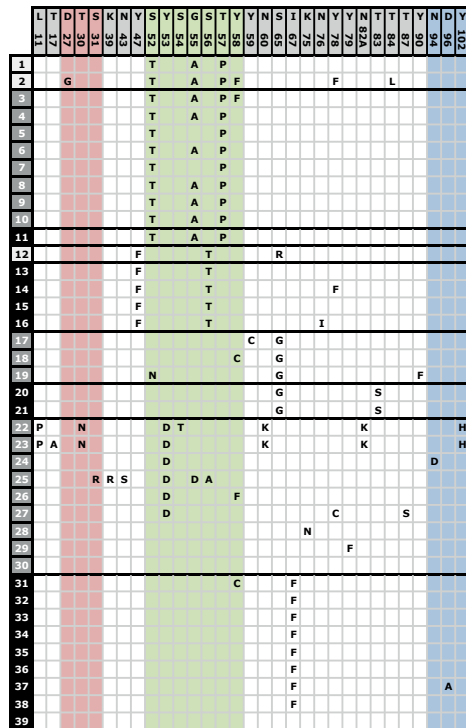


HyHEL10 variant	K_A for HEL ^{3X} (M ⁻¹)	K_A for HEL ^{WT} (M ⁻¹)
Unmutated ("germ-line")	1.3×10^7	$> 10^{10}$
Basic mutant**	$< 10^5$	2.0×10^8
Basic + Y53F	$< 10^5$	1.5×10^8
Basic + Y58C	6.4×10^5	5.5×10^6
Basic + Y53N, Y58C A93T, S112T	$< 10^5$	$< 10^5$

**S35N, K39R, Y58F, A93S, D96H (presumed precursor clone)

B

Mouse 71.02



- IgG1+ GC clones
- IgG1+ PC clones
- IgE+ PC clones

Group 1

Group 2

Group 3

Figure S5, Related to Figure 5. Analysis of clonally-related, donor-derived cells in single recipient mice given SW_{HEL}.*Fas*^{lpr/lpr}.*Rag1*^{Δ/Δ} B cells and challenged with HEL^{3X}-SRBC.

(A) (Left Panel) Ig heavy chain variable region replacement mutations in clonally-related cells obtained from a single recipient mouse (57.69) on day 21 of the response of SW_{HEL}.*Fas*^{lpr/lpr}.*Rag1*^{Δ/Δ} B cells to HEL^{3X}-SRBC. Percentages indicate the frequency of these clones within the respective responder populations and mirror those seen in the independently analyzed mouse 71.06 (Figure 5E). (Right Panel) Affinity for HEL^{3X} and HEL^{WT} of recombinant HyHEL10 soluble IgG1 carrying the indicated heavy chain variable region replacement mutations together with HyHEL10 κ light chain. Clones corresponding exactly to the mAbs analyzed are indicated by the colored dots. The presumed ancestral clone carried the S35N, K39R, Y58F, A93S and D96H substitutions.

(B) All sequenced IgG1⁺ and IgE⁺ PC clones and related IgG1⁺ clones from a single recipient mouse (71.02) obtained on day 21 of the response of SW_{HEL}.*Fas*^{lpr/lpr}.*Rag1*^{Δ/Δ} B cells to HEL^{3X}-SRBC. Three groups of related clones that span two or more cell subsets can be seen with additional related clones apparent within the IgG1⁺ and IgE⁺ PC compartments.

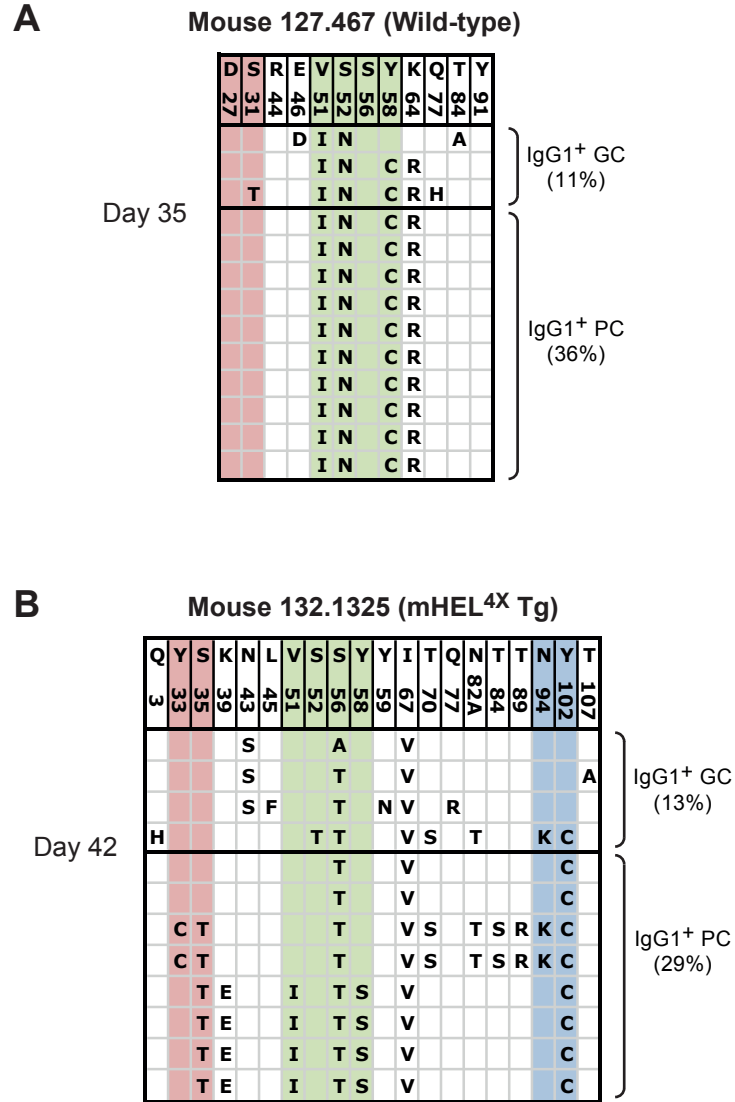


Figure S6, Related to Figure 6. Expanded populations of clonally-related GC_R B cells form large fractions of the late SW_{HEL}.Fas^{lpr/lpr} response.

Ig heavy chain variable region replacement mutations in clonally-related cells obtained from a single recipient (A) wild-type mouse (127.467) on day 35 or (B) mHEL^{4X} Tg mouse on day 42 of the response of SW_{HEL}.Fas^{lpr/lpr} B cells to HEL^{3X}-SRBC. Percentages indicate the frequency of these clonal families within the respective responder populations. These rogue GC (GC_R) B cells show increased representation in the GC at these later time-points compared to day 21 (see Figure S5). IgE⁺ PCs were present but were not sorted at these late time-points.

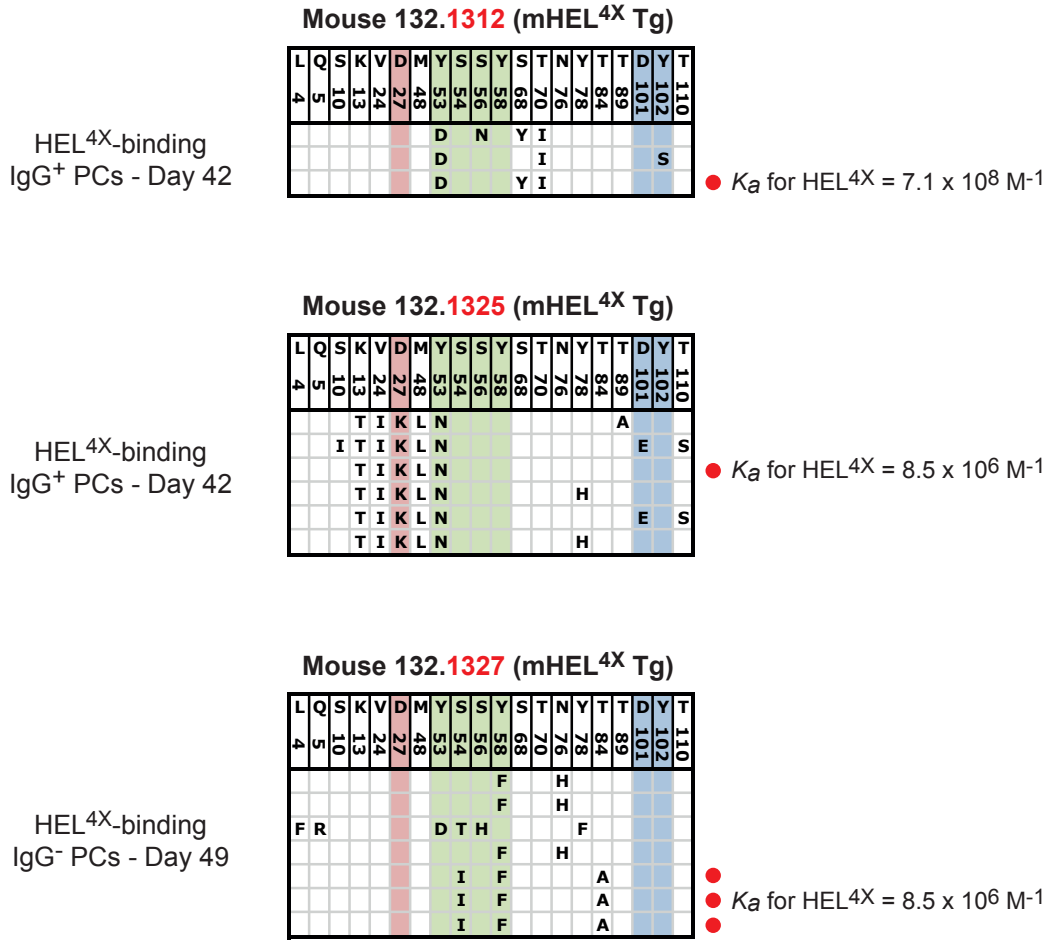


Figure S7, Related to Figure 7. Autoantibodies generated by FAS-deficient PC_R late in the response carry clonally-related Ig heavy chain variable regions.

Single cell sorting and Ig heavy chain variable region gene sequencing was carried out on donor-derived PCs exhibiting high HEL^{4X}-binding from individual mHEL^{4X} Tg recipient mice that had received SW_{HEL}.*Fas*^{lpr/lpr}.*Rag1*^{Δ/Δ} and HEL^{3X}-SRBC. HEL^{4X}-binding gates were applied to either IgG1⁺ PCs at days 42 (mice 132.1312 or 132.1325) or to IgG1⁻ PCs at day 49 (mouse 132.1327) for SHM analysis of the Ig heavy chain variable region gene (see Figure 7C). Self-reactive PCs in individual mice almost exclusively formed clonally-related families. The indicated core clones (red dots) were expressed as soluble IgG1 with the HyHEL10 κ light chain and subjected to binding analyses. Their affinities for HEL^{4X} as measured by Blitz analysis are indicated.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice. SW_{HEL} mice (Phan et al., 2003) were maintained on a CD45.1 congenic (*Ptprc*^{a/a}) C57BL/6 background. *Fas*^{lpr/lpr} (Adachi et al., 1993), *Fas*^{Δ/Δ} (Adachi et al., 1995), *Fasl*^{gld/gld} (Takahashi et al., 1994), *Fasl*^{Δm/Δm} (O' Reilly et al., 2009) and *Rag1*^{Δ/Δ} (Mombaerts et al., 1992) mice were either maintained on a C57BL/6 background or crossed with SW_{HEL} mice on a CD45.1 congenic C57BL/6 background for use in adoptive transfer experiments. mHEL^{4x} transgenic mice (Chan et al., 2012) were maintained on a C57BL/6 background. Wild-type C57BL/6 mice were purchased from either Australian BioResources (Moss Vale, Australia) or Animal Resources Centre (Perth, Australia). All mice were bred and maintained in specific pathogen-free conditions at Australian BioResources and the Garvan Institute of Medical Research Biological Testing Facility. Animal experiments were performed under the approved guidelines of the Garvan Institute of Medical Research/St. Vincent's Hospital Animal Ethics Committee.

Single cell RT-qPCR. RNA was isolated from sorted single cells using Ambion Single Cell to CT kit (Life Technologies) according to manufacturers protocol. Briefly, single IgE⁺ PC, IgG1⁺ PC or IgG1⁺ GC were sorted into 10 μL of single cell lysis/DNAse I solution in 96-well skirted plates chilled to 4°C in the collection chamber of a FACS AriaII (BD Biosciences). Plates were spun and brought to room temperature for 5 min, after which 1 μL of stop solution was added and samples incubated at room temperature for a further 2 min. To perform reverse transcription, 4.5 μL of RT mix was added and samples incubated for 10 min at 25°C, 60 min at 42°C and 5 min at 85°C. For pre-amplification, 11 μL of the PreAmp Mix/Taqman

assays was added to the samples which were then incubated at 95°C for 10 min followed by 14 cycles of 95°C for 15 sec plus 60°C for 4 min, followed by a final incubation at 99°C for 10 min. Incubation steps were performed in a C1000 Thermocycler (BioRad). RT-qPCR on the diluted pre-amplified product was performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems).

Pre-processing and analysis of single cell gene expression data. dCt values were obtained by subtracting raw cycle threshold (Ct) values from a cutoff value of 35. These were then expressed as Relative Quantitation (RQ) values ($2^{\Delta Ct}$). Data was then log₂ transformed using the LogTransform module in GenePattern (<https://pwbcr.garvan.org.au/gp>) thereby setting any negative values, where the gene expression levels are below the threshold Ct35, to zero. The NormalizeColumns module in GenePattern allowed for quantile normalization of the single cell expression data sets. For the purpose of normalization, zero values were removed from the dataset and replaced afterwards. Heatmaps were generated using the HeatMapView module in GenePattern. Gene expression analyses were carried out on log-transformed normalized data. LimmaGP, a GenePattern implementation of the Limma (Wettenhall and Smyth, 2004) BioConductor library module in GenePattern, was used to identify differentially expressed genes between the sorted populations, with a false discovery rate (FDR) of <0.05.

ELISAs. Total IgE levels in sera were measured by direct ELISA. 384-well plates (Corning) were coated with purified anti-IgE (R35-72, BD Biosciences) at 5 µg/mL in NPP buffer (35 mM NaHCO₃, 19 mM Na₂CO₃, 3 mM NaN₃, 1 mM MgCl₂) and stored overnight at 4°C. A plate washer (BioTek) was used to wash plates with ELISA

wash buffer (0.05% Tween20/PBS) and all subsequent steps performed at 37°C for 1 h. Wells were blocked with 1% BSA/PBS. Dilutions of serum samples and the standard, IgE isotype control (MEB-38, Biolegend), were prepared in 0.1% BSA/PBS and added in duplicate. Bound IgE antibodies were detected using an anti- κ light chain antibody (187.1, BD Bioscience) diluted in 0.1% BSA/1% skim milk powder/PBS. SA-alkaline phosphatase (Roche) in PBS was added and visualized with the substrate *p*-nitrophenyl phosphate (MP Biomedicals Australia) prepared in NPP buffer. Absorbance at 405 nm was read and a standard curve was constructed to determine the concentration of IgE antibodies in serum. To quantitate anti-HEL IgE serum antibody, 384-well plates were coated with purified anti-IgE (R35-72) at 5 μ g/mL in NPP buffer and stored overnight at 4°C. A plate washer was used to wash plates with ELISA wash buffer and all subsequent steps were incubated at 37°C for 1 h. Wells were blocked with 1% BSA/PBS followed by the addition of serum samples diluted in 0.1% BSA/PBS. Biotinylated HEL^{WT} was prepared using NHS-Biotinylation reagent (Sigma-Aldrich), diluted in 0.1% BSA/1% skim milk powder/PBS and used to detect bound anti-HEL IgE antibodies. SA-alkaline phosphatase was added and then visualized with the substrate *p*-nitrophenyl phosphate as for total IgE ELISA. Absorbance at 405 nm was read and endpoint titers were used to determine serum antibody concentrations (Frey et al., 1998) based on 99.9% confidence levels.

Immunofluorescence histology. Spleens were frozen in Tissue Tek OCT compound (Sakura). 6-7 μ m sections were cut using a CM3050 S cryostat (Leica). Prior to staining for IgE, CD23-bound IgE was removed using an acid wash (Katona et al., 1983). Briefly, sections were incubated for 1 min in a pH 4.0 acetate buffer followed

by neutralization in PBS. Sections were then fixed in ice-cold acetone and blocked with 30% normal horse serum (Invitrogen). Sections were stained with the following antibodies: anti-IgG1 biotin (A85-1, BD Biosciences) or anti-IgE biotin (R35-72, BD Biosciences), anti-IgD AlexaFluor647 (11-26c.2a, Biolegend). Biotinylated antibodies were detected using SA-AlexaFluor555 (Invitrogen). To stain HEL-binding cells, sections were incubated with 200 ng/mL of HEL^{WT} and detected with polyclonal rabbit anti-HEL IgG (Rockland Immunochemicals Inc.) followed by goat anti-rabbit FITC (Southern Biotech). Slides were analyzed with a Leica DM5500 using the x10 or x40 objective and Adobe Photoshop software.

Statistical Analysis. Statistical significance of data was calculated with Prism 6.0 software (Graphpad). For comparisons between two normally distributed groups a two-tailed unpaired *t*-test with Welch's correction was used whilst non-parametric data was analyzed using Mann-Whitney *U* test. For comparison between more than two groups statistical analysis was performed using a one-way ANOVA with the post-hoc Bonferroni test whereas the Kruskal-Wallis test with Dunn's multiple comparison post-test was used in cases where data was non-parametric. Two-way ANOVA with the post-hoc Bonferroni's test was used to compare responses with more than two parameters. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001.

SUPPLEMENTAL REFERENCES

Adachi, M., Suematsu, S., Kondo, T., Ogasawara, J., Tanaka, T., Yoshida, N., and Nagata, S. (1995). Targeted mutation in the Fas gene causes hyperplasia in peripheral lymphoid organs and liver. *Nat Genet* *11*, 294-300.

Adachi, M., Watanabe-Fukunaga, R., and Nagata, S. (1993). Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of lpr mice. *Proc Natl Acad Sci U S A* *90*, 1756-1760.

Frey, A., Di Canzio, J., and Zurakowski, D. (1998). A statistically defined endpoint titer determination method for immunoassays. *J Immunol Methods* *221*, 35-41.

Katona, I.M., Urban, J.F., Jr., Scher, I., Kanellopoulos-Langevin, C., and Finkelman, F.D. (1983). Induction of an IgE response in mice by *Nippostrongylus brasiliensis*: characterization of lymphoid cells with intracytoplasmic or surface IgE. *J Immunol* *130*, 350-356.

Mombaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., and Papaioannou, V.E. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* *68*, 869-877.

Wettenhall, J.M., and Smyth, G.K. (2004). limmaGUI: a graphical user interface for linear modeling of microarray data. *Bioinformatics* *20*, 3705-3706.