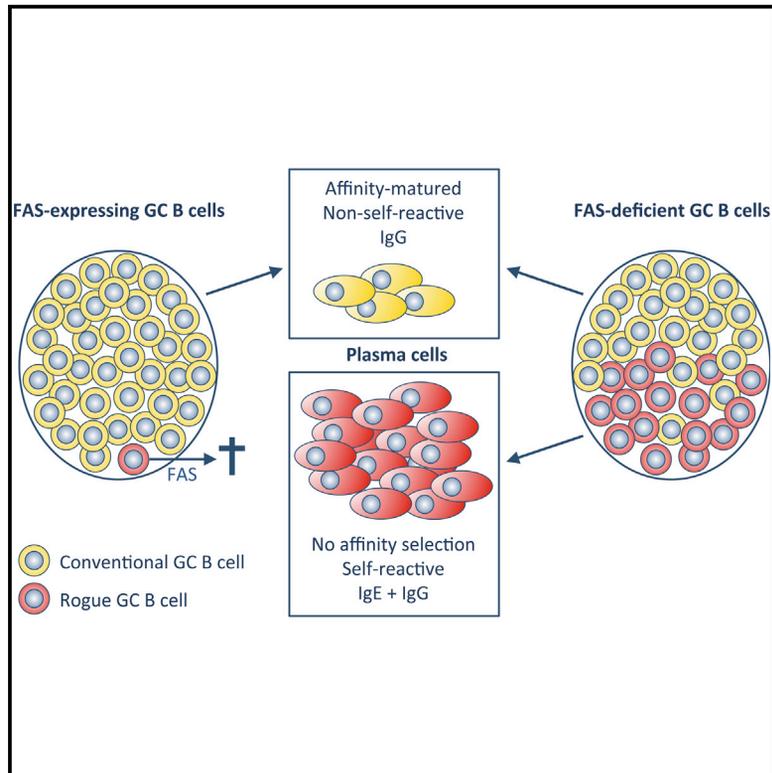


Immunity

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

Graphical Abstract



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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

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<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 (Victoria 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

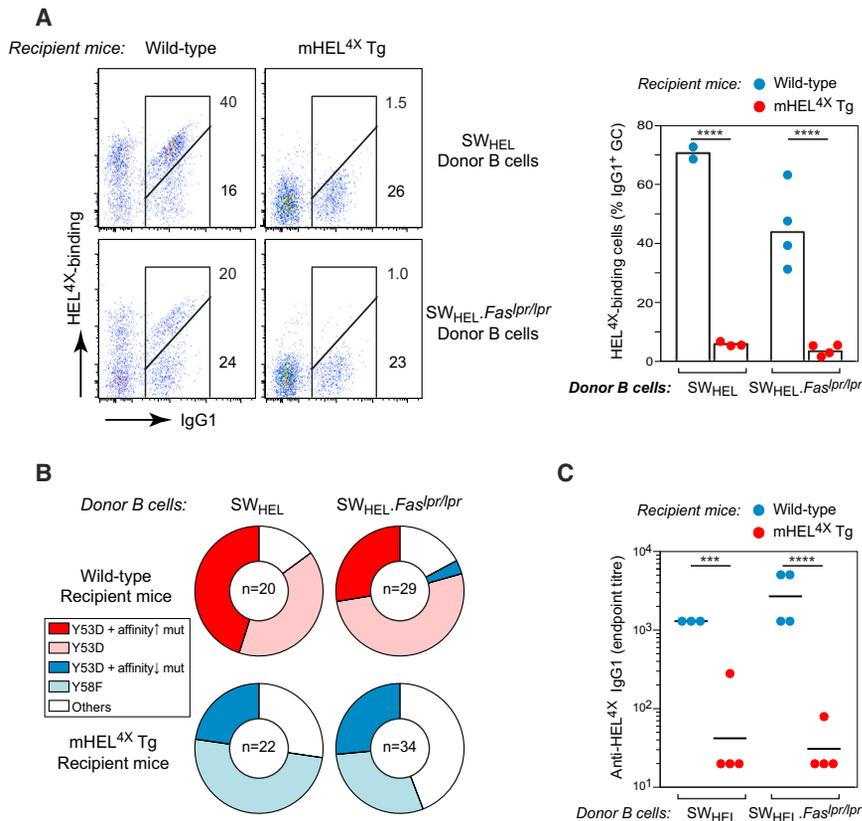


Figure 1. FAS-Mediated Apoptosis Is Not Required to Remove Conventional Self-Reactive GC B Cells

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

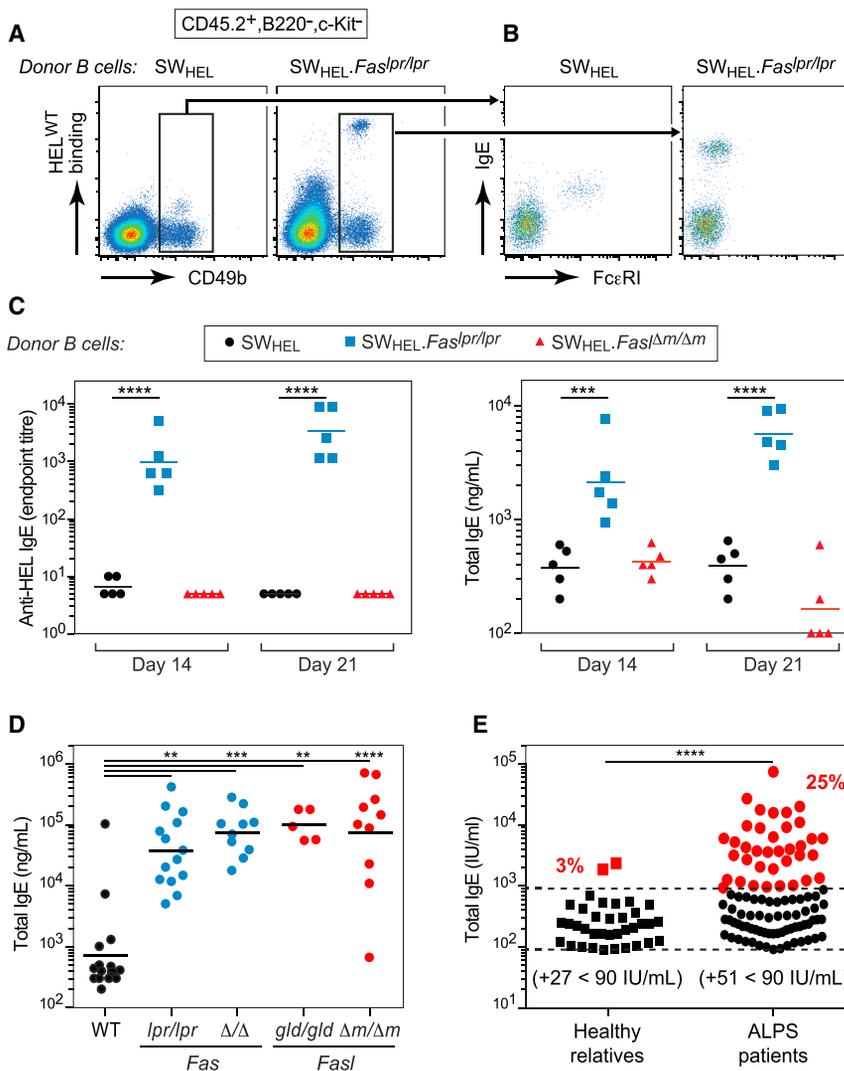


Figure 2. FASL-FAS Suppress IgE Antibody Production in Mice and Humans

(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 was 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.

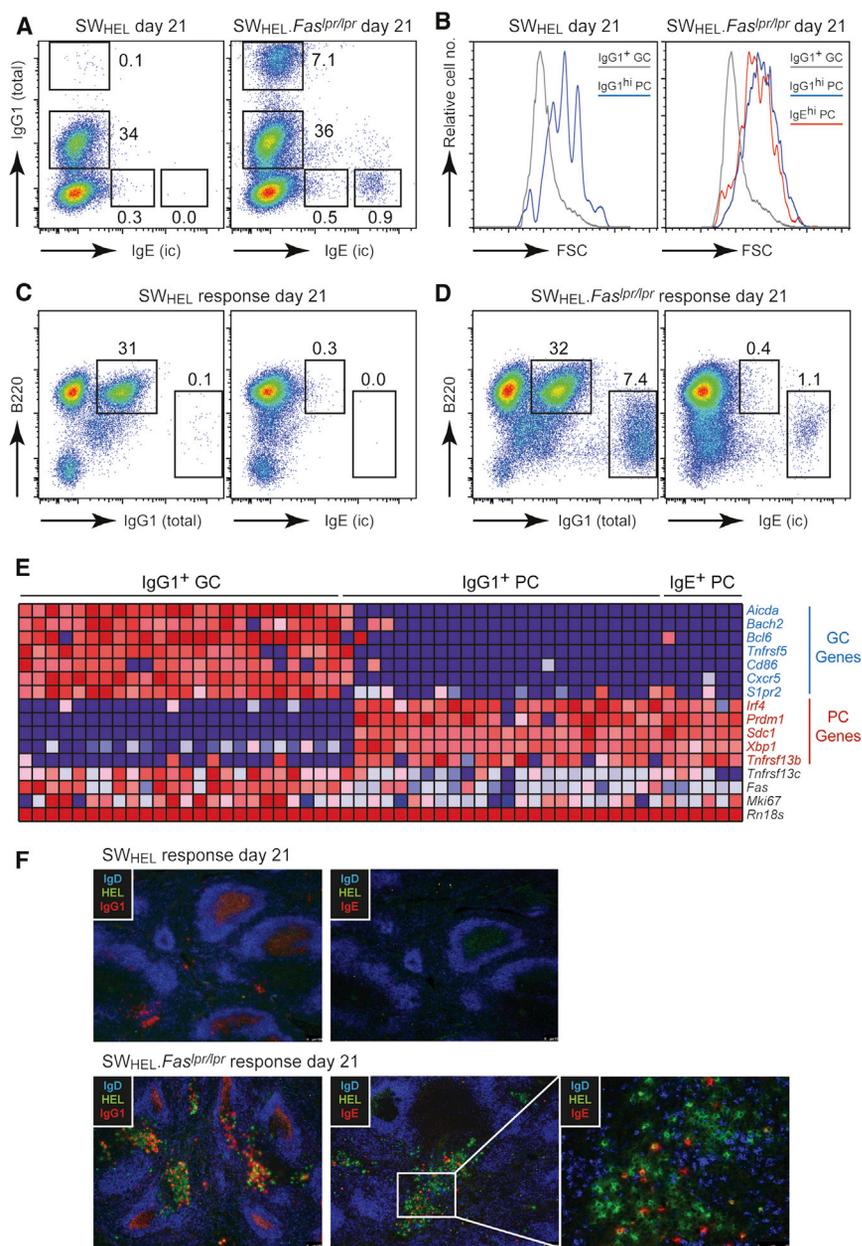


Figure 3. FAS-Deficient B Cells Generate Large Numbers of IgG1⁺ and IgE⁺ PCs

SW_{HEL} or SW_{HEL.Fas^{Ipr/Ipr}} 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^{Ipr/Ipr}} response. Relative mRNA expression is indicated from high (red) to low (blue).

(F) Immunofluorescence histology of SW_{HEL} and SW_{HEL.Fas^{Ipr/Ipr}} 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^{Ipr/Ipr}} 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^{Ipr/Ipr}} 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^{Ipr}* mutation (SW_{HEL.Fas^{Ipr/Ipr}}) 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^{Ipr/Ipr}} 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

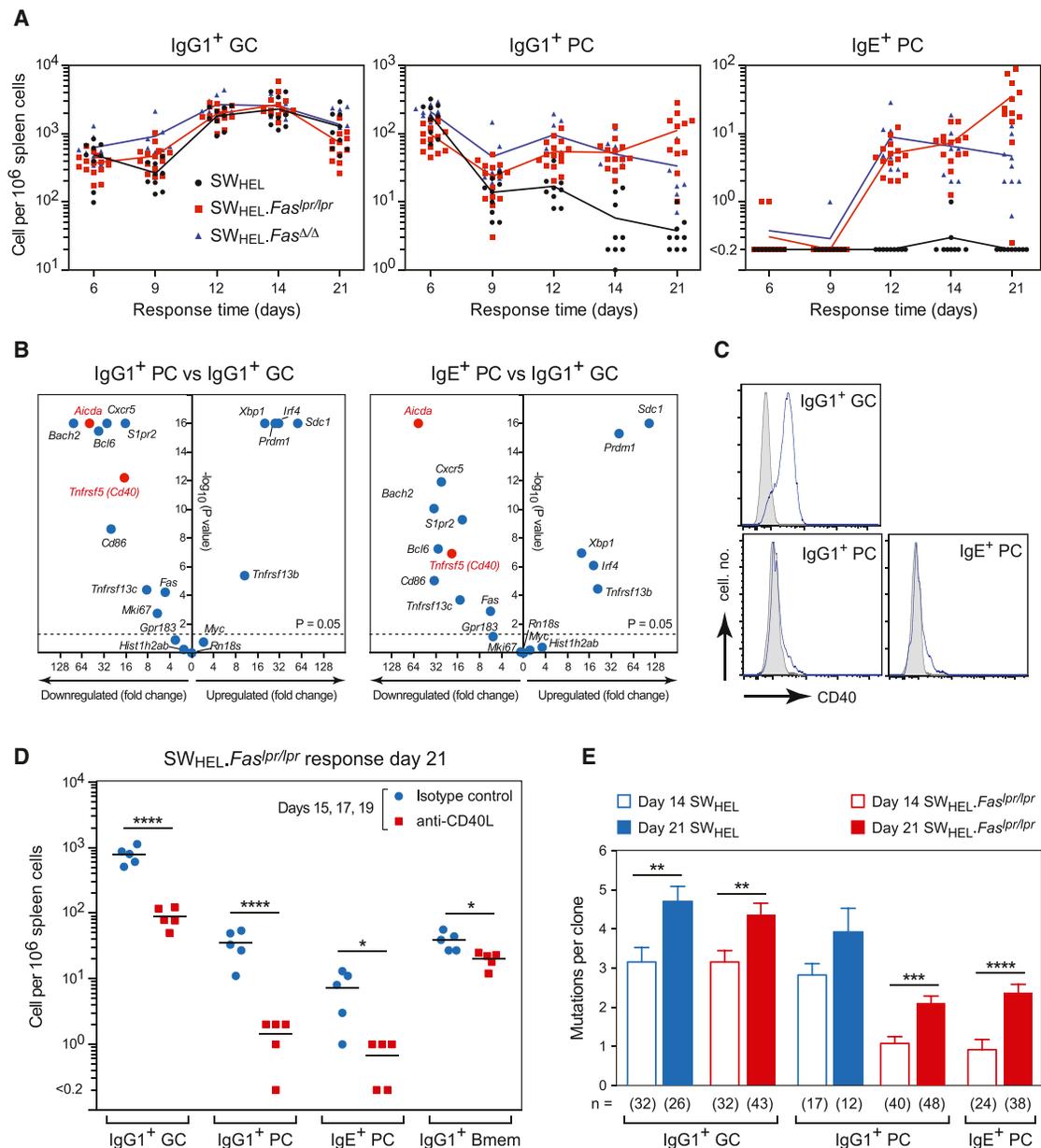


Figure 4. FAS-Deficient IgG1⁺ and IgE⁺ PCs Derive from GC B Cell Precursors

(A) Time course of donor-derived splenic responses of SW_{HEL}, SW_{HEL}.Fas^{Ipr/Ipr}, 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^{Ipr/Ipr} 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^{Ipr/Ipr} B cells to HEL^{3X}-SRBCs. (D) Frequency of splenic donor-derived subsets from SW_{HEL}.Fas^{Ipr/Ipr} 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^{Ipr/Ipr} 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 GCr 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 GCr 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 GCr B cells and their PC derivatives (PCr). This analysis revealed that the generation of PCr from GCr 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 ($K_a < 10^5 M^{-1}$) 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 GCr 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

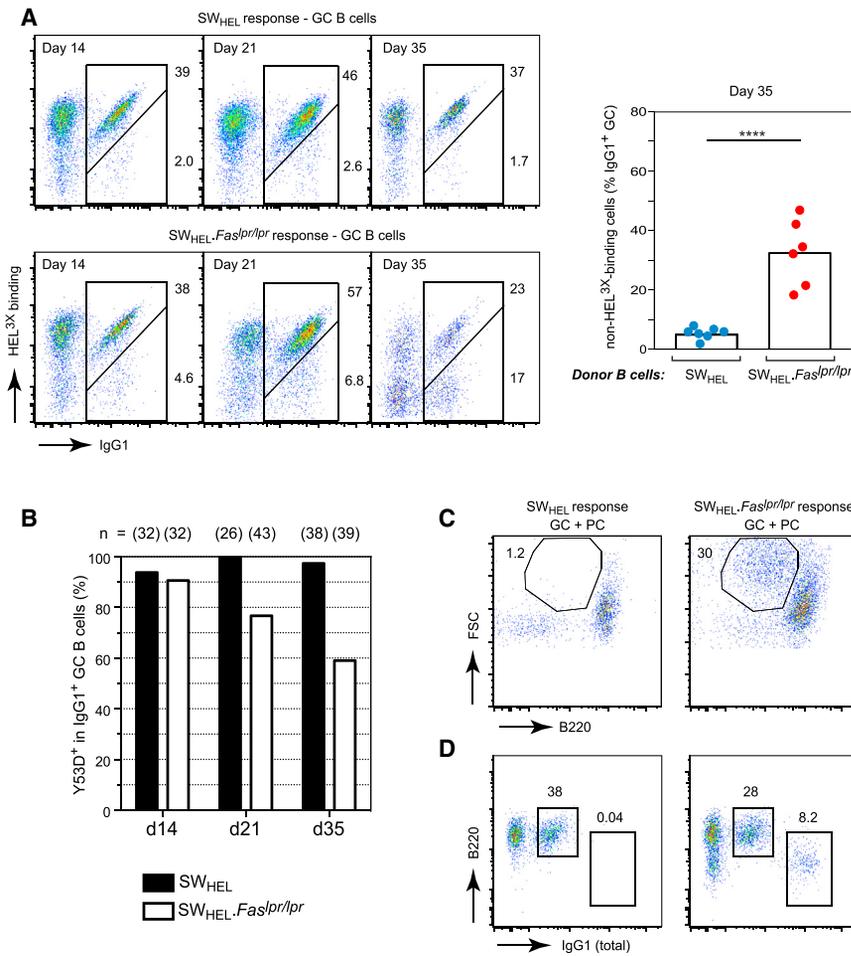


Figure 6. GCr B Cells Accumulate, Display Abnormal Antigen Selection, and Continue to Generate PCr

SW_{HEL} or SW_{HEL}.Fas^{lpr/lpr} 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^{lpr/lpr} 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^{lpr/lpr} 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).

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^{lpr/lpr} 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^{lpr/lpr} 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^{lpr/lpr} 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^{lpr/lpr}.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).

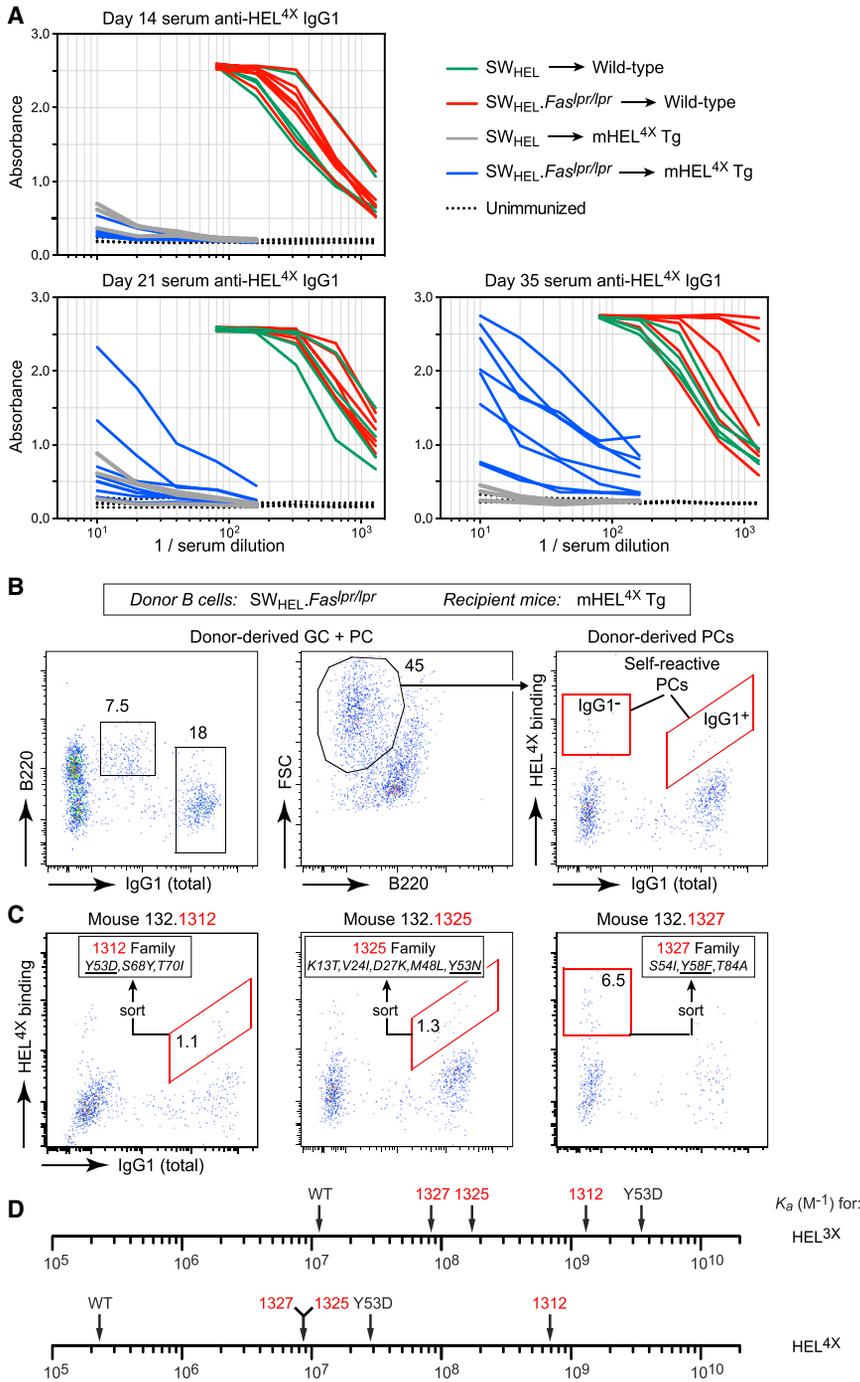


Figure 7. GCr B Cells Produce Autoantibody-Secreting PCr Late in the Response of SW_{HEL}.Fas^{lpr/lpr} B Cells to HEL^{3X}-SRBCs

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 PCr 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 PCr.

(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, GCr 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 PCr 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 GCr 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 GCr and PCr 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 GCr 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 GCr 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 Tfh cells, could be required to maintain GCr 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 Tfh cells.

Whatever the specific mechanism is that drives the production of GCr 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 GCr 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), Tfh/Tfr CD4⁺ cells (Rathmell et al., 1995), or even CD8⁺ T cells (Afshar-Sterle et al., 2014). What feature of GCr 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 GCr 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 GCr 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 GCr 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 GCr 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 GCr 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 GCr 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 × 10⁶ HEL-binding B cells were transferred i.v. into wild-type or mHEL^{4X} Tg recipient mice together with 2 × 10⁸

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 R1 (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 immunometric 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

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