IMMUNOLOGY

The microanatomic segregation of selection by apoptosis in the germinal center


INTRODUCTION: Germinal centers (GCs) are transient microanatomic structures that form in lymphoid organs during an immune response. They are the sites of B cell clonal expansion and affinity maturation, a process that leads to the production of high-affinity antibodies. GCs are highly dynamic and contain activated B cells, specialized T follicular helper (Tfh) cells, and antigen-trapping follicular dendritic cells. GCs are organized into two functionally distinct compartments: a dark zone (DZ) and a light zone (LZ). The DZ is the site of rapid cell division and random antibody-gene mutation, which is initiated by activation-induced cytidine deaminase (AID). The mutation process leads to the accumulation of a large number of chromosome translocations. It can also produce immunoglobulin (Ig) gene missense mutations and deletions or create self-reactive antibodies. These deleterious mutations need to be selected against. Indeed, histologists have long appreciated large numbers of apoptotic nuclei in the specialized tingible body macrophages found in GCs. However, beyond histology, little is known about the exact rate of GC B cell apoptosis and whether it differs in the DZ and LZ of the GC. Moreover, the mechanisms that cause apoptosis, their relative importance in each GC compartment, and their role in GC B cell selection have not been defined. To examine these questions, we created fluorescent apoptosis-indicator mice and used them to enumerate, isolate, and characterize dying cells in the GC.

RESULTS: We found that apoptosis is prevalent in both the DZ and LZ compartments of GCs throughout the immune response: up to 50% of GC B cells undergo programmed cell death every 6 hours. Single dying GC B cells were isolated, and their antibody genes were cloned, expressed by transient transfection, and tested for antigen binding and other properties. Apoptotic DZ GC B cells were highly enriched for Ig genes damaged by AID, including missense mutations and deletions. By contrast, dying LZ cells primarily expressed intact antibodies with a range of affinities indistinguishable from GC B cells in the live LZ compartment. By experimentally blocking positive selection and by using reporter mice for Myc, a proto-oncogene, as an indicator of positive selection, we found that apoptosis is the default fate for LZ GC B cells that are not actively positively selected. Thus, LZ GC B cells carrying low-affinity BCRs do not preferentially undergo apoptosis. Instead, apoptosis occurs irrespective of BCR-affinity, and LZ B cells carrying high-affinity BCRs are simply more likely to be positively selected.

CONCLUSION: Apoptosis is a major feature of GC B cell biology and is required to counterbalance the high rate of proliferation and purge B cells that carry deleterious mutations. Although apoptosis occurs in both the DZ and LZ, the underlying mechanisms of apoptosis in each zone are distinct and microanatomically segregated. These insights into GC B cell biology are relevant for vaccine design, particularly for pathogens that normally evade effective antibody responses.

The list of author affiliations is available in the full article online.

*Corresponding author. Email: nussenzweig@rockefeller.edu

Downloaded from http://science.sciencemag.org/ on January 13, 2019

Mayer et al., Science 358, 193 (2017) 13 October 2017
The microanatomic segregation of selection by apoptosis in the germinal center


B cells undergo rapid cell division and affinity maturation in anatomically distinct sites in lymphoid organs called germinal centers (GCs). Homeostasis is maintained in part by B cell apoptosis. However, the precise contribution of apoptosis to GC biology and selection is not well defined. We developed apoptosis-indicator mice and used them to visualize, purify, and characterize dying GC B cells. Apoptosis is prevalent in the GC, with up to half of all GC B cells dying every 6 hours. Moreover, programmed cell death is differentially regulated in the light zone and the dark zone: Light-zone B cells die by default if they are not positively selected, whereas dark-zone cells die when their antigen receptors are damaged by activation-induced cytidine deaminase.

Germinal centers (GCs) are divided into two anatomic compartments: the light zone (LZ) and the dark zone (DZ). B cells divide and undergo somatic hypermutation (SHM) in the DZ and are positively selected for affinity-enhancing mutations by interacting with T follicular helper (TFH) cells in the LZ [1, 2, 3]. Cell division is a dominant feature of the GC, with rapid cell division rates of 4 to 6 hours and up to 30% of cells in cycle at any time [1, 2, 3, 4]. Despite extensive cell division, the size of the GC compartment can be relatively constant for weeks or months [5]. Equilibrium is attributed to a combination of cell death by apoptosis (negative selection) and emigration of memory and plasma cells from the GC. Emigration rates are estimated to be relatively low (<0.1% for plasma cells [6] and <2% for memory cells [7]). By contrast, cell loss by apoptosis is reported to be high [6, 8], but the precise rate and causes of apoptosis, its contribution to GC B cell selection, and whether it is differentially regulated in the LZ and DZ of the GC has not been determined.

Negative selection in the DZ and LZ

We used a monoclonal antibody against active caspase-3 (aCasp3) to identify apoptotic cells in the GCs of C57BL/6J mice immunized with either 4-hydroxy-3-nitrophenylacetyl (NP)-conjugated ovalbumin (NP-OVA) or an HIV-1 envelope antigen (BG505 SOSIP.v4.1-GT1.1 trimers, (GT1.1)). Wherefore follicular (FO) B cells expressing aCasp3 were nearly absent, 3.6% to 5.7% of GC B cells were aCasp3+ irrespective of the time of analysis or immunogen (Fig. 1, A and B). Similarly, 3% of B cells in chronic GCs in Peyer’s patches were aCasp3+ (Fig. S1, A and B). When compared to nonapoptotic cells, aCasp3+ GC B cells expressed slightly reduced levels of the B cell lineage and activation markers B220, Fas, CD19, CD86, and GL7, but comparable levels of activation-induced cytidine deaminase (AID), as indicated by an AID-green fluorescent protein (GFP) knock-in gene [10], (fig. SIC).

Analysis of apoptosis among DZ and LZ GC B cells revealed that 3.7 to 5.7% of the DZ and 2.6 to 5.6% of the LZ were aCasp3+ at all time points analyzed (Fig. 1, C and D). Similar results were also obtained for Peyer’s patch GC B cells (fig. SIE). Thus, the frequency of apoptotic GC B cells is relatively constant over time and nearly equivalent in LZ and DZ compartments.

The size of GCs in Peyer’s patches is relatively constant over time in mice housed under specific pathogen-free conditions, and thus, the number of dividing cells should equal the number of dying cells plus a small number that leave the GC to become memory B or plasma cells [6, 7, 11]. To estimate the proportion of dividing cells in GCs, we performed kinetic labeling experiments with the nucleoside analog 5-ethyl-2′-deoxyuridine (EdU), which is incorporated into DNA during the S phase of the cell cycle. About 50% of all Peyer’s patch GC B cells were labeled by EdU in 5.3 hours (Fig. 1, E and F), suggesting that a large number of GC B cells are lost to cell death during this time.

Dynamics of dying GC B cells

To further characterize these events, we produced transgenic and knock-in mice that express an apoptosis reporter (indicator of apoptosis (INDIA), as follicular (FO) B cells expressing aCasp3 were nearly absent, 3.6% to 5.7% of GC B cells were aCasp3+ irrespective of the time of analysis or immunogen (Fig. 1, A and B). Similarly, 3% of B cells in chronic GCs in Peyer’s patches were aCasp3+ (Fig. S1, A and B). When compared to nonapoptotic cells, aCasp3+ GC B cells expressed slightly reduced levels of the B cell lineage and activation markers B220, Fas, CD19, CD86, and GL7, but comparable levels of activation-induced cytidine deaminase (AID), as indicated by an AID-green fluorescent protein (GFP) knock-in gene [10], (fig. SIC).

Analysis of apoptosis among DZ and LZ GC B cells revealed that 3.7 to 5.7% of the DZ and 2.6 to 5.6% of the LZ were aCasp3+ at all time points analyzed (Fig. 1, C and D). Similar results were also obtained for Peyer’s patch GC B cells (fig. SIE). Thus, the frequency of apoptotic GC B cells is relatively constant over time and nearly equivalent in LZ and DZ compartments.

The size of GCs in Peyer’s patches is relatively constant over time in mice housed under specific pathogen-free conditions, and thus, the number of dividing cells should equal the number of dying cells plus a small number that leave the GC to become memory B or plasma cells [6, 7, 11]. To estimate the proportion of dividing cells in GCs, we performed kinetic labeling experiments with the nucleoside analog 5-ethyl-2′-deoxyuridine (EdU), which is incorporated into DNA during the S phase of the cell cycle. About 50% of all Peyer’s patch GC B cells were labeled by EdU in 5.3 hours (Fig. 1, E and F), suggesting that a large number of GC B cells are lost to cell death during this time.

Dynamics of dying GC B cells

To further characterize these events, we produced transgenic and knock-in mice that express an apoptosis reporter (indicator of apoptosis (INDIA), as follicular (FO) B cells expressing aCasp3 were nearly absent, 3.6% to 5.7% of GC B cells were aCasp3+ irrespective of the time of analysis or immunogen (Fig. 1, A and B). Similarly, 3% of B cells in chronic GCs in Peyer’s patches were aCasp3+ (Fig. S1, A and B). When compared to nonapoptotic cells, aCasp3+ GC B cells expressed slightly reduced levels of the B cell lineage and activation markers B220, Fas, CD19, CD86, and GL7, but comparable levels of activation-induced cytidine deaminase (AID), as indicated by an AID-green fluorescent protein (GFP) knock-in gene [10], (fig. SIC).

Analysis of apoptosis among DZ and LZ GC B cells revealed that 3.7 to 5.7% of the DZ and 2.6 to 5.6% of the LZ were aCasp3+ at all time points analyzed (Fig. 1, C and D). Similar results were also obtained for Peyer’s patch GC B cells (fig. SIE). Thus, the frequency of apoptotic GC B cells is relatively constant over time and nearly equivalent in LZ and DZ compartments.

The size of GCs in Peyer’s patches is relatively constant over time in mice housed under specific pathogen-free conditions, and thus, the number of dividing cells should equal the number of dying cells plus a small number that leave the GC to become memory B or plasma cells [6, 7, 11]. To estimate the proportion of dividing cells in GCs, we performed kinetic labeling experiments with the nucleoside analog 5-ethyl-2′-deoxyuridine (EdU), which is incorporated into DNA during the S phase of the cell cycle. About 50% of all Peyer’s patch GC B cells were labeled by EdU in 5.3 hours (Fig. 1, E and F), suggesting that a large number of GC B cells are lost to cell death during this time.

Dynamics of dying GC B cells

To further characterize these events, we produced transgenic and knock-in mice that express an apoptosis reporter (indicator of apoptosis (INDIA), as follicular (FO) B cells expressing aCasp3 were nearly absent, 3.6% to 5.7% of GC B cells were aCasp3+ irrespective of the time of analysis or immunogen (Fig. 1, A and B). Similarly, 3% of B cells in chronic GCs in Peyer’s patches were aCasp3+ (Fig. S1, A and B). When compared to nonapoptotic cells, aCasp3+ GC B cells expressed slightly reduced levels of the B cell lineage and activation markers B220, Fas, CD19, CD86, and GL7, but comparable levels of activation-induced cytidine deaminase (AID), as indicated by an AID-green fluorescent protein (GFP) knock-in gene [10], (fig. SIC).

Analysis of apoptosis among DZ and LZ GC B cells revealed that 3.7 to 5.7% of the DZ and 2.6 to 5.6% of the LZ were aCasp3+ at all time points analyzed (Fig. 1, C and D). Similar results were also obtained for Peyer’s patch GC B cells (fig. SIE). Thus, the frequency of apoptotic GC B cells is relatively constant over time and nearly equivalent in LZ and DZ compartments.

The size of GCs in Peyer’s patches is relatively constant over time in mice housed under specific pathogen-free conditions, and thus, the number of dividing cells should equal the number of dying cells plus a small number that leave the GC to become memory B or plasma cells [6, 7, 11]. To estimate the proportion of dividing cells in GCs, we performed kinetic labeling experiments with the nucleoside analog 5-ethyl-2′-deoxyuridine (EdU), which is incorporated into DNA during the S phase of the cell cycle. About 50% of all Peyer’s patch GC B cells were labeled by EdU in 5.3 hours (Fig. 1, E and F), suggesting that a large number of GC B cells are lost to cell death during this time.
the complementary DNA of which was embedded into immunoglobulin κ regulatory elements (Iκκ<sup>ΔNDA</sup>) and the wild-type Rosa26 locus (Rosa26<sup>ΔNDA</sup>, fig. S2, A and B). This reporter consists of an optimized fluorescence (Förster) resonance energy transfer (FRET) pair, mNeonGreen and mRuby2, linked by a peptide containing an aCasp3-cleavage site (DEVDG) (fig. S2C). To validate the reporter, transgenic B cells were activated in vitro and induced to undergo apoptosis by incubation with staurosporine (22). Flow cytometry revealed two distinct populations based on the mNeonGreen/FRET ratio (termed “FRET loss”; Fig. 2B, left). Whereas FRET<sup>+</sup> B cells were alive, FRET<sup>−</sup> B cells were apoptotic, as confirmed by aCasp3, TUNEL, or Annexin V–DAP staining (TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling; DAPI, 4′,6-diamidino-2-phenylindole) (Fig. 2B and fig. S2D).

To examine the kinetics of activated B cell death, we tracked FRET loss in real time in cultured Igκκ<sup>ΔNDA</sup> B cells (Fig. 2C and fig. S2E). On average, the first morphological signs of apoptosis were observed within 12.5 min of FRET loss, including cell shrinkage, bleb formation, and changes in motility (Fig. 2, C and D, fig. S2E, and movies S1 to S3). Secondary necrosis, as revealed by loss of membrane integrity and leakage (Fig. 2C, fig. S2E, and movies S1 to S3), was observed on average of 68 min after FRET loss (Fig. 2D). Similar results were obtained in vivo by tracking Rosa26<sup>ΔNDA</sup> knock-in GC B cell death using two-photon laser scanning microscopy. GC B cell fragmentation occurred on average 20.6 min after FRET loss and was observed in both DZ and LZ compartments (Fig. 2, E to G; Movies 1 to 3; and fig. S3, A and B). Although the precise relationship between caspase staining, FRET loss, and cell fragmentation has not been defined, it is clear that the apoptotic compartment in GCs turns over with rapid kinetics. At an apoptosis rate of 3% every 20.6 min (fig. S1, A and B), 46% of GC B cells in Peyer’s patches are estimated to be lost in 5.3 hours, which agrees with our measurements made by EdU labeling (Fig. 1, E and F). Thus, apoptosis is a major feature of the B cell program in the GC.

Negative selection against damaged B cell receptors (BCRs) in the DZ

What causes the high level of GC B cell apoptosis? GC B cells express AID, an enzyme that initiates class-switch recombination (CSR) and SHM by polymerase chain reaction. (I) and (J) Pie charts show the fraction of nonfunctional BCRs (red) in live and apoptotic GC B cells (top) or in the LZ and DZ compartments (bottom) after (I) NP-OVA and (J) GT1.1 immunization. The number in the center indicates the number of Ig pairs analyzed. Data are from at least two independent experiments in all cases. ****P < 0.0001; Fisher’s exact test.
AID introduces random mutations in Ig genes that can increase antibody affinity, but can also be deleterious. To determine how Ig mutation impacts apoptosis, we cloned antibodies from single FRET Ig<sup>DNNA</sup> GC B cells that had started undergoing apoptosis (Fig. 2H and fig. S5A). Ig heavy-chain (Igh) and light-chain (Igk and Igλ) sequencing revealed that 94 and 83% of live NP-OVA- and GTL1-elicited GC B cells carried intact BCRs, respectively (Fig. 2, I and J; top). By contrast, only 68 and 59% of apoptotic NP-OVA- and GTL1-elicited GC B cells carried BCR transcripts capable of producing Ig (Fig. 2, I and J; top). The loss of BCR expression in the apoptotic compartment was confirmed by flow cytometry in NP-OVA-specific GCs and Peyer’s patches and was AID-dependent (fig. S5, B and C). Apoptotic GC cells with nonfunctional BCRs were highly enriched in the DZ as compared to the LZ: 43 and 58% of apoptotic DZ BCRs and 9 and 14% of apoptotic LZ GC B cells in NP-OVA- or GTL1-immunized mice, respectively, carried nonproductive Ig transcripts (Fig. 2, I and J; bottom). This observation is consistent with reports that AID is expressed at higher levels and accesses DNA in proliferating DZ B cells (5, 16, 17). Although most nonfunctional apoptotic GC BCRs carried stop codons (63 and 69% in NP-OVA- and GTL1-elicited GCs, respectively), a significant fraction (37 and 31%, respectively) was out of frame because of nucleotide insertions or deletions (fig. S5, D and E).

Thus, apoptotic DZ B cells are enriched for nonfunctional Ig transcripts as a consequence of AID activity. We hypothesize that the small number of apoptotic LZ GC B cells with nonfunctional BCRs derive from recent LZ emigrants in which a delay between aberrant Ig gene mutation, loss of BCR expression, and apoptosis has occurred, as has been reported for naïve B cells (18).

**Characterization of monoclonal antibodies cloned from dying GC B cells**

In addition to compromising the integrity of the BCR, SHM can also alter antibody affinity, produce autoreactive or polyreactive BCRs, or render Ig heavy and light chains incompatible. To measure the contribution of each of these effects to GC B cell apoptosis, we produced Ig<sub>H</sub><sup>1+72</sup> Ig<sub>δ</sub>−NP antibodies cloned from GC B cells of NP-OVA-immunized Ig<sub>δK<sup>DNNA</sup></sub> mice as determined by aCasp3 staining. Results are combined from two independent experiments each involving three to five mice. ****<i>p</i> < 0.0001; paired Student’s <i>t</i> test.

Thus, apoptotic DZ B cells are enriched for nonfunctional Ig transcripts as a consequence of AID activity. We hypothesize that the small number of apoptotic LZ GC B cells with nonfunctional BCRs derive from recent LZ emigrants in which a delay between aberrant Ig gene mutation, loss of BCR expression, and apoptosis has occurred, as has been reported for naïve B cells (18).

**Characterization of monoclonal antibodies cloned from dying GC B cells**

In addition to compromising the integrity of the BCR, SHM can also alter antibody affinity, produce autoreactive or polyreactive BCRs, or render Ig heavy and light chains incompatible. To measure the contribution of each of these effects to GC B cell apoptosis, we produced Ig<sub>H</sub><sup>1+72</sup> Ig<sub>δ</sub>−NP antibodies cloned from GC B cells of NP-OVA-immunized Ig<sub>δK<sup>DNNA</sub></sub> mice as determined by aCasp3 staining. Results are combined from two independent experiments each involving three to five mice. ****<i>p</i> < 0.0001; paired Student’s <i>t</i> test.

Thus, apoptotic DZ B cells are enriched for nonfunctional Ig transcripts as a consequence of AID activity. We hypothesize that the small number of apoptotic LZ GC B cells with nonfunctional BCRs derive from recent LZ emigrants in which a delay between aberrant Ig gene mutation, loss of BCR expression, and apoptosis has occurred, as has been reported for naïve B cells (18).

**Characterization of monoclonal antibodies cloned from dying GC B cells**

In addition to compromising the integrity of the BCR, SHM can also alter antibody affinity, produce autoreactive or polyreactive BCRs, or render Ig heavy and light chains incompatible. To measure the contribution of each of these effects to GC B cell apoptosis, we produced Ig<sub>H</sub><sup>1+72</sup> Ig<sub>δ</sub>−NP antibodies cloned from GC B cells of NP-OVA-immunized Ig<sub>δK<sup>DNNA</sub></sub> mice as determined by aCasp3 staining. Results are combined from two independent experiments each involving three to five mice. ****<i>p</i> < 0.0001; paired Student’s <i>t</i> test.

Thus, apoptotic DZ B cells are enriched for nonfunctional Ig transcripts as a consequence of AID activity. We hypothesize that the small number of apoptotic LZ GC B cells with nonfunctional BCRs derive from recent LZ emigrants in which a delay between aberrant Ig gene mutation, loss of BCR expression, and apoptosis has occurred, as has been reported for naïve B cells (18).

**Characterization of monoclonal antibodies cloned from dying GC B cells**

In addition to compromising the integrity of the BCR, SHM can also alter antibody affinity, produce autoreactive or polyreactive BCRs, or render Ig heavy and light chains incompatible. To measure the contribution of each of these effects to GC B cell apoptosis, we produced Ig<sub>H</sub><sup>1+72</sup> Ig<sub>δ</sub>−NP antibodies cloned from GC B cells of NP-OVA-immunized Ig<sub>δK<sup>DNNA</sub></sub> mice as determined by aCasp3 staining. Results are combined from two independent experiments each involving three to five mice. ****<i>p</i> < 0.0001; paired Student’s <i>t</i> test.
Fig. 4. Apoptosis in the GC LZ. (A) Graphs show the fraction of GC B cells among CD19 + B cells (left column), fraction of Edu + GC B cells (second column), and fraction of aCasp3 + cells in GC LZ and DZ (right columns) in the draining lymph nodes of C57BL/6J mice 14 days after NP-OVA immunization. Hamster IgG or α-CD40L was injected 24, 48, or 72 hours before analysis. Results are combined from two independent experiments, each with five mice per condition. Red lines indicate means. **P = 0.007 (24 hours, GC size); NS, P > 0.05 (not statistically significant); two-tailed Mann-Whitney U rank sum test. (B and C) Analysis of apoptosis in GC B cells in Nur77-GFP or Myc-GFP mice 14 days after immunization with NP-OVA. (B) Representative flow-cytometry plots show the frequency of aCasp3 + cells in Nur77-GFP + and Nur77-GFP − (left graph), or Myc-GFP + and Myc-GFP − LZ GC B cells (right graph). (B) and (C) Two to three independent experiments each involving three to six Nur77-GFP or Myc-GFP mice with three mice pooled per data set. Nur77-GFP, *P = 0.0355; Myc-GFP, **P = 0.006; paired Student’s t test.

Nearly all Iggs cloned from live GC B cells produced secreted antibodies in transient transfection experiments. By contrast, a significant fraction of the antibodies derived from apoptotic IgH717,72 Igδ GC B cells did not, despite functional Ig genes (Fig. 3F). This phenomenon was particularly prominent among apoptotic DZ cells, where approximately half of the clones did not produce secreted antibodies in transient transfection experiments. Despite the lack of secreted Ig, immunoblot analysis of transfected cell pellets showed normal levels of IgH and Igδ expression, suggesting that structural problems and/or defective Ig pairing interfered with normal antibody secretion (fig. S7). Thus, a significant fraction of the apoptotic cells in NP-specific GCs express antibodies that are structurally compromised (28%), and this phenomenon is exclusive to DZ GC B cells. In summary, selection against SHMs that introduce nucleotide insertions or deletions, produce stop codons, change the reading frame, or otherwise compromise IgH717,72 Igδ expression or stability account for 75% of all the apoptotic cells in the DZ of NP-specific GCs (Fig. 3G).

Isotype switching

AID initiates both SHM and CSR. To examine the role of CSR in regulating apoptosis independently of SHM, we analyzed Peyer’s patches from AIDflvFlv -/+ IgH717,72Gδ656YFP Rosa26+/- mice in which ~50% of GC B cells undergo Cre-mediated CSR to IgG1 in the absence of AID and SHM (fig. S8, A and B; (22)). IgG1 + GC B cells were enriched among apoptotic cells in both the LZ and DZ. By contrast, IgM + cells were overrepresented in the
live compartments (Fig. 3H and fig. S8B). Similar results were also obtained in Peyer’s patches and in lymph nodes of NP-OVA-immunized AID-deficient C57BL/6J mice (fig. S8, C and D). Thus, IgG1+ GC B cells are more prone to apoptosis than IgM-expressing cells irrespective of AID expression or SHM. This effect may be due to altered IgG1 BCR signaling, as indicated by lower levels of ion or SHM. This effect may be due to altered nuclear receptor and (LZ B cell apoptosis remained similar [Fig. 4A](#fig4a)). Absence of a measurable increase in apoptosis (26) in α-CD40L antibody–treated mice is consistent with the relatively small number of LZ B cells (5 to 10%) undergoing positive selection at any time (27).

To gain further insights into the mechanisms responsible for cell death in the LZ, we measured apoptosis in cells undergoing BCR signaling and positive selection using Nur77-GFP and Myc-GFP (myelocytomatosis oncogene) reporters, respectively (27–29). A fraction of LZ B cells expressed Nur77, but only a subset of these cells were protected from apoptosis (Fig. 4, B and C, and fig. S9, B and C). By contrast, positive selection, as indicated by Myc-GFP expression, was associated with nearly complete protection from apoptosis (Fig. 4, B and C, and fig. S9, B and C). Thus, positive selection, as measured by Myc expression, protects B cells from apoptosis, but BCR cross-linking alone, as measured by Nur77, appears to be insufficient.

Conclusions

We have investigated the causes of apoptosis and their contribution to cell death in the LZ and DZ of GCs by combining a FRET-indicator of apoptosis, single-cell sorting, and antibody cloning. Only a small fraction of LZ GC B cells are positively selected to return to the DZ and undergo additional rounds of division, and this process is, in part, stochastic (1, 30). The element of chance appears to be introduced by random encounters between LZ B cells displaying high levels of peptide–major histocompatibility complex II and cognate T cells. Consistent with this notion, the apoptotic compartment in the LZ contained an arbitrary assortment of B cells, including those with high-affinity BCRs (Fig. 3B). Thus, both low- and high-affinity B cells undergo apoptosis in the LZ, but high-affinity cells are more likely to become positively selected after they encounter cognate Tfh cells (1, 31, 32). By contrast, the LZ is the microanatomic site of antibody quality control by selection against deleterious mutations introduced by AID. B cells expressing Igs damaged by SHM undergo apoptosis. The largest group of apoptotic B cells in the DZ arises by AID-mediated introduction of stop codons, insertions, or deletions into BCR genes (33). Thus, GC B cells resemble developing and naïve B cells (in that they require BCR expression for survival (18, 34–36).

GC B cells are among the most rapidly dividing eukaryotic cells with cell-cycle times as short as 4 to 6 hours. Despite rapid cell division and limited export of memory and plasma cells, the size of the GC is relatively stable over periods of weeks. Our experiments indicate that GC homeostasis is maintained by high rates of cell death. Moreover, whereas positive selection occurs in the LZ, negative selection by apoptosis occurs in both GC zones but is differentially regulated in the LZ and DZ.

Materials and methods

Generation of IgκINDIA transgenic mice and Rosa26INDIA knock-in mice

Indicator of apoptosis (INDIA) cDNA was assembled from sequence-optimized N-terminally Myc-tagged mNeonGreen (37), an 18-aminomycin acid linker bearing the aCas9 cleavage site DEVD (SSSSESGDEVDTGSQSEF; (38)) and mRuby2 (39) by gBlocks Gene fragment synthesis (Integrated DNA Technologies), overlap PCR, and standard cloning procedures.

To produce IgκINDIA transgenic mice, INDIA was placed under transcriptional control of mouse Igs regulatory elements as previously described (40, 41), with the modification that the entire open reading frame was placed after the non-coding Vκ exon followed by the Igs polyadenylation signal. After sequence and vector integrity confirmation, the vector backbone was eliminated by NotI/MluI digestion, and the resulting 7.2-kb fragment was injected into the pronuclei of fertilized C57BL/6J oocytes. Transgenic founder mice were identified by PCR (table S1; annealing temperature 55°C) on tail DNA. The transgenic founder line IgκINDIA was selected and maintained by mating to C57BL/6J mice or by intercrossing. Genotyping was performed by flow cytometry on peripheral blood.

To generate Rosa26INDIA knock-in mice, INDIA was cloned into the Asel site of the CTV targeting vector (42) that was a gift from Klaus Rajewsky (Addgene plasmid #15912). The frt-flanked IRES-eGFP sequence was deleted by l-arabinose-inducible Flp recombineering in SWI05 bacteria (43). In brief, Flp expression was induced by 0.09% (w/v) l-arabinose for 1 hour at 32°C, followed by electroporation of 1 ng vector and growth on LB agar plates containing ampicillin over night at 32°C. Vector integrity and deletion of IRES-eGFP in the resulting clones were confirmed by restriction-enzyme digestion and sequencing. CY2.4 albino C57BL/6J-Tyrc-2J−/− mice were targeted with the modified CTV vector at the Gene Targeting Resource Center (The Rockefeller University) and homologous recombination was verified by Southern blot and PCR. Chimeric males obtained after blastocyst injection were crossed to B6(Cg)-Tyrc-2J−/− J females. Rosa26SL−INDIA mice exhibiting germine transmission were identified by PCR and crossed to B6.C-Tg(CMV-cre)1Cgn/J mice to induce germine deletion of the LSL casette. Rosa26SL−INDIA CMV−cre− offspring were crossed to C57BL/6J mice. The resulting CMV−cre− offspring ubiquitously expressing INDIA were intercrossed to establish Rosa26SLINDIA mice. Genotyping was performed by flow cytometry on peripheral blood.

Mice

B6.C-Tg(CMV-cre)1Cgn/J, B6.Cg-Tyr−/-J, B6.SJL and C57BL/6J mice were purchased from Jackson Laboratories. AIDCre/CreIgH96K/96KRosa26LSL−YFP, AID−/−, AID-GFP, Nur77-GFP, Myc-GFP and B1−/− mice were described previously (10, 13, 22, 28, 44, 45). Bone marrow chimeras were generated as described (46). All animal experiments were approved by the Institutional Review Board and the IACUC at The Rockefeller University.

Immunizations and treatments

Primary GCs were elicited by immunizing mice of the indicated genotypes subcutaneously with 25 µL of PBS containing 12.5 µg of NP6-OVA (Biosearch Technologies) or 4 µg of HIV envelope antigen (BG505 SOSIP.v4.1 GT1.1 trimers, (GT1.1); provided by Rogier W. Sanders, Weil Medical College of Cornell University, New York) precipitated in alum (Immune, ThermoFisher Scientific) at a 2:1 ratio. IgκINDIA mice were also immunized intraperitoneally with 100 µL of PBS containing 50 µg of NP6-OVA precipitated in alum. For blocking CD40L/CD40 interactions, immunized mice were injected intravenously with 300 µg of α-CD40L (MR-1, Bio X Cell) or 300 µg of Armenian Hamster IgG (Bio X Cell). To
estimate dynamic GC B cell loss by apoptosis, mice were injected intraperitoneally with 1 mg of 5-ethyl-2'-deoxyuridine (EdU; ThermoFisher Scientific) every 2 hours for up to 10 hours. For identifying GC B cells in S phase, mice were given a single intravenous pulse of 1 mg of EdU 1.0 to 2.5 hours prior to sacrifice. To elicit secondary GCs, mice were immunized intraperitoneally with 100 μl of PBS containing 50 μg of OVA (Grade V, Sigma) precipitated in alum. Two weeks later, mice received 5 × 10^6 B1-8^hi B cells intravenously (~5 × 10^5 Igk gl−NODA)/95% Rosa26^FRT^ followed one day later by a subcutaneous boost with 25 μl of PBS containing 25 μg of NPg-ova (Biosearch Technologies). Follicular dendritic cells (FDC) were labeled by intravenous injection of 0.5 to 1.0 mg of polyclonal rabbit α-B-Phycoerythrin (α-B-PE, Rockland) 4 days after boost immunization, followed 1 day later by subcutaneous injection of 0.05 μg B-PE (Thermo Fisher Scientific).

**B cell isolation and culture**

B cells were purified from spleens and subcutaneous lymph nodes as previously described (2). Igk^−NODA^ B cells were stimulated for 4 days with 25 μg/ml of LPS (1-2630, Sigma) and 5 ng/ml of IL-4 (11020, Sigma) in vitro as described (47). Activated B cells were harvested, washed and cultured at 2 × 10^5 cells/ml with LPS/IL-4-free media for 3 hours in the presence of 1 μM staurosporine (1285, Tocris) to induce apoptosis. GC B cells were enriched from immunized Igk^−NODA^ mice by incubating single-cell suspensions with 1.25 μg/ml of biotinylated α-IgD for 10 min on ice followed by incubation with α-Biotin- and mouse CD43 MicroBeads (Miltenyi Biotec). Cells were passed through a magnetized LS column (Miltenyi Biotec) and enriched GC B cells were collected in the flow-through.

**Live imaging of cultured B cells**

On day 4, LPS/IL-4-activated Igk^−NODA^ B cells were washed and placed in a 35-mm μ-Dish (Ibidi). After the addition of 1 μM staurosporine, cells were imaged in an environmental chamber set to 37°C using a DeltaVision Inverted Olympus IX-71 Image Restoration Microscope (GE Healthcare) with an Insight SSI 7 color solid state illumination system and a 20× dry objective. Separate excitation and emission filter wheels were employed to collect data for mNeonGreen (488-nm excitation; 525/50 BP), FRET (488-nm excitation; 530/30 BP) and standard brightfield images for each time point. 512 × 512 pixel images were taken every 60 s for 3 hours using Ultimate Focus and a Prior SYZ piezo stage for multiple point visiting. Images were processed and analyzed with ImageJ 1.48q (National Institutes of Health) and videos were generated at a frame rate of 10 fps.

**Intravital imaging and image analysis**

Intravital imaging of popliteal lymph nodes and image acquisition were essentially performed as described previously (31). Mice were anesthetized by the inhalation of 4% isoflurane in pure oxygen, placed on a stage warmer set to 37°C and maintained on anesthesia by inhalation of 1.25% isoflurane in pure oxygen. Popliteal lymph nodes in shaved hind legs were exposed by microsurgery and animals were placed under the heated Olympus 25× 10.5 NA Plan objective of an Olympus BX61 upright microscope fitted with a Coherent M staurosporine, cells were pulsed multiphoton laser was tuned to 900 nm. A pulsed multiphoton laser was used to split the emission from either 2 GaAsp detectors (with a 500-550-nm filter for mNeonGreen fluorescence and a 575- to 630-nm filter for FRET fluorescence) and a PMT detector (with a 460- to 500-nm filter for CFP/autofluorescence). Images were acquired every 30 s as 75-μm Z-stacks (5-μm steps) with 1.4× zoom and with 512 × 512 X-Y resolution. Imaris software (Bitplane) was used to process data. Collapsed Z-stacks were exported as TIFF series through a 70-μm cell strainer (BD Biosciences).

**Flow cytometry**

Spleens and lymph nodes were collected in RPMI media containing 6% of serum on ice. Single-cell suspensions were obtained by forcing the tissue through a 70-μm cell strainer (BD Biosciences). All centrifugation steps were performed at 4°C and cells were otherwise handled on ice to minimize apoptosis. Erythrocytes were lysed with 1 ml of ACK lysing buffer (Gibco) for 3 min on ice. After incubation with 5 μg/ml of α-CD16/32 (rat mAb 2.4G2, Bio X Cell) for 20 min at 4°C, biotinylated α-CXC4R4 was incubated for 45 min at 4°C. Biotin and additional surface antigens were detected by staining for 30 min at 4°C. The fixation/Permeabilization Solution Kit (BD Biosciences) was used for intracellular staining. α-CD19 (rat mAb eBio3D), α-CD38 (rat mAb 90), α-CD45R/B220 (rat mAb RA3-2B2) and α-IgM (rat mAb 11/41) were from eBioscience. α-CD19 (rat mAb 6D5) and α-Cd86 (rat mAb 6D5) were from Biolegend. α-aCasp3 (Alexa Fluor 647-conjugated; rabbit mAb CJ9-605), α-CXCR4 (rat mAb 2B11), α-Fas (hamster mAb Jo2), α-igx (rat mAb 187.1), α-Igk_A4, α-IgG1 (rat mAb R6-46), α-IgG2a (rat mAb 2A5-1), streptavidin conjugated to V500, and α-T- and B cell activation antigen (rat mAb GL7) were from BD Biosciences. Alexa Fluor 647-conjugated polyclonal rabbit IgG was from Cell signaling. EdU was detected with the Click-IT Plus EdU Alexa Fluor 488 Flow Cytometry Assay Kit (ThermoFisher Scientific). Alexa Fluor 647-conjugated Annexin-V and 5a Annexin Binding Buffer for flow cytometry were from ThermoFisher Scientific. Annexin-V staining was performed for 15 min at room temperature (1:50 dilution) and DAPI (Sigma) was added at 0.04 μg/ml prior to acquisition. TUNEL staining was carried out with the Apo-BrdU Apoptosis Detection Kit and Alexa Fluor 647-conjugated a-BrdU (mouse mAb MoBu1-1, both from ThermoFisher Scientific) according to the manufacturer’s instructions.

Flow cytometry data were acquired on a BD Fortessa (BD Biosciences) and data were analyzed with Flowjo (Tristar). Intact cells and singlets were identified by their SSC/SFC profiles and in the case of Igk^−NODA^ B cells additionally by mRuby2 expression (561-nm excitation; 582/15 BP), FO B cells were gated CD19^+CD38^+Fas and GC B cells were gated CD19^+CD38^+Fas and additionally GL7 where indicated. Live and apoptotic fractions were discriminated by aCasp3 staining, or by mNeonGreen (488-nm excitation; 505LP and 530/30 BP) and FRET (488-nm excitation; 600LP and 610/20 BP) for Igk^−NODA^ mice. FRET loss was derived as a separate parameter in Flowjo defined as the ratio of mNeonGreen and FRET fluorescence among intact mRuby2 B cells. GC B cell fractions were differentiated into LZ (CXCR4^+CD86^+ and LZ) and DZ (CXCR4^−CD86^-) where indicated. Due to lower expression of CD86 in apoptotic compared to live GC B cells (see fig. S1), DZ and LZ were separately gated for live and apoptotic GC compartments. Apoptosis rates in DZ and LZ were calculated as: (% aCasp3^+ of GC) / (% DZ or LZ of total GC).

**Cell sorting**

Cell sorting was carried out on a FACS Aria II (BD Biosciences). For bulk sorting, cultured Igk^−NODA^ B cells were washed and directly re-suspended in PBS containing 1% serum and 2 mM EDTA. For single-cell sorting, Igk^−NODA^ GC B cells were identified as B220^+DUMP^+CD38^+Fas (DUMP = TCR/β/γ/CD4/CD8/) and selected as B220^+DUMP^+CD38^+Fas and additionally GL7 where indicated. Due to lower expression of CD86 in apoptotic compared to live GC B cells (see fig. S1), DZ and LZ were separately gated for live and apoptotic GC compartments. Apoptosis rates in DZ and LZ were calculated as: (% aCasp3^+ of GC) / (% DZ or LZ of total GC).
Single-cell cloning and recombinant antibody expression

Reverse transcription, nested PCR amplification, sequencing, and ligation-independent cloning of IgL, IgA, and IgG were as described (49), with minor modifications. IgM sequences were amplified in the same reaction as IgG by adding specific reverse primers (First PCR: 5′-AGGGGCCTCTCGCGAGAGCAGGAG-3′; sequencing PCR: 5′-AGGGGGAAGACATTTGGGAAGGAC-3′). A specific forward primer (5′-CTTATGAACGTCGGAAGCATTGCATGACTCTTACGTCGACTGCGAGGATC-3′) was used to amplify and clone IgH4-T2. Sequences were analyzed with IMGT/V-QUEST and IgBlast. Some Ig sequences were directly ordered from GenScript (11). E. C. Butcher et al. (342929a0; pmid: 2594086).

**Immunoblot analysis**

Transfected HEK293-6E cell cultures were collected on day 7, centrifuged and the supernatant was harvested. The cell pellet was lysed (1% SDS, 10 mM EDTA, 50 mM Tris/HC1, pH 8) and sonicated for 10 min. Antibodies were analyzed in the matched supernatants and cell pellets by SDS-PAGE and immunoblot. HRP-conjugated goat α-human IgG (H+L) antibody (Jackson Immunoresearch, cat# 109-056-088) was detected with HyGLO Quick Spray (Denville Scientific).

**ELISAs**

Auto-reactivity against nuclear and cytoplasmic self-antigens was determined with QUANTA Lite ANA ELISA (Inova Diagnostics) as described (52). Polyclonality against ssDNA, dsDNA, Keyhole limpet hemocyanin (KLH), human insulin, and lipopolysaccharide (LPS) was determined as described (29). To measure NP binding, high-binding 96-well plates (Corning) were coated overnight with 50 μl of PBS containing 10 μg/ml of NP-BSA or NP2-BSA (Biosearch Technologies). After washing with PBS containing 0.05% of Tween 20 (Sigma), wells were blocked with PBS containing 1% of BSA for 2 hours at room temperature. Monoclonal antibodies were incubated at 4 μg/ml or 7 consecutive 1:4 dilutions in PBS for 2 hours at room temperature. After washing, HRP-conjugated goat α-human IgG (Jackson Immunoresearch) was added at 0.16 μg/ml for 1 hour at room temperature. After additional washing, HRP was revealed with 1-step ABTS Substrate Solution (ThermoFisher Scientific). Absorbance was measured at 405 nm after incubation for 20 min at room temperature.

**Statistical analyses**

Statistical significance was determined with Graphpad Prism Version 6.0 using the tests indicated in each figure.


51. C.-H. Yao for technical help; K. M. Gordon and N. M. Thomas for advice on mouse colony management; and in the supplementary materials. C.T.M. was supported by a European Molecular Biology Organization (EMBO) long-term fellowship (ALT 456-2014) and by the European Commission Seventh Framework Programme (FP7) (Marie Curie Actions, EMBODFUNDU2012, GA-2012-620034). A.D.G. was supported by the NIH Medical Scientist Training Program grant T32GM07739 and the National Institute of Allergy and Infectious Diseases (NAID) of the NIH, grant T32-AI109903-03. M.M.-R. and R.W.S. were supported by AIDSfonds Netherlands. M.C.N. was supported by the Bill and Melinda Gates Foundation Collaboration for AIDS Vaccine Discovery (OPPI1033115 and OPPI112406B), the NIH Center for HIV/AIDS Vaccine Immunology and Immunogen Discovery (CHAVI-ID, 1UM1 AI100663), the NAID of the NIH (AI100148, AI637526), the Robertson Foundation, and the Rockefeller University. M.C.N. is an HHMI investigator. M.M.-R. and R.W.S. are inventors on a patent application submitted by the University of Amsterdam that covers GT1.1. This work is licensed under a Creative Commons Attribution 4.0 International (CC BY 4.0) license, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. To view a copy of this license, visit http://creativecommons.licenses/by/4.0/. This license does not apply to figures/photos/artwork or other content included in the article that is credited to a third party; obtain authorization from the rights holder before using such material.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/358/6360/eaao2602/suppl/DC1

Figs. S1 to S9

Table S1

References

Movies S1 to S3

30 June 2017, accepted 29 August 2017

Published online 21 September 2017

10.1126/science.aac2602

Mayer et al., Science 358, eaao2602 (2017) 13 October 2017 8 of 8
The microanatomic segregation of selection by apoptosis in the germinal center


Science 358 (6360), eaao2602.
DOI: 10.1126/science.aao2602 originally published online September 21, 2017

Light- and dark-zone death dynamics

Germinal centers (GCs) are areas within lymphoid organs where mature B cells expand and differentiate during normal immune responses. GCs are separated into two anatomic compartments: the dark zone, where B cells divide and undergo somatic hypermutation, and the light zone, where they are selected for affinity-enhancing mutations after interacting with T follicular helper cells. Mayer et al. studied apoptosis reporter mice and found that both GC zones experience very high rates of apoptosis (see the Perspective by Bryant and Hodgkin). However, the underlying mechanisms were distinct and microanatomically segregated. Light-zone B cells underwent apoptosis by default unless they were rescued by positive selection. In contrast, apoptotic dark-zone B cells were highly enriched among cells with genes damaged by random antibody-gene mutations.

Science, this issue p. eaao2602; see also p. 171