Broad Volume Distributions Indicate Nonsynchronized Growth and Suggest Sudden Collapses of Germinal Center B Cell Populations

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Broad Volume Distributions Indicate Nonsynchronized Growth and Suggest Sudden Collapses of Germinal Center B Cell Populations

Nicole Wittenbrink,† Tom S. Weber,† Anke Klein,† Armin A. Weiser,† Werner Zuschratter,‡ Michael Sibila,§ Johannes Schuchhardt,|| and Michal Or-Guil*†

Immunization with a T cell-dependent Ag leads to the formation of several hundred germinal centers (GCs) within secondary lymphoid organs, a key process in the maturation of the immune response. Although prevailing perceptions about affinity maturation intuitively assume simultaneous seeding, growth, and decay of GCs, our previous mathematical simulations led us to hypothesize that their growth might be nonsynchronized. To investigate this, we performed computer-aided three-dimensional reconstructions of splenic GCs to measure size distributions at consecutive time points following immunization of BALB/c mice with a conjugate of 2-phenyl-oxazolone and chicken serum albumin. Our analysis reveals a broad volume distribution of GCs, indicating that individual GCs certainly do not obey the average time course of the GC volumes and that their growth is nonsynchronized. To address the cause and implications of this behavior, we compared our empirical data with simulations of a stochastic mathematical model that allows for frequent and sudden collapses of GCs. Strikingly, this model succeeds in reproducing the empirical average kinetics of GC volumes as well as the underlying broad size distributions. Possible causes of GC B cell population collapses are discussed in the context of the affinity-maturation process. The Journal of Immunology, 2010, 184: 1339–1347.

The humoral arm of the adaptive immune response is crucially important for fighting invading pathogens because it leads to the generation of high-affinity Abs. These Abs arise as a consequence of Ag-driven B cell differentiation within the unique environment of germinal centers (GCs), where B cells undergo clonal expansion, somatic hypermutation (SHM), antigenic selection, class switch recombination, and affinity maturation (1–5). In addition, plasma cell commitment and memory B cell formation are linked to GCs (6–8).

During T cell-dependent immune responses, following a first exposure to Ag, GCs form de novo in secondary lymphoid organs, grow, and eventually regress. The overall GC response involves an ensemble of coexisting GCs, several hundreds in the case of the murine spleen (9). Flow cytometric and histological observations of formation and decay of GCs in rodents revealed a commonly accepted temporal evolution of the primary GC response; the primary GC response spans ~3 wk, first becoming detectable by day 4 postimmunization, peaking at around days 10 to 12, and then gradually decaying (9–14). However, the data generated so far [e.g., number of GCs (9, 11, 14), relative volume occupied by GCs (10, 14), mean area and diameter of GCs (15), and frequency of lymphocytes with a GC B cell phenotype (12)], can only determine the average kinetics of the GC ensemble (eGC) with certainty, but not necessarily the growth kinetics of individual GCs (iGCs). The growth kinetics of iGCs can only be inferred from the average eGC kinetics if iGC growth is synchronized (i.e., if all iGCs show a kinetic behavior following average eGC kinetics). Therefore, the so-far unaddressed issue of synchronization of GC growth is of major importance. If GCs grow in a synchronized manner, their volume distribution should be quite narrow at each time point of the immune response. By contrast, broad GC volume distributions would indicate nonsynchronized growth, thereby unequivocally ruling out the possibility of inferring iGC growth kinetics from average eGC kinetics.

In this study, we analyzed GC size distributions and the occupation of follicular niches within the microenvironment of the murine spleen following primary immunization of BALB/c mice with the hapten 2-phenyl-oxazolone (phOx) coupled to the carrier protein chicken serum albumin (CSA). Time-dependent changes in the frequency of follicular niches occupied by GCs indicated that newly formed GCs emerge over an extended period of time. Moreover, three-dimensional (3D) reconstruction of splenic GCs revealed that the volume distributions of iGCs are strikingly broad and are further characterized by high frequencies of rather small GCs. Taken together, our results demonstrate that the growth of iGCs is nonsynchronized; thus, all attempts to deduce iGC growth kinetics from average eGC kinetics are prone to failure. In addition, comparing our empirical data on GC volume distributions with simulation data from a stochastic mathematical GC growth model revealed that sudden, fast, and frequent collapses of GCs explain the empirical GC volume distribution and might play an important role in the affinity-maturation process.
Mice and immunization

Six to eight-week-old BALB/c mice were immunized with a single i.p. injection of 100 µg phOx coupled to CSA at a ratio of 10:1 and precipitated onto alum, as described previously (16). To ensure that the examined GCs are the result of the immunization protocol, we drew samples and examined spleens of unimmunized mice that were housed in the same cage with immunized mice. In unimmunized controls, we were not able to record GC size distributions, because environmental Ag-induced GCs were observed only occasionally, in very low numbers, and were of very small size. BALB/c mice were bred and maintained under specific pathogen-free conditions at the facilities of the Bundesinstitut für Risikobewertung, Berlin. All animal experiments were performed in accordance with institutional, state, and federal guidelines.

Immunofluorescence

For two-dimensional cross-sectional evaluation of GC growth kinetics, cohorts of immunized mice (n = 4–24) were killed at days 4, 6, 8, 10, 12, 14, 16, 18, and 21 postimmunization. Spleens were removed, bisected, frozen in Tissue-Tek OCT compound (Sakura Finetek, Zoeterwoude, The Netherlands), and stored at −70°C. Longitudinal sections of 10-µm thickness were cut on a cryostat microtome and mounted onto Superfrost Plus glass slides (Roth, Karlsruhe, Germany). The sections were air-dried for 1 h, fixed in cold acetone for 10 min, air-dried again for ≥2 h, and stored at −20°C until further analysis. To establish the 3D volumetric growth kinetics of splenic GCs, cohorts of immunized mice (n = 3) were killed at days 6, 10, and 14 postimmunization. Spleens were removed and processed identically, except that whole spleen sections were cut as longitudinal sections of 25-µm thickness.

Prior to staining, spleen sections were pretreated by fixation in ice-cold 1% paraformaldehyde for 30 min and permeabilization in ice-cold 1% sodium citrate containing 1% Triton X-100 (Promega, Mannheim, Germany) for 2 min. Spleen sections were blocked in PBS containing 3% BSA for 30 min. For two-dimensional cross-sectional evaluation of GC growth kinetics, spleen sections were triple stained with unconjugated rat anti-mouse Ki-67 (clone TEC-3, Dako), Alexa Fluor 488-labeled anti-mouse FDCs (clone FDC-M2, labeled anti-rat IgG (Invitrogen), 3) Alexa Fluor 488-labeled anti-mouse 

For each spleen specimen, seven serial sections (s01–s07), spaced at intervals of 50 µm and spanning a total thickness of 300 µm, were quadruple stained for proliferating cells, FDC networks, T cells, and macrophages by sequentially incubating them with mixtures of Abs as follows: 1) unconjugated rat anti-mouse Ki-67 (clone TEC-3, Dako), 2) Alexa Fluor 647-labeled anti-rat IgG and Alexa Fluor 564-conjugated streptavidin (both from Invitrogen, Karslruhe, Germany), respectively.

Stochastic simulation of GC seeding and growth kinetics

To model the B cell population kinetics of iGCs, we used an extended Ricker discrete population model that involves two phases of iGC growth: free growth and competitive growth.

For free growth phase:

\[ P_{i,t+1} = P_{i,t} \exp \left[ \frac{\ln(2)}{\tau_{\text{free}}} \right] \]  

For competitive growth phase:

\[ P_{i,t+1} = P_{i,t} \exp \left[ \frac{\ln(2)}{\tau_{\text{comp}}} \left( 1 - \frac{P_{i,t}}{K_i} \right) \right] \]  

During the free growth phase, iGCs that were founded by a defined number \( M \) of B cells grow exponentially and B cells proliferate with a constant division time \( \tau_{\text{free}} \) (Equation 1) until iGCs exceed the critical size \( P_{\text{critical}} \) and subsequently enter the competitive growth phase. The critical size \( P_{\text{critical}} = M^2 \) is defined by the number of generations \( \xi \), where \( \xi \) is drawn from a normal distribution \( N(\mu, \sigma^2) \). After iGCs have entered the competitive growth phase, they grow according to a Ricker discrete time population model (18) extended by a multiplicative noise term (\( \chi \)) to account for frequently large and unpredictable impacts on the B cell population size due to SHM and (e)migration of B cells. In Equations 1 and 2, \( P_t \) is the B cell population size of an iGC at time \( t \), \( K_i \) is the carrying capacity of the follicular niche, and \( \chi_{\text{comp}} \) is the maximal division time. The carrying capacities \( K_i \) were linearly interpolated between days 6, 10, 14. The noise term \( \chi \) is drawn from a uniform distribution \( UN(0, 1) \) that represents a random variable between [0, 1].

The ensemble kinetics of GCs was attained assuming a constant seeding probability \( \epsilon \) of follicular niches and by carrying out consecutive Monte Carlo simulations of 100,000 iGCs according to the two-phased model specified above, in which the time resolution \( \Delta t \) was 1.2 h for the free growth and 24 h for the competitive growth phase. Simulated B cell population sizes of iGCs were retrieved and recorded in the form of rank plots at days 6, 10, and 14 after immunization. These rank plots were subsequently compared with rank plots of the experimentally obtained data at the corresponding time points. To enable comparison of simulated and experimental data, rank plots were adapted so that the index of the smallest-sized GC from the experimental data set and the simulated GC with the most similar size and the maximal index values of both sets had the same values. The first measure accounts for the smallest safely empirically detectable GCs. The fitted model parameters, as summarized in Table I, resulted in an excellent match over two orders of magnitude.

Results

Two-dimensional cross-sectional evaluation reveals robust average growth kinetics of eGCs based upon a notable size distribution of iGCs

The kinetics of GC growth was initially assessed by quantitative analysis of cross-sectional GC size in spleens of phOx-CSA-challenged BALB/c mice at different time points after immunization.
GCs were identified as clusters of Ki-67\(^+\) proliferating cells in close proximity to FDC networks and adjacent to T cell zones (Fig. 1A). After manual assignment of GC boundaries (Fig. 1A), enclosed cross-sectional areas and numbers of Ki-67\(^+\) cells were automatically measured as described in Materials and Methods. The number of Ki-67\(^+\) cells correlated with the cross-sectional area of GCs at all time points analyzed (Supplemental Fig. 1). As a result, the density of B cells per cross-sectional GC area was estimated as 0.0116 ± 0.0008 cells/µm\(^2\). In total, we evaluated 1093 GCs in 44 sections derived from 23 mice (Table II).

Well-established GCs, typically of very small size (3786 ± 271 µm\(^2\)), were first detected on day 4 after immunization (Fig. 1B). The mean size of GCs subsequently increased steeply, reaching a maximum at day 10 (18,551 ± 2,134 µm\(^2\)) and then gradually declining until day 21 (10,397 ± 1,274 µm\(^2\)) (Fig. 1B). The variance among mice at the same time point or spleen sections obtained from the same animal was small (Fig. 1B and data not shown). However, a wide range of GC sizes was observed within individual spleen sections (e.g., 1,300–70,000 µm\(^2\) at day 8; Fig. 1C). Notably, the cross-sectional area distribution of splenic GCs was very robust for the cohorts of mice analyzed at each time point (example for day 8 after immunization shown in Fig. 1C). Additionally, the area distribution of cross-sectional GCs was shown to be subject to time-dependent changes. We observed a marked shift toward higher frequencies of large GCs from days 4–8 after immunization (Fig. 1D); the area distribution remained in an almost steady-state between days 8 and 14. After day 14, the area distribution gradually shifted back toward smaller GCs. In accordance with other studies, the background level of GCs without immunization was minimal, indicating that the examined GCs were induced by immunization (19).

### 3D approach to evaluating GC growth kinetics

Our previous computer simulations showed that the experimentally recorded cross-sectional profile of GC growth is consistent with different hypothetical ensemble kinetics, including marked non-synchronization of GC formation and growth (20). Therefore, cross-sectional profiling alone is inconclusive and, in general, is insufficient to assess the growth behavior of GCs. Because we were particularly interested in assessing the synchronization of GC formation and growth, we turned to 3D evaluation of murine spleens. For this purpose, series of serial longitudinal spleen sections were quadruple stained for proliferating cells, FDC networks, T cells, and macrophages. Subsequently, composite microphotographs of whole spleen sections were obtained by confocal microscopy in conjunction with meander scan technology (Fig. 2A). Three spleens were examined each at days 6, 10, and 14 after primary immunization with phOx-CSA, with sampled splenic volumes ranging between 9.2 and 14.9 mm\(^3\) (Table III). The quadruple staining resulted in high morphological resolution of the spleen, including demarcation of red and white pulp regions and authentic identification of follicular niches (empty FDC networks or FDC networks occupied by GCs) (Fig. 2). The total number of follicular niches identified per spleen sample ranged between 206 and 322, with no obvious differences related to the time after immunization (Table III). The high morphological resolution of quadruple-stained spleen sections also contributed positively to tracing follicular niches throughout the sampled splenic volumes (Fig. 2B), with its overall efficiency rated as >95% (Table III).

### Newly formed GCs emerge over an extended period of time

The induction of GC formation after antigenic challenge is still poorly defined, in that the time frame when new GCs arise is unknown. To address this, we examined the density and occupation of follicular niches within murine spleens at days 6, 10, and 14 after primary immunization with phOx-CSA, using the 3D approach illustrated in Fig. 2 and outlined above. The time courses of the densities of total, occupied, and empty follicular niches (or those in which occupation remained below the detection level) are illustrated in Fig. 3. Densities are given as the numbers of follicular niches per 12 mm\(^3\), corresponding to the average volume analyzed per spleen specimen (Table III). The overall density of follicular niches proved to be remarkably fixed, not even changing during progression of the immune response (231 ± 13, 234 ± 35, and 225 ± 24 at days 6, 10, and 14, respectively; Fig. 3). However, the density of follicular niches occupied by GCs increased significantly from 128 ± 25 at day 6 to 189 ± 27 at day 10 (p = 0.024; Student t test); thereafter, the density decreased slightly to 177 ± 29 at day 14. Accordingly, the frequency of follicular niches occupied by GCs continued to increase from day 6 (56%) to day 10 (81%) and was rather stable by day 14 (79%). Interestingly, the frequency of empty follicular niches was high, even at days 10 and 14 after immunization (19% and 21%, respectively; Fig. 3). Thus, the formation of new GCs was not restricted to the very early phase of the immune response; it continued for an extended period of time, even between days 6 and 10 postimmunization.

### Table I. Parameters for the two-phased model of GC seeding and growth kinetics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of founder B cells</td>
<td>M</td>
<td>3</td>
</tr>
<tr>
<td>Seeding probability of empty follicular niches per 1.2 h</td>
<td>ε</td>
<td>0.013</td>
</tr>
<tr>
<td>Free growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Division time (h)</td>
<td>τ(_{\text{free}})</td>
<td>9.65</td>
</tr>
<tr>
<td>Mean number of generations</td>
<td>μ(_t)</td>
<td>6.35</td>
</tr>
<tr>
<td>Variance of the mean number of generations</td>
<td>σ(^2)</td>
<td>1.8</td>
</tr>
<tr>
<td>Competitive growth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Division time (h)</td>
<td>τ(_{\text{comp}})</td>
<td>13.20</td>
</tr>
<tr>
<td>Carrying capacity of follicular niches at day 6</td>
<td>K(_6)</td>
<td>21,445</td>
</tr>
<tr>
<td>Carrying capacity of follicular niches at day 10</td>
<td>K(_{10})</td>
<td>35,420</td>
</tr>
<tr>
<td>Carrying capacity of follicular niches at day 14</td>
<td>K(_{14})</td>
<td>11,914</td>
</tr>
</tbody>
</table>

The parameter value \(M\) was chosen according to the literature (10, 42). Assuming reasonable initial values, all other parameters were fitted by minimizing the sum of the mean distances between simulated and experimental rank plots (\(D = \sum_{d=4}^{14} \sqrt{\frac{\text{simulated} - \text{measured}}{\text{simulated} + \text{measured}}}^2\)), \(d = 4, 6, 10, 14\). Day 4 GCs were assumed to be spherical, and their volume \((V)\) was calculated according to their cross-sectional area \((A)\) as \(V = \frac{\pi}{4} A\). Notably, the values of the fitted model parameters for the division times of GC B cells \((τ\(_{\text{free}}\)\) and \(τ\(_{\text{comp}}\)) fully agree with published experimental values (34–36).
The actual numbers of three-dimensionally reconstructed GCs, identified as Ki-67+ cells and by anatomical location in triple immunofluorescence stainings of proliferating cells (blue, mAb Ki-67), T cells (green, mAb CD3), and FDC networks (red, mAb FDC-M2). The cross-sectional size of GCs was measured after manual assignment of GC boundaries (white selection) using Imaged3 software (17). The image shown is representative of a GC at day 8 after immunization (original magnification ×40). Scale bar, 50 μm. B, BALB/c mice were immunized with phOx-CSA, and spleens were removed at the indicated time points. Two independent sections were stained for each spleen, and the cross-sectional size of GCs was recorded as illustrated in A (for details, see Table II). Open circles represent mean cross-sectional GC sizes of individual mice; the solid line indicates the average course over time. C, Size distributions of day 8 GCs plotted as cumulative frequencies of cross-sectional GC areas. Each curve refers to one individual mouse at day 8 postimmunization. D, Time-dependent transitions of the size distribution illustrated by plotting the cumulative frequencies of cross-sectional GC areas at different time points after immunization. Each curve is representative of the entire recorded GCs at the indicated time point after immunization.

**3D volumetric evaluation reveals a considerable volume distribution of GCs.**

We performed 3D reconstructions of GCs on serial sections from three spleen specimens at days 6, 10, and 14 postimmunization. For reasons of feasibility and to facilitate inspection, 3D reconstructions were confined to those GCs completely contained within or spanning the overall series of serial sections. Lateral views of 3D reconstructions and detail enlargements from representative spleen specimens at days 6, 10, and 14 postimmunization are shown in Fig. 4A. The actual numbers of three-dimensionally reconstructed GCs, as well as their mean, range, and total volume, are summarized in Table III. Although the numbers of reconstructed GCs fluctuated with respect to the three spleen specimens analyzed per time point (e.g., 23, 27, and 42 at day 10), no striking differences were detected for the different time points analyzed. Naturally, fluctuations in GC numbers were also reflected by the total volume of the GCs themselves (e.g., 55.0, 80.8, and 112.9 × 10⁶ μm³ at day 10). However, the total volume of reconstructed GCs was significantly lower on day 6 (21.2, 23.1, and 41.1 × 10⁶ μm³) than on day 10 (55.0, 80.8, and 112.9 × 10⁶ μm³) or day 14 postimmunization (107.4, 40.8, and 80.9 × 10⁶ μm³). Likewise, the estimated mean GC volume was small for day 6 (0.5 ± 0.6, 0.6 ± 0.8, and 1.4 ± 1.8 × 10⁶ μm³) compared with day 10 (2.4 ± 3.5, 3.0 ± 4.4, and 2.7 ± 3.7 × 10⁶ μm³) and day 14 (2.6 ± 2.8, 2.0 ± 2.1, and 1.6 ± 1.9 × 10⁶ μm³). The particularly high SDs were due to a nonnormal, right-skewed distribution of GC volumes (Fig. 5). A striking characteristic common to all analyzed spleens is the concomitance of very differently sized GCs (Fig. 4A). The smallest and largest GCs differed by a maximal factor of 220 on day 6, 2070 on day 10, and 555 on day 14 (Fig. 4, Table III). Although the smallest GC volume remained rather constant during progression of the immune response (0.01, 0.01, and 0.02 × 10⁶ μm³ at days 6, 10, and 14, respectively (i.e., equivalent to GCs comprising ~25 Ki-67+ cells); the largest value increased from day 6 to days 10 and 14 (4.0, 20.7, and 11.1 × 10⁶ μm³). Hence, GCs of rather small volume are not restricted to the early phase but were also frequently found at the peak of the immune response. By contrast, larger-volume GCs only occurred at the peak of the immune response.

Collectively, the 3D reconstructions of GCs showed that a broad volume distribution of splenic GCs exists at any time point analyzed (Fig. 5). Although the volume distribution of GCs proved very robust for the three mice analyzed at each time point (data not shown), it is subject to fundamental changes over the duration of the immune response (Fig. 5). The volume distribution of day 6 GCs is narrow, with 91% of GCs within the smallest size class (between 0.01 and 3.5 × 10⁶ μm³) or between 15 and 4400 GC B cells) (Fig. 5). Toward day 10, the GC volume distribution broadens and shifts in favor of larger-sized GCs. However, the frequency of GCs assigned to the smallest size class is still high, although decreased (59%). The volume distribution of splenic GCs at day 14 is similar to day 10, but there has been a minor shift back toward smaller-sized GCs (i.e., 71% of GCs fall into the smallest size class).

The distributions of GC volumes were not normal; they were strongly right-skewed as the result of a small frequency of very large GCs (Fig. 5). In contrast, GCs comprising <1254 B cells account for as much as 55%, 40%, and 29% of all GCs at days 6, 10, and 14, respectively. The volume distribution of small GCs is best resolved in logarithmic histograms (Supplemental Fig. 2). Simulation of GC population kinetics indicates that frequent and large collapses of iGCs might account for the empirically observed broad volume distributions of GCs.

To further address the origin of broad volume distributions of GCs, we compared our empirical data with simulation results from a two-phased stochastic Ricker population growth model. In the free growth phase of the model, iGCs are first seeded and subsequently grow until they finally reach a critical size and enter the competitive growth phase. During this second phase, the B cell population kinetics of iGCs suffer from frequent collapses in population size. Although the classical Ricker map is widely used for modeling population growth under strong competition for resources (21, 22), it turned out to be unsuitable for reproducing experimental data on GC size distributions because it underestimated the frequency of small-sized GCs (data not shown). Therefore, we extended the classical Ricker map during the competitive growth phase by a stochastic component accounting for effects other than competition for resources, such as unpredictable events possibly due to SHM and migration. A defining characteristic of the extended Ricker map is that iGCs grow constantly but can suffer multiple, and sometimes, large collapses at any stage during the competitive growth phase. During this second phase, the B cell population kinetics of iGCs suffer from frequent collapses in population size. Although the classical Ricker map is widely used for modeling population growth under strong competition for resources (21, 22), it turned out to be unsuitable for reproducing experimental data on GC size distributions because it underestimated the frequency of small-sized GCs (data not shown). Therefore, we extended the classical Ricker map during the competitive growth phase by a stochastic component accounting for effects other than competition for resources, such as unpredictable events possibly due to SHM and migration. A defining characteristic of the extended Ricker map is that iGCs grow constantly but can suffer multiple, and sometimes, large collapses at any stage during the competitive growth phase.
growth phase (for details see Materials and Methods). Compared with other simple deterministic growth models, the extended Ricker map proved to be the mathematical model that best reproduced the experimental data on GC growth (Supplemental Fig. 3).

Monte Carlo simulations of the extended two-phased population growth model reproduce the empirical data on GC volume distributions at days 4, 6, 10, and 14 after immunization (Fig. 6 A–D) for a suitable parameter set as specified in Table I. Our simulations further emphasize that growth kinetics of iGCs can differ substantially from each other, deviating strongly from the average kinetics (Fig. 6E), which explains the empirically observed broad volume distributions of GCs. The model’s response to representative variations of the chosen parameter values is shown in Supplemental Table I.

**Discussion**

We addressed the issue of synchronization of de novo formation and growth of murine splenic GCs by examining the occupation of follicular niches and size distributions of GCs over the duration of the primary phOx-CSA response. We showed that the overall GC response is characterized by a marked size distribution of GCs that is robust between individuals but subject to substantial time-dependent changes. Moreover, the occupation of follicular niches by newly forming GCs was not restricted to the very early stage of the response but occurred over an extended period of time, even between days 6 and 10 postimmunization.

The reported average growth kinetics of the phOx-CSA–induced GC response (Fig. 1B) is in line with previous studies of the GC response in rodents using different Ags (9–11, 15, 23, 24). GCs are

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**Table II. Survey of cross-sectional GC size evaluation**

<table>
<thead>
<tr>
<th>Day</th>
<th>No. of Evaluated GCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M1</td>
</tr>
<tr>
<td></td>
<td>S1</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>11</td>
</tr>
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</tr>
<tr>
<td>18</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>17</td>
</tr>
</tbody>
</table>

*Fig. 2.* 3D approach to evaluation of GC growth kinetics. A, Serial longitudinal spleen sections (s01–s07) spaced at intervals of 50 μm and spanning a total thickness of 300 μm, were quadruple stained for proliferating cells (blue, mAb Ki-67), FDC networks (white, mAb FDC-M2), T cells (red, mAb CD3), and macrophages (green, mAb CD68). Entire areas of spleen sections were imaged by meander scans using a ×10 objective. Follicular niches, as identified by FDC-M2 reactivity and anatomical location, were numbered consecutively, and each niche was traced throughout the series of imaged spleen sections. During tracing, follicular niches were marked as occupied or empty by virtue of the concomitant existence or absence of GCs. B, Follicular niche tracing for the boxed region in A. Illustrated are image details of the image series s01–s07 shown in A; three occupied niches (GC105, GC108, and GC112) and one empty niche (N129) are highlighted. Occupied and empty niches were consistently found to span different numbers of spleen sections. The image series is representative of a spleen obtained 10 d after primary immunization with phOx-CSA. Scale bar, 1 mm (A, B).
first observed by day 4 after immunization, peak at around day 10 to 12, and then gradually decline. Despite agreeing with previous reports, our data support the notion that GCs might not be present for only ~3 wk, as initially proposed by earlier studies (8, 10), but might be present for several weeks after immunization (25, 26).

The mean cross-sectional size of GCs at day 21 postimmunization for only 12, and then gradually decline. Despite agreeing with previous first observed by day 4 after immunization, peak at around day 10 to 12, and then gradually decline. Despite agreeing with previous studies performed with SRBCs (11, 23). Likewise, al-

Table III. 3D evaluation of GC growth kinetics

<table>
<thead>
<tr>
<th>Daya</th>
<th>Volumeb (mm3)</th>
<th>FDCc</th>
<th>Efficiency (%d)</th>
<th>GCf</th>
<th>Mean ± SD</th>
<th>Range</th>
<th>∑</th>
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<tr>
<td>6</td>
<td>10.8</td>
<td>234</td>
<td>97.0</td>
<td>43 (44)</td>
<td>0.5 ± 0.6</td>
<td>0.01–2.2</td>
<td>21.2</td>
</tr>
<tr>
<td>6</td>
<td>13.3</td>
<td>260</td>
<td>95.4</td>
<td>37 (28)</td>
<td>0.6 ± 0.8</td>
<td>0.03–3.7</td>
<td>23.1</td>
</tr>
<tr>
<td>6</td>
<td>14.9</td>
<td>322</td>
<td>90.7</td>
<td>29 (15)</td>
<td>1.4 ± 1.8</td>
<td>0.06–4.0</td>
<td>41.1</td>
</tr>
<tr>
<td>10</td>
<td>9.2</td>
<td>226</td>
<td>95.6</td>
<td>23 (14)</td>
<td>2.4 ± 3.5</td>
<td>0.02–11.7</td>
<td>55.0</td>
</tr>
<tr>
<td>10</td>
<td>14.1</td>
<td>260</td>
<td>99.2</td>
<td>27 (14)</td>
<td>3.0 ± 4.4</td>
<td>0.01–20.7</td>
<td>80.8</td>
</tr>
<tr>
<td>10</td>
<td>13.5</td>
<td>247</td>
<td>97.6</td>
<td>42 (22)</td>
<td>2.7 ± 3.7</td>
<td>0.01–11.8</td>
<td>112.9</td>
</tr>
<tr>
<td>14</td>
<td>11.3</td>
<td>240</td>
<td>98.7</td>
<td>41 (21)</td>
<td>2.6 ± 2.8</td>
<td>0.02–11.1</td>
<td>107.4</td>
</tr>
<tr>
<td>14</td>
<td>10.4</td>
<td>206</td>
<td>87.4</td>
<td>20 (14)</td>
<td>2.0 ± 2.1</td>
<td>0.04–9.4</td>
<td>40.8</td>
</tr>
<tr>
<td>14</td>
<td>13.8</td>
<td>264</td>
<td>97.0</td>
<td>50 (28)</td>
<td>1.6 ± 1.9</td>
<td>0.02–8.1</td>
<td>80.9</td>
</tr>
</tbody>
</table>

aMice were killed at the indicated time points after immunization, and spleens were collected. For each spleen, a series of seven serial sections, spaced at intervals of 50 μm and spanning a total thickness of 300 μm, were stained and analyzed as described in Materials and Methods and illustrated in Fig. 2.
bThe volume of each series of serial sections was estimated by multiplying their mean area by their total thickness (300 μm × 50 μm and spanning a total thickness of 300 μm).
cMean, range and total volume (∑) of GCs wholly contained within or spanning the overall series of serial sections. The volumes of GCs were estimated as described in Materials and Methods.
dTotal number of follicular niches (FDC networks) identified within splenic volumes.
eEfficiency of 3D tracing of follicular niches. Indicated are the percentages of follicular niches that could be unambig-

At first glance, broad GC volume distributions leave us with various potential explanations regarding the underlying kinetics of iGC growth. The abundance of small GCs might be readily attributed to the extended emergence period of new GCs (Fig. 3). However, because this scenario fails to reproduce the high frequencies of small GCs, it has to be ruled out. If all GCs existed for ~3 wk and all reached the maximal size, then around the peak of the response, the majority of GCs would be large, as we illustrated previously by computer simulations (20). Instead, we provide two alternative explanations: 1) all iGCs follow the same average growth kinetics, but they differ considerably in their attained maximal size and most of them stay small or 2) the growth and decay of iGCs is nonsynchronized and substantially “faster” than the average. With the latter, every iGC may achieve maximal size,
but it remains large for only a short period of time. In support of the first explanation, Kleinstein and Singh (32) introduced a stochastic version of the Oprea-Perelson GC growth model (33) in 2001. In this study, we propose a new stochastic population growth model, new GCs were performed using meander scans of immunofluorescence-stained serial longitudinal spleen sections (Fig. 2). Briefly, GC outlines were segmented, and GCs were aligned slice-wise according to their center of mass. After alignment, ellipsoid fitting was performed on GCs (turquoise). A, Images are representative of spleens obtained at days 6, 10, and 14 after immunization. 3D reconstructions were restricted to the fractions of GCs that were wholly contained within or spanned the overall analyzed spleen volumes. B, Detail enlargement of the 3D reconstruction of the day 10 splenic volume relating to the series of meander scans shown in Fig. 2. The positions of the operated niches GC103, GC108, and GC112 are indicated by arrows, with their volumes and estimated numbers of B cells shown. For orientation purposes, staining of the respective region (proliferating cells [blue, mAb Ki-67], FDC networks [white, mAb FDC-M2], T cells [red, mAb CD3], and macrophages [green, mAb CD68]; scale bar, 1 mm) is superimposed for serial section s03, and s03 is highlighted in the 3D reconstruction.

FIGURE 4. 3D reconstructions of splenic GCs during the primary immune response. 3D reconstructions of GCs were performed using meander scans of immunofluorescence-stained serial longitudinal spleen sections (Fig. 2). A, Images are representative of spleens obtained at days 6, 10, and 14 after immunization. 3D reconstructions were restricted to the fractions of GCs that were wholly contained within or spanned the overall analyzed spleen volumes. B, Detail enlargement of the 3D reconstruction of the day 10 splenic volume relating to the series of meander scans shown in Fig. 2. The positions of the occupied niches GC105, GC108, and GC112 are indicated by arrows, with their volumes and estimated numbers of B cells shown. For orientation purposes, staining of the respective region (proliferating cells [blue, mAb Ki-67], FDC networks [white, mAb FDC-M2], T cells [red, mAb CD3], and macrophages [green, mAb CD68]; scale bar, 1 mm) is superimposed for serial section s03, and s03 is highlighted in the 3D reconstruction.

According to the model of Kleinstein and Singh (32), many GCs remain small if the survival of GC B cells depends on the affinity of their BCRs, if the probability of generating high-affinity mutants is low, and when selection is driven by escape from apoptosis. However, a strong, but experimentally unvalidated, prediction of their model is that iGC size is positively correlated with the level of affinity maturation (32).

In our proposed stochastic population growth model, new GCs emerge over a prolonged period of time and subsequently grow by B cell proliferation. With increasing size, B cell proliferation becomes more and more restrained by competition for a common resource, whereas sudden and fast collapses of iGCs may occur stochastically. In this scenario, GCs may achieve maximal size, but remain large for only a short period of time. In contrast to the model of Kleinstein and Singh (32), our model neither includes an explicit selection mechanism nor does it assess the fraction of high-affinity B cells in GCs. Although being deliberately simple, the presented model succeeds in reproducing our empirical data. Its most characteristic feature, the extended Ricker map, best reproduced the experimental data compared with other deterministic growth models (Supplemental Fig. 3). Notably, the values of the fitted model parameters for GC B cell division times (\(t_{\text{free}}\) and \(t_{\text{comp}}\)) (Table I) completely agree with experimental values from the literature (34–36). Volume distributions of splenic GCs at days 6, 10, and 14 after primary immunization are well reproduced by our model over two orders of magnitude (Fig. 6B–D).

The simulations of our model further emphasize that growth and decay kinetics of iGC size are much more dynamic than average eGC size kinetics (Fig. 6E). The abundance of small GCs at the peak of the GC reaction is due to iGCs attaining maximal size for only a short time because they frequently collapse. In fact, for the chosen parameter set, ~11% of all GCs shrank to one third of their size within 1 d. However, the same model without the stochastic feature of sudden collapses does not fit the experimental data for any parameter value. If GC B cell populations grew continuously, all GCs would be large at the peak of the immune response.

According to our model, iGCs of small size serve as indicators for newly formed GCs or recent collapses. Several mechanisms might lead to collapses after a dramatic selection, emigration period, or both. Such collective B cell behavior can result from intercalated phases of proliferation and selection or from intercalated phases of SHM and mutation-free expansion, as suggested by Kepler and Perelson (37). Alternatively, the unpredictable appearance of cells bearing very high-affinity BCRs, due to SHM or immigration, combined with winner-takes-all selection mechanisms, as suggested by Keşmir and de Boer (38), can lead to the substantial and quick loss of iGC B cells.
Although the currently available data do not permit speculation on detailed selection mechanisms, one can discuss the functional context of sudden collapses of GCs. As pointed out by Or-Guil et al. (20), sudden massive B cell death followed by vigorous proliferation provides for the fast and effective takeover of GCs and explains why GCs are often found to be oligoclonal (10, 39, 40). Rapid takeover of GCs was first described by Radmacher et al. (41), who observed that within a GC, usually all or none of the BCRs are selected for the fast and effective takeover of GCs and explains why GCs are often found to be oligoclonal (10, 39, 40).

As implemented in the extended Ricker model, the actual proliferation rate of GC B cells decreases with an increasing population size because resources within a GC run low (resource-limited competition) (Equation 2). Consequently, GCs enter a state of “stagnation” when their population size reaches their maximal carrying capacity. In this scenario, high-affinity GC B cells cannot prevail because their expansion is detained. Sudden collapses of GCs counteract this stagnation, leading to a quick release of resources, thereby allowing unrestricted proliferation of the few remaining or newly entering GC B cells. If one assumes that sudden collapses of GCs are affinity dependent, this scenario provides a basis for unrestricted expansion and diversification of high-affinity GC B cells, a hallmark of an effective selection mechanism.

Although the actual growth behavior of iGCs cannot be directly monitored in vivo and remains enigmatic, our results challenge the current perception about affinity maturation. The experimental data presented herein showed that iGCs grow nonsynchronously and that their growth behavior strongly deviates from the average eGC growth kinetics. Our mathematical model further suggests that these deviations are due to frequent collapses of GC B cell populations, after which the populations resume growth by B cell proliferation.

The results presented in this study underscore the need for extending the perception of the GC response to a more systemic description level, from individual GCs toward ensembles of GCs and from deterministic toward stochastic processes. By implication, this also advises caution in interpreting averaged GC development and growth data, because very different GC responses might seem to be similar based on averaged data.

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**Disclosures**

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