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CUTTING EDGE

Cutting Edge: Back to "One-Way" Germinal Centers[1]

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***The present status of germinal center (GC) research is revisited using in silico simulations based on recent lymphocyte motility data in mice. The generally adopted view of several rounds of somatic hypermutations and positive selection is analyzed with special emphasis on the spatial organization of the GC reaction. We claim that the development of dark zones is not necessary for successful GC reactions to develop. We find that a recirculation of positively selected centrocytes to the dark zone is rather unlikely. Instead we propose a scenario that combines a multiple-step mutation and selection concept with a "one-way" GC in the sense of cell migration. The Journal of Immunology,* 2005, 174: 2489–2493.**

In the germinal center (GC)[3] reaction (GCR) preplasma and memory cells are generated. The B cells undergo a process of affinity maturation, i.e., the affinity of encoded Abs is optimized with respect to a specific Ag during GCR. The GCR can be divided into four stages, namely 1) the initiation of the reaction, 2) monoclonal expansion of activated clones, 3) hypermutation of centroblasts including differentiation to centrocytes and selection, and 4) production of preplasma and memory cells and depletion of the reaction.

The GCR initiation is oligoclonal, i.e., the B cells as found in later stages of the GCR can be ascribed to a small number of activated B cells by clonal tree analysis (1, 2). T cells can facilitate this initiation, however GCR in mice without T cells have been observed for specific Ags (3, 4) and are characterized by a rapidly declining total B cell population: B cells rapidly die by apoptosis and their positive selection is inhibited.

The small number of activated B cells expands monoclonally within a network of follicular dendritic cells (FDC). Typically the proliferating centroblasts fill the FDC network and even more space (5). In later stages of the expansion phase, the process of somatic hypermutation is set in motion (6). Centroblasts differentiate into centrocytes that stop proliferating and present the newly encoded Ab on their surface. The characteristic dark and light zones emerge (7). The dark zone comprises proliferating and hypermutating centroblasts, and a small fraction of FDC (5). The light zone mainly consists of the FDC network, centrocytes, and T cells. The existence of proliferating cells in the light zone has not been addressed quantitatively so that the extent of their presence has to be considered uncertain. The general view is that proliferation takes place in the dark zone and selection in the light zone (8). The production of preplasma and memory cells begins after about 2 days following the start of somatic hypermutation. This delay guarantees the high quality (i.e., affinity) of preplasma and memory cells summed over the whole GCR (9). Mechanisms that terminate the GCR are still under discussion (10–13).

A probability analysis of finding a "better" clone within the shape space concept (14) leads to the conclusion that most mutations give rise to a "worse" Ab (in the sense of its affinity for the Ag), and these less "optimal" clones die by apoptosis for lack of positive selection. Assuming that every competitive clone is selected and differentiates further results in the production of only a very small number of output cells (15). A rough estimation in a four-dimensional shape space infers that 10,000 B cells are needed to find one optimal clone (9). This analysis leads to the prediction of a recycling process (16), i.e., positively selected centrocytes restart proliferation. In this way the GC is able to learn and restart the whole process from a better initial position in subsequent rounds of mutation and selection. Until now there is only indirect evidence for recycling (17) and a quantitative analysis of this experiment shows that four of five positively selected centrocytes have to be recycled (9). A more direct experimental test of the existence of recycling has been proposed (18). The widely accepted view is that B cells are selected to re-enter the cell cycle in the light zone and then return to the dark zone for proliferation (17, 19).

In this article we discuss the space-time dynamics of the GC on the basis of recent lymphocyte motility data (20, 21) with special emphasis on the locus of re-entry into the cell cycle of positively selected centrocytes.

Materials and Methods

Cellular automaton rules

We developed a hybrid cellular automaton for simulation of GCR. Each cell is represented as a single node on a lattice and evolves according to interaction rules which are listed as follows:

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[3] Abbreviations used in this paper: GC, germinal center; GCR, GC reaction; FDC, follicular dendritic cell.

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The mean velocity of B and T cells is 2 and 10.8 μm per minute, respectively, according to recently measured two-photon imaging data on in vivo lymphocyte motility in lymph nodes of mice (20, 21). These velocities define displacement probabilities to a neighboring node. Cell velocities may become smaller when neighboring nodes are occupied.

Monoclonal expansion of centroblasts in an FDC network lasts for 3 days with 6 h cycle time (22). Proliferation is only possible if a neighboring node is empty to provide space for the daughter cell.

Mutation is set "on" at day 3 with mutation probability of 0.5 (23). Mutations are represented by jumps in a four-dimensional shape space (9).

As it is unknown how centroblast differentiation into centrocytes is initiated a corresponding assumption has to be made in silico. Here an FDC-derived soluble differentiation signal is hypothesized (13).

Centrocytes die by apoptosis in 10 h (24).

Centrocytes are rescued from apoptosis by interaction with Ag presented on FDC. The binding probability depends on the Ab affinity to the Ag. Affinity is described by a Gauss function in the shape space with a width of 2.8 mutations centered at the clone of highest affinity (9). The rescue signal is provided within 2 h (25), and centrocytes are immobile during this period. Retesting of centrocytes remains possible during their lifetime.

Rescued centrocytes have to find T cell help for further differentiation. The number of T cells in the GC is fixed to 3% of the peak cell population. If centrocytes find a T cell their interaction lasts for 3 h. After that a probabilistic decision is taken as to whether the centrocytes differentiate to preplasma, memory cells or recycle to centroblasts. The recycling probability of positively selected centrocytes is 1 between days 3 and 5, and 0.8 from day 5 after immunization, implying a delayed production of preplasma and memory cells (9).

Parameter values and lattice dimension

The parameters of the model are based either on accessible data (mostly stemming from mice) or on general properties of the GCR that restrict the possible parameter ranges. All parameters have a physiological meaning and their values are in reasonable physiological ranges.

The simulations have been performed in two- and three-dimensional lattices. As the results are widely independent of space dimension (26), the following results are based on two-dimensional simulations. For more detail concerning the model please refer to Refs. 13 and 27. Note that modeling of lymphocyte motility and the inclusion of T cells are new ingredients of the present simulations.

Results

General features of GC simulations

The starting point of all simulations is an FDC network and three activated B cells in cell cycle with low binding probability for the Ag of 0.04. The cells then act according to the rules defined in *Materials and Methods*. The simulations capture the key features of GCR:

1) The general population kinetics (22, 28) are correctly reproduced (Fig. 1). After a phase of monoclonal expansion, apoptosis becomes a dominant process and substantially reduces the total cell population. When high affinity clones dominate, the population slowly decreases until the end of the reaction.

2) The model predicts that high affinity cells will dominate after 10 days as demonstrated in experimental settings (6). Note that in this model affinity maturation is not a result of competition for Ag but for interaction sites on FDC and T cells. Also, no affinity classes are introduced (as in e.g., Ref. 15): the relation of affinity and mutation is a Gauss-function in the shape space.

3) The total number of accumulated mutations in output cells was found to be 9, which is in agreement with experimental results (2, 29).

4) The number of output cells increases by ~6-fold between days 6 and 12 after immunization (30) as a result of the delayed production of preplasma and memory cells.

5) The whole in silico GCR simulation has a duration of 21 days (31), which is determined by the balance of cell cycle time, centroblast differentiation duration, and recycling probability.

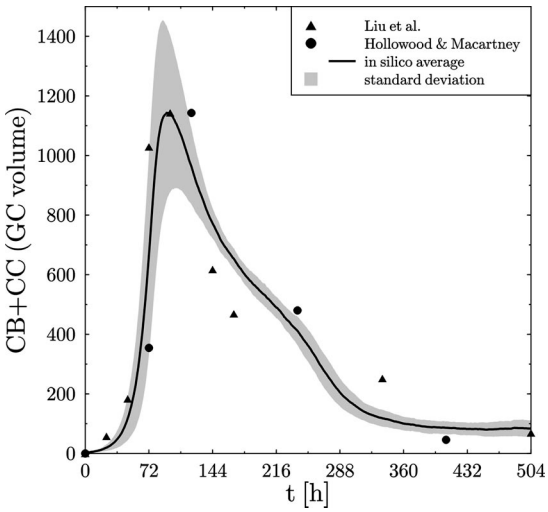

FIGURE 1. GC kinetics. The time course of the GC cell population is shown as the sum of centroblasts and centrocytes. The shaded area denotes one SD from 50 simulations. For comparison, data from primary immune response in rat and mice are included (read-off from Refs. 22 and 28).

6) The population kinetics as found for T cell-independent GCR (3) are correctly reproduced by switching off positive selection in the model.

Affinity maturation occurs in four phases

The model predicts that affinity maturation occurs in four phases (Fig. 2, and Refs. 13 and 26): 1) no affinity maturation during monoclonal expansion; 2) moderate affinity maturation during the phase of two zones being present; 3) steep affinity maturation during depletion of the dark zone; 4) saturation of the final state of achieved affinity during the depletion of the whole reaction.

Dark zones are transient

Within this model the dark zone is defined as the domain with domination of proliferating B cells.

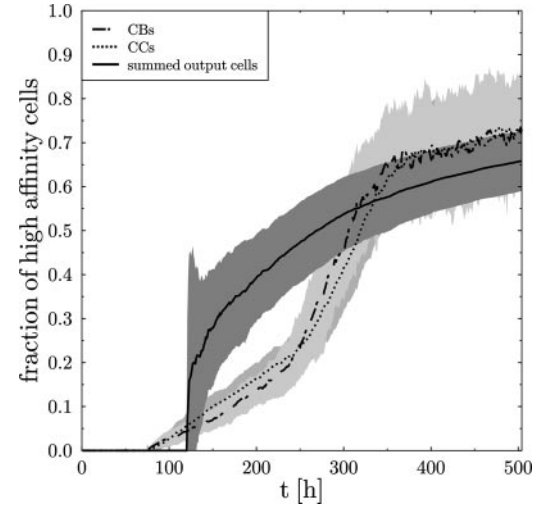

FIGURE 2. Affinity maturation. The time course of the fraction of high affinity cells is shown for centroblasts, centrocytes, and for the summed output cells. The shaded areas denote one SD from 50 simulations. Four phases of affinity maturation are observed (see *Results* section "Affinity maturation occurs in four phases").

The B cells monoclonally expand beyond the FDC network. After onset of centroblast differentiation by an FDC-derived signal, centrocytes become the dominant population in the FDC network and the dark zone emerges in the domain of low FDC density. As the differentiation signal is soluble the dark zone is then reduced and finally depleted. Note that it is not essential that the differentiation signal is derived from FDC, as any soluble light zone-derived signal would suffice. Under this assumption the correct GC kinetics are reproduced. If, alternatively, centroblasts would differentiate after a number of cell divisions, chemotaxis or cell adhesion had to be introduced to describe dark zone formation (27).

Recycled B cells do not return to the dark zone

The in silico experiments predict that positively selected B cells re-entering the cell cycle do not return to the dark zone but proliferate within the FDC network (Fig. 3). They perform a random walk through the GC and return to the dark zone by chance only. When the dark zone is dissolved around day 9 after immunization, recycled B cells become homogeneously distributed in the GC area. In silico recycled B cells might return to the dark zone only on the basis of at least two different chemokines and alternating chemokine receptor expression on B cells.

GC without dark zone may work

In silico, GC without a dark zone display affinity maturation to a similar extent as GC with dark zones (data not shown). In GC with dark zones B cells pass the FDC network unidirectionally while in GC without dark zones B cells isotropically migrate within the FDC network.

Discussion

The prediction of a steep increase in affinity when the dark zone is depleted has important implications concerning the role of the dark zone for affinity maturation: its function is to produce a large pool of clones which is important in the early stages of the GCR. The dark zone consists of cells that undergo poor selection if at all because the FDC density is substantially smaller compared with the light zone. In later stages of the GCR this infers a growing gap between the affinity of encoded Abs in the dark and light zones. Therefore the dark zone has to be depleted in time to optimize affinity maturation in GC. Indeed, the affinity and the number of produced plasma and memory cells is optimized for dark zones that vanish around days 8–9 after immunization (13).

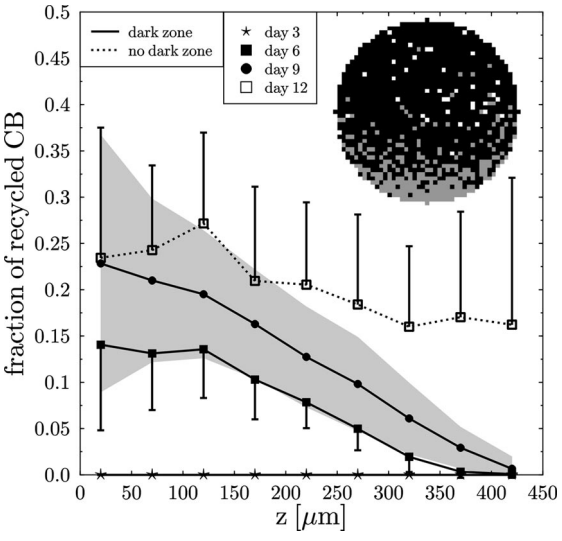

FIGURE 3. Distribution of recycled B cells. The fraction of recycled B cells is shown at different places within the GC (one SD is shown as half error bar or shaded area). The light zone (0–300 μm) hosts most recycled B cells whereas in the dark zone (300–450 μm) recycled B cells are rarely found (full lines). The dark zone is dissolved around day 9 and in later stages the former dark zone area contains equal amounts of recycled centroblasts (dotted line). An example of the distribution of B cells in cell cycle is shown at day 7 after immunization (*inset*, *top right*). The light zone is at the top. White cells are recycled, gray cells nonrecycled centroblasts (FDC, T cells, and centrocytes are not shown).

The in silico experiments predict that the dark zone is not even necessary for a GCR to develop. It mainly facilitates the onset of the GCR. GC without dark zones have been observed and their appearance depends on the species and the organ under consideration (32, 33). However, this does not sufficiently substantiate the existence of GCR without dark zones because these GC have not been monitored during their whole development. The present results support the view that GC without dark zones may exist and be equally successful in terms of affinity maturation.

The most striking result is that in silico B cells move in a one-way direction through the FDC network. B cells switching between the dark and light zones after every recycling round (as often assumed) would not only imply huge cell movements within the follicle, but also the distribution of chemotactic signals would have to be rather specific and this seems unlikely. A more realistic scenario for B cells circulating between the dark and light zones would be two concurring chemotactic gradients with the corresponding receptors expressed and down-regulated by B cells in subsequent cycles (34).

A much more simple proposal for recycling would be that re-entry into the cell cycle is performed at the same place where the rescue signals have been provided. Different rounds of proliferation and mutation would then occur in higher and higher regions of the light zone (i.e., in regions with increasing distance from the dark zone). It has already been proposed on the basis of GC snapshots in mice that selection and re-entry into the cell cycle may take place in the light zone (5). Our present in silico experiments show that such a one-way GC is the most likely scenario for GCR.

B cells moving in a one-way GC may be interpreted as performing a random walk (i.e., an undirected active movement) in the secondary follicle. In silico the unique direction of cell migration is determined by the position of the dark zone (if present). The proliferative pressure exerted by centroblasts in the dark zone pushes cells into the light zone. This breaks the isotropic random walk of B cells. Note that recent in vivo studies using two-photon imaging techniques revealed that B and T cell migration in the periphery of lymph nodes in mice is consistent with a random walk (21). We claim that this may equally apply to B cell migration in secondary follicles. The measured B cell migration would still be anisotropic because of GC polarity, which results in different proliferative pressure in the dark and light zones. However, Ags in secondary follicles may induce other deviations from a random walk (20).

The present one-way GC model has two major implications: first, a certain number of proliferating B cells should be found throughout the light zone. Indeed, at least in mice, proliferating B cells are found throughout the FDC network (5, 35). In addition, the majority of GC B cells in mouse spleen have been

found to be Ki67-positive in the whole GC (36). It seems as if B cell selection in mice can occur within a rather phenotypically homogeneous population of actively proliferating B cells (8), thus supporting the view that re-entry into the cell cycle takes place in the light zone.

Second, T cells have to be distributed within the light zone if they are considered to be necessary for providing the differentiation signal that leads either to re-entry into the cell cycle or to final differentiation into preplasma or memory cells (37). However, re-entry into the cell cycle may also be induced by Ag presenting FDC only. In fact, various cytokines have been reported to regulate B cell differentiation. IL-4 is produced by T cells and associated with B cell differentiation (38). Both IL-2 and IL-4 have been seen to promote in vitro differentiation to memory B cells (39); IL-6 is secreted by FDC and promotes B cell proliferation (40); IL-10 is required for differentiation to plasma cells (37, 39). CD40L promotes B cell proliferation, regulates Fas-induced apoptosis (39), and has a role in B cell differentiation (38) favoring differentiation to memory B cells (41). Both FDC and T cells have been shown to express FasL and in this way to promote negative selection via Fas-induced apoptosis (42). More generally, FDC seem to provide signals for rapid proliferation of B cells while T cells are more likely to regulate B cell differentiation and inhibit apoptosis (38). However, the relative role of FDC and T cells in GC still remains to be elucidated (8, 43).

We conclude that it is likely that T cells play a role in initiation of re-entry into the cell cycle of selected centrocytes. Then, according to the one-way GC model, T cells are expected to be present throughout the FDC network. In fact T cells have been found to be concentrated in the outer zone at least in the case of human tonsils (44). However, this does not exclude T cells from being present in other regions of the light zone. If T cell help were limiting for affinity maturation, it would be reasonable to expect T cells to be present more frequently with increasing distance from the dark zone. Apoptosis would then be most frequent in the basal light zone, which, indeed, has been observed (44). T cells have also been found throughout the FDC network of human tonsils (45). A homogeneous distribution of T cells within the FDC network has also been proposed in mice (5). Note that if cells re-entering the cell cycle would return to the dark zone then the T cell distribution would be less important.

The appearance of dark zones depends on the species and organ under consideration. If, according to the classical view, recycled B cells are attracted by chemotactic signals back to the dark zone and if they have to do so to re-enter the cell cycle, then recycling would behave very differently in GC with or without dark zones. We would have to distinguish between two types of re-entry mechanisms. Note that in the one-way GC model proposed here, re-entry of B cells into the cell cycle is not affected by the presence of a dark zone. The one-way GC model can cope with GC with or without dark zones.

A decisive test of this new GCR model is to measure time courses of spatial distributions of proliferating B and T cells. It is important to include data on how pronounced the dark zone is in the GC. This would have to be conducted on a statistically relevant basis using a well-defined model system to obtain a definite answer for a specific species and organ. An analysis of average distributions including their width would have to be undertaken. A result in which proliferating B cells as well as T cells are found rather frequently within the FDC network would support the present model of a "one-way" GC.

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Disclosures

The authors have no financial conflict of interest.

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