Mechanisms of organogenesis of primary lymphoid follicles

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Abstract

Primary lymphoid follicles (PLFs) in secondary lymphoid tissue (SLT) of mammals are the backbone for the formation of follicular dendritic cell (FDC) networks. These are important for germinal center reactions during which affinity maturation creates optimized antibodies in adaptive immune responses. In the context of organogenesis, molecular requirements for the formation of follicles have been identified. The present study complements these findings with a simulation of the dynamics of the PLF formation, and a critical analysis of the relevant molecular interactions. In contrast to other problems of pattern formation, the homeostasis of cell populations in SLTs is not governed by a growth-death balance but by a flow equilibrium of migrating cells. The influx of cells into these tissues has been extensively studied. However, less information is available about the efflux of lymphocytes from SLTs. This study formulates the minimal requirements for cell efflux that guarantee a flow equilibrium and, thus, a stable PLF. The model predicts that in addition to already identified regulatory mechanisms, a negative regulation of the generation of FDCs is required. Furthermore, a comparison with data concerning the microanatomy of SLTs yields the conclusion that dynamical changes of the lymphatic endothelium during the formation of FDC networks are necessary to understand the genesis and maintenance of follicles.

Introduction

The primary lymphoid follicle (PLF) is the precursor state of germinal centers in secondary lymphoid tissue (SLT), which are essential for antibody optimization in adaptive immune responses (1). The main constituents of PLFs are naive B cells and follicular dendritic cells (FDCs). PLFs serve as a filter for antigen and bring B cells into contact with antigen presented by FDCs in order to start a germinal center reaction (2).

In mammals, PLFs first develop around birth (3). The development involves members of the tumor necrosis factor (TNF) superfamily such as TNF-α, lymphotoxin (LT)-α1β2, LTα3, LIGHT and TRANCE [reviewed in (4, 5)] and homeostatic chemokines CCL19, CCL21, and CXCL13 that guide lymphocytes to their compartments (6). CXCL13 is secreted by FDCs and acts as chemotactant for B cells via the CXCR5 chemokine receptor. CCL19 and CCL21 are produced by cell populations in the T zone of SLT. The common receptor CCR7 is expressed by T cells and, at a lower level, by B cells. The expression of these chemokines and the presence of FDCs depend on LTα1β2, TNF-α and related molecules. In most SLT, a persistent LTα1β2 stimulus is needed for both the induction and the maintenance of FDC networks (7). Further studies suggest that the maintenance of the PLF structure is mediated by a positive feedback loop (8): B cells are stimulated by CXCL13 to express high levels of LTα1β2. This in turn stimulates FDCs to produce CXCL13.

An interesting property of PLFs is their equal size of several hundred micrometers in all mammals ranging from mice to horses (9–12). Thus, the follicle size does not scale with the size of the animals or of the organs under consideration, which is considered to be a strong boundary condition for simulations of PLF formation and maintenance. The formation of PLFs is studied with the Delaunay-Object-Dynamics method—a multi-scale three-dimensional modeling framework (13, 14)—which is based on a Delaunay triangulation (15, 16). A clear separation of B and T cells is achieved using chemoattraction of lymphocytes to exit spots. Formation of PLFs with adjacent T zone is found assuming suitable signaling mechanisms between B cells and stromal cells (14).
However, the model (14) is incomplete because the location of the PLF relative to lymphatic exit paths is not in agreement with experiment (17–22).

The present study concentrates on the correct formation of the lymphatic pathways and the PLF without considering the dynamics of the T zone and T cells. The model includes the dynamics of lymphatic structures, which strongly changes the dynamics of the PLF formation, its geometry and also the regulation mechanisms involved in PLF maintenance and size regulation. It is found that a mechanism is required that prevents the formation of new FDCs and does not affect existing FDCs. The simulations suggest that chemokine receptor internalization dynamics are not at the origin of follicle size regulation. Furthermore, it is demonstrated that a chemotactic activity of B cells for sphingosine 1-phosphate (S1P) is in contradiction to PLF morphology, suggesting that S1P chemotaxis is not active during PLF formation and maintenance in vivo, which was also found in experiment (23).

Method

Simulation framework

The PLF is simulated using an agent-based off-lattice model architecture (15, 24, 25), the Delaunay-Object-Dynamics (13, 14, 16). A regular triangulation, which is a generalization of the Delaunay triangulation, is used to provide the neighborhood topology for the cells of continuous size and position. The simulations are performed in a three-level model. The first level is the dynamics of the internal state representing the changing phenotype of the cell. The second level models the contact interaction between cells including mechanical interactions with the environment and exchange of signals by membrane-bound molecules like LTα1β2. The forces generated at this level enter Newtonian equations of motion for each cell. The third level incorporates long-range interactions via diffusive substances, which is used to describe the chemokine distribution in the PLF. A reaction-diffusion system for the solubles is solved on an overlayed grid, with the cells (depending on their internal state) being the sources and sinks. More details of the model framework can be found in (14) and in the supplementary material (Text-S1 available at International Immunology Online).

Flux of naive B cells

The essential ingredients to study the formation of the PLF are the B cells, the FDCs and the FDC progenitor cells. The B cells are constantly entering SLT via high endothelial venules (HEVs) in mucosa-associated lymphoid tissue and lymph nodes (LNs), or along the central arterioles through the peri-arteriolar sheath of the spleen (6). The exit route of lymphocytes through lymphatic endothelium (LE) is less clearly identified. Recent experimental data (6) suggest that B cells leave mucosa-associated lymphoid tissues via efferent lymphatic vessels. In LN and spleen, the lymphatic sinuses guide lymphocytes to draining efferent lymphatic vessels. As the sinuses merge with the lymphatic vessels, they are considered to belong to functionally similar lymphatic exit paths in the model. Both will be referred to as LE in the following.

The location of the LE is very similar across the different SLT and species (17–22). The LE is formed around the follicles ranging from half-open baskets around the follicle base to almost closed shells enclosing the whole follicle. In the non-follicular area, the LE forms a dense network.

S1P may be involved in the regulation of lymphocyte egress from SLT (26, 27). S1P acts as a chemoaatractant for lymphocytes in vitro (26). Thus, the dynamics of the S1P receptor 1 (S1PR1) is considered in the simulation: because the LE is widely engulfing follicles, lymphocytes have to migrate around or across these structures in order to reach the follicles. Therefore, a minimal transit time through SLT shall be achieved by a mechanism that prevents the lymphocytes from entering the LE. In the simulation it is assumed that S1PR1 is down-regulated when B cells enter SLT. Up-regulation is required for lymphocyte egress, which is supported by studies using the S1PR1 agonist FY720 (26, 27). S1PR1 is assumed to be up-regulated after 3 h (27), which coincides with the minimal transit time of lymphocytes in SLT (20, 28).

Origin of FDCs

One fundamental question in the PLF formation is as follows: Where do the FDCs come from? The most commonly accepted view is that FDCs are derived from stromal cells, which may be related to the stromal cells observed in the T zone. These stromal cells are fibroblastic reticular cells (FRCs) (29, 30). Comparative reviews support the mesenchymal origin of FDCs (31). Evidence for the relation of FDCs to FRCs is also given by shared markers (32) and a gradual change from the ‘classical fibroblastic’ to the typical FDC morphology (33). The analysis of typical FDC marker expression indicates a smooth transition from stromal progenitor cells to FDCs by subsequent accumulation of ‘FDC-ness’ of the stromal cells (11).

Indirect evidence for the reverse transition from FDCs to stromal cells is provided by culture experiments in which purified FDCs gradually lose characteristic FDC markers, and finally return to a stromal cell-like state (34, 35). This suggests that persistent stimuli are required to maintain the FDC phenotype. However, a clear relationship between FRCs and FDCs was not confirmed on the basis of newly developed markers (30). In an alternative view, bone marrow-derived progenitors of FDC (36, 37) may migrate into SLT (38–40) and complete their maturation program.

The present study will not decide on local differentiation versus immigration of FDC progenitors. It is assumed that newly developed FDCs replace existing FRCs at their site of generation, which can be interpreted as FRC displacement by FDC or by FRC to FDC differentiation (see also Fig. 1).

In a previous simplified PLF model, it was shown that sufficiently large aggregates of B cells around stromal cells can induce FDCs (14). This involves a positive feedback loop between FDC and B cell signaling (8), which is included in the present model: B cell LTα1β2 induces CXCL13 production by FDC which in turn increases LTα1β2 expression on B cells. However, the positive feedback loop is not mandatory: The migration properties of B cells derived from the experiment are such that the contact duration and contact area of the B cell–FRC interaction allow for a strong LTα1β2 stimulus.
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sumed that the anti-correlation between FDCs and LE is not considered explicitly but summarized in a time delay of LE remodeling of several hours.

Model of dynamic lymphatic pathways
A phenomenological model of dynamic lymphatic pathways is included in the model in order to generate an LE distribution that matches microanatomical data (17–22). It is as-

sumed that the LE is not preformed, which would determine the shape and position of PLFs a priori. A preformed LE gap would immediately raise the question what generates this specific LE pattern prior to follicle formation. Thus, in the simulation, the generation of FDCs is anti-correlated with the LE dynamics to produce follicle and LE pattern in one step: LE is degraded when FDCs are formed in the vicinity. It is as-

sumed that the anti-correlation between FDCs and LE is local: The LE degradation process is not governed by a long-ranged diffusion of molecular messengers. LE re-forms at places where FDCs have transformed back into FRC.

More detailed angiogenesis and lymphangiogenesis mod-

elns are still under development (42, 43). Therefore, a phe-

nomenological model of vessel formation and degradation is used omitting details of the underlying mechanisms. Presumably, remodeling is organized by the exchange of chemicals inducing behavioral changes of the LE cells. This is not considered explicitly but summarized in a time delay of LE remodeling of several hours.

Regulation of chemotaxis
The B cells have to leave the PLF before they can exit from the SLT. In the simulation, the aforementioned model of ves-

sel dynamics will induce LE, which encloses the follicles. B cells that reach the surface of the follicle either find a lymphatic exit path for exit or return into the follicle. It is assumed that this search for exit points is regulated by B cell chemotaxis.

Optionally, in the model the chemotactic response of lymphocytes might be modulated by receptor internalization (44). This mechanism is modeled for the chemokine receptors CCR7 and CXCR5. Receptor internalization is supported by experimental data for lymphocytes in SLT (6, 45–48). This implies that lymphocytes may be unresponsive to chemokines despite proper receptor expression and observed in vitro responses. Note that, within the model framework, regulation of chemotaxis by other mechanisms like transcriptional regulation is not considered.

The integrated action of the chemoattractants CCL19,
CCL21, CXCL13, and S1P on a cell is assumed to be a vector sum of the individual responses of this cell (49): The speed of lymphocytes is set to a constant value derived from two-photon imaging experiments (48, 50, 51). The direction of the chemotactic response is the weighted average direction of the chemokine gradients. The weight is provided by the gradient of the bound chemokine at both ends of the cell assuming that the cell senses the difference of bound molecules across its diameter.

Results
The minimal set of assumptions entering the model is:

• a fixed number of non-migrating stromal cells,
• constant entrance of B cells via a small number of HEVs,
• B cells use the LE for egress from the SLT,
• generation of FDCs by B cell LTα1β2 signaling,
• replacement of FRCs by generated FDCs,
• removal of FDCs (replaced by FRCs) in case of lack of LTα1β2 stimulation,
• secretion of CXCL13 by FDCs,
• secretion of CCL21 by FRCs,
• secretion of S1P by LE,
• chemotactic activity of B cells to CXCL13, CCL21 and S1P and
• B cells leaving the SLT via LE when S1P1 levels are sufficient to dominate CXCL13 response.

These assumptions alone lead to reasonable cell dy-

namics. With the simplifying assumption that lymphocytes use only focal lymphatic exit spots, instead of the whole LE, a PLF is generated that is stable in size, shape and position (14) using physiological values for the parameters (a table of parameters is provided as supplementary material, Table-S1 available at International Immunology Online). However, the position of the follicle is in contradiction to microanatomical data (17, 18, 20–22). Introducing the correct relative position of PLF and LE using dynamic LE, dramatically changes the follicle stability. Thus, the stability of follicle shape and size crucially depends on further mechanisms.

Positive and negative feedbacks are required to form a stable follicle (52). The positive regulation by B cells induc-

ing FDC is well known (8). In the following, possible mecha-

nisms for the negative regulation are discussed. The most intuitive hypothesis is related to internalization dynamics of chemotaxis receptors. It is shown that this hypothesis leads to unphysiological results and is therefore unlikely to be the relevant mechanism of follicle formation and maintenance. Realistic results could only be achieved by the assumption of a so far unknown mechanism of negative regulation of
FDC generation. This mechanism is a prediction of the model.

**Including internalization dynamics**

The simulations reveal that internalization of CXCR5 (model equation in the supplementary material, Text-S1 available at *International Immunology* Online) destabilizes the shape of the follicle (Fig. 2A–D). In a previous work, the internalization dynamics was analyzed with a dynamic FDC network but constant B cells numbers (14). In this article, it is shown that neither the B cell flux nor a non-dynamic pre-formed FDC network can compensate for the instability.

The instability is caused by the uptake of CXCL13 when the CXCR5–CXCL13 complex is internalized: The chemotactically responding B cells act as a sink for CXCL13. This generates steep gradients in the chemokine distribution which guide the B cell movement. Interestingly, these local gradients, induced by the responding lymphocyte population itself, can become such strong that they locally reverse the chemokine gradient. Cellular movement becomes not strictly directed towards the chemokine source anymore.

The instability persists when the CXCL13 production by FDCs is at maximum. Within the concept of a positive feedback loop (see Origin of FDCs), maximum production is normally achieved when an FDC is completely surrounded by B cells which happens for virtually all FDCs in the PLF. The same applies if maximal CXCL13 production is enforced as soon as the minimal required \( \text{LT}_{\alpha_1\beta_2} \) threshold is reached.

A CXCL13 production exceeding the known physiologic values can compensate for locally inverted gradients and shape fluctuations induced by the CXCR5 internalization dynamics (data not shown). This, however, implies that the resulting CXCL13 concentration remains high, such that B cells in the PLF rapidly internalize CXCR5 and become insensitive to CXCL13. Consequently, CXCL13 is not able to hold B cells for >15–30 min (44), which contradicts the observed transit times of at least 3–5 h (20, 28).

Locally inverted gradients due to the CXCR5 internalization dynamics can induce quasi-periodical alterations of the follicle shape. The destabilization is also observed when the CXCL13 production by FDCs is at maximum, even when the FDC network is of fixed size and LEs are densely distributed in the T zone (A: 48 h, B: 48.5 h, C: 49 h, D: 50 h). The dynamics of the LE destabilizes the follicle shape (E: 5 h, F: 122 h, G: 5 h, H: 57 h). Although the morphology of the follicle is correct (LE surrounds a spherical follicle), the follicle grows infinitely. This is independent of the density of the LE (E, F: low density; G, H: high density). Note that at high LE density, the distinct spheres representing the exit spots merge into a seemingly homogeneous background in the pictures. Introducing negative regulation of FDC generation, the follicle forms around the HEV (sequence I: 10 h, J: 60 h). If B cells chemotax towards CCL21, the follicle may elongate towards the center of the stromal network (J). If an FDC network with high-density LE is preformed distant to the HEV, the follicle rapidly adopts its final shape (K: 5 days). The follicle keeps its position and does not relocate to the HEV. The negative regulation prevents the induction of FDC between B cell entry and PLF. When S1P is acting in the dynamic PLF formation [compare with panels (I) and (J)], a disturbed follicular border occurs which is already clearly visible after 1 day (L). The PLF appears larger due to the lower density of B cells, whereas the number of B cells is not significantly larger (data not shown).

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**Fig. 2.** Three-dimensional slice projections from simulations (rendered with POVRay, http://www.povray.org). Objects are shown as colored spheres (yellow: FDC, green: FRC, white: naive B cells, red: HEV, dark gray: LE). The internalization process destabilizes the follicle shape, even when the FDC network is of fixed size and LEs are densely distributed in the T zone (A: 48 h, B: 48.5 h, C: 49 h, D: 50 h). The dynamics of the LE destabilizes the follicle shape (E: 5 h, F: 122 h, G: 5 h, H: 57 h). Although the morphology of the follicle is correct (LE surrounds a spherical follicle), the follicle grows infinitely. This is independent of the density of the LE (E, F: low density; G, H: high density). Note that at high LE density, the distinct spheres representing the exit spots merge into a seemingly homogeneous background in the pictures. Introducing negative regulation of FDC generation, the follicle forms around the HEV (sequence I: 10 h, J: 60 h). If B cells chemotax towards CCL21, the follicle may elongate towards the center of the stromal network (J). If an FDC network with high-density LE is preformed distant to the HEV, the follicle rapidly adopts its final shape (K: 5 days). The follicle keeps its position and does not relocate to the HEV. The negative regulation prevents the induction of FDC between B cell entry and PLF. When S1P is acting in the dynamic PLF formation [compare with panels (I) and (J)], a disturbed follicular border occurs which is already clearly visible after 1 day (L). The PLF appears larger due to the lower density of B cells, whereas the number of B cells is not significantly larger (data not shown).
chemokine profile. A small aggregate of B cells will down-regulate their receptors and thereby locally deplete CXCL13. The cells lose their chemokine responsiveness and start a persistent random walk. While the B cells are exposed to low chemokine concentrations, they up-regulate CXCR5 again, follow the inverted gradients and eventually aggregate around newly generated concentration peaks where the cycle starts again.

The internalization-induced instability is independent of the FDC dynamics. The position of new CXCL13 concentration peaks might be located outside the FDC network. Then new FDCs would be generated at the border of or outside the follicle. In addition, locally depleted CXCL13 might leave FDCs without a sufficient number of supporting B cells in contact. Then FDCs would disappear because of the missing LTα1β2-maintenance signal. We conclude that the FDC dynamics amplifies, but is not required for, the shape instability of follicles induced by CXCR5 internalization dynamics.

The simulations show that internalization dynamics of CXCR5 induce unstable PLFs in a physiological parameter regime. As such instabilities are not observed in nature, the present model predicts that receptor internalization is not the dominant process in PLF size regulation. Even though it cannot be excluded that internalization is active and that the instability is only attenuated by further interactions, it is unlikely that we lose essential features of PLF formation when neglecting internalization dynamics in the following. However, the robustness of all results with respect to internalization dynamics was checked.

**Including LE dynamics**

LE dynamics may be considered to stabilize follicle shape and size by locating the exit points for B cells. However, LE dynamics turn out to destabilize the follicle system independently of the internalization dynamics (Figs 2E–H and 3): If sufficient B cells approach the LE—either by random motion or S1P-directed migration (see Why S1P Chemotaxis Is Not Likely to Occur in PLFs)—they stimulate the generation of novel FDCs right next to the exit spots. As FDCs and LE are anti-correlated, that is the vicinity of LE is free of FDCs, the generation of FDCs leads to the degradation of the LE. The exit spots for B cells are pushed farther away. The B cells need to approach the LE and ‘follow’ the distant exit spots. Due to the increased distance of the exit spots from the follicle center, the B cells take longer time to reach them, thus increasing their transit time. Consequently, the follicle is enlarged and the density of B cells close to the exit spots again reaches concentrations sufficient to induce new FDCs. This self-perpetuating process drives the border between FDCs and LE leading to a constantly growing PLF size.

Even though the model for the genesis of lymph endothelium is rather simplistic, it nevertheless covers a reasonable idea of the dynamics of LE. The destabilizing effect of this model points towards an additional process that prevents the PLF from constant growth by inhibition of the generation of FDCs at the right follicle size.

**Negative regulation of FDC generation**

A negative regulation mechanism that inhibits the generation of FDCs may be realized in two distinct ways. First, FDCs induction is prevented when some factor is missing, that is FDC progenitor activation gets lost. Cells located in the T zone but not in the follicle secrete a substance that keeps the FDC progenitors in a state in which they are able to become FDCs. The production and sensitivity for the signal has to be such that only a sufficiently large number of cells induces the signal concentration that permits FDC generation. This scenario seems rather unlikely given the fact that under certain conditions separated PLFs can be generated in the absence of a well developed T zone (4).

In a second option, the generation of FDCs leads to the secretion of a substance X that inhibits their own generation and counterbalances the effect of LTα1β2. The negative regulation must not induce dedifferentiation of already existing FDCs to FRCs. Also the signal X has to be diffusible. A local inhibition mechanism can prevent FDC generation only independently of the follicle size. A signal sensitive to the follicle size needs to be accumulating and dispersing the information in the vicinity of the PLF.

The negative regulation is included in the model by solving the Poisson equation with the FDCs as sources of the signal. In addition, the signal has a limited life time characterized by the decay constant $\kappa$. The time independence of this approach is justified considering the low mobility and slow dynamics of stromal cells. The threshold at which the signal X inhibits FDC generation is chosen to be 20% of the signal concentration that would be generated by a follicle of infinite size: $c_{\text{threshold}} = 0.2 \times \rho Q / \kappa$, with the density $\rho$ of FDCs producing the signal X at rate $Q$. The chosen threshold is such that an area of sufficient width around the FDC network is kept free of FDC once the typical PLF size is reached.
Lymphoid follicle organogenesis

The simulations show that negative regulation of FDC induction, indeed, leads to the formation of stable follicles (Fig. 2I–L). Both aforementioned scenarios have similar effects. The PLF forms close to the HEV as expected from the high B cell concentration resulting from the cell influx. There is no other B cell aggregation center. Neither S1P nor CCL21 can lead to B cell aggregation as they are broadly distributed providing only shallow gradients for B cell chemotaxis. The chemotactic activity of B cells in response to CCL21 can, however, lead to elongated follicles when a CCL21 concentration peak is nearby the PLF center (Fig. 2J).

The negative regulation mechanism has influence on the morphology of the PLF now exhibiting two zones. One B cell-rich zone with FDCs and without efferent vessels and a shell of B cells with low or vanishing FDC density but with LE. Within this shell, B cells are exiting from the PLF and SLT.

An additional stability test is done using a preformed FDC network that is placed in a certain distance from the HEVs. The simulation of this configurations shows that the preformed FDC network rapidly extents to its final size and, most importantly, remains at its location (Fig. 2K) because of the negative regulation mechanism. The signal suppressing the generation of FDC acts also on the FRCs between the follicle and the HEV. The B cells are not able to induce FDCs while passing through the LE and the FRC network before reaching the PLF.

Why S1P chemotaxis is not likely to occur in PLFs

An important result of this study is that the chemotaxis towards S1P is not required. The LE is distributed homogeneously around the follicle. This allows B cells to find the LE by random migration within typical transit times.

In addition, S1P chemotaxis in the PLF disturbs the border of the lymphoid follicle (Fig. 2L). The unexpected irregular border results from a rather low concentration of CXCL13 at the boundary of the follicle, which allows S1P to influence the direction of motion of B cells at the follicle border. This is only slightly affected by the internalization dynamics of CXCR5 because the cells at the border are mainly sensitive for CXCL13 due to low CXCL13 concentration. The follicle morphology caused by S1P suggests that B cells in the follicle are not chemotactically responding to S1P.

Discussion

A simulation of the formation and maintenance of PLFs was presented using the Delaunay-Object-Dynamics (13, 14). The initial formation of FDCs from the stromal progenitor cells is governed by B cell aggregates with low LTαβ levels. B cells are sensitive to the chemokine CXCL13 via their receptor CXCR5. CXCL13 is secreted by FDCs. The positive feedback loop that enhances B cell LTαβ levels by CXCL13 stimulation (8) is included in the model but not required to understand PLF formation. It only speeds up the growth of the follicle. PLFs are found to be unstable when either CXCR5 internalization dynamics or FDC-dependent LE dynamics are included. A mechanism is predicted by the model which is discussed in the context of related experiments in the following.

Internalization of chemokine receptors does not drive PLF formation

The internalization of CXCR5 has been introduced in the simulation to allow B cells to leave the follicle. Attraction of B cells to the center of the FDC network is desensitized and make B cells approach the follicular border either by random or chemotactic migration in response to other chemokines like CCL21, CCL19 and/or S1P. The instability generated in the simulation by the CXCR5 internalization dynamics suggests that this process may not be relevant in the PLF.

A recent experimental finding suggests that the chemotactic response of lymphocytes in SLT is predominantly towards chemokines bound to the surface of FRC and FDC (53). This may prevent the uptake of the chemokine by the cells and thus counterbalance the destabilizing effects of CXCR5 internalization seen in the model. Whether and how the bound receptors are internalized under these conditions remains unclear. Chemokine ligand–receptor interactions are of high affinity (54, 55). Thus, upon internalization, the chemokine receptor–ligand complex might be intact and the complex would be cleaved from FRC or FDC. Indeed, dipeptidyl peptidases like CD26 are known to cleave chemokines (56). However, assuming only bound chemokines to be relevant for B cell chemotaxis, the cleavage of CXCL13 would have similar effects like the degradation of CXCL13 by internalization as considered in the model. In order to internalize the receptor alone, the bond would have to be broken physically by sufficiently strong forces during receptor endocytosis. So far, cleavage of chemokine receptors from their ligand has not been demonstrated.

It cannot be ruled out that another modification of the CXCR5 receptor levels, for example on the transcriptional level, leads to a desensitization of B cells for CXCL13 which enables them to leave the PLF. Also it cannot be excluded that mechanisms exist that compensate for the internalization-induced instability of the PLF. This may even be suggested when considering that the internalization dynamics can induce cycling of naive B cells between the center of the PLF and its border. The cycling is due to the strong desensitization in the center of the PLF where CXCL13 concentrations are high. B cells can then approach the border by random migration where the lower CXCL13 concentrations permit a resensitization for CXCL13 chemotaxis. These B cells migrate back to the center of the PLF. Such a cycling may contribute to the relocation of antigen-stimulated B cells to the follicular border to acquire T-cell help (6). It would be interesting to investigate in experiments whether a block of internalization dynamics would inhibit germinal center induction.

Follicular B cells are not responsive to CCL19, CCL21 and S1P

The morphological data of the simulations suggest that B cells in the PLF are not chemotactic responsive for S1P. A superposition of the two chemoattractants S1P and CXCL13 generates a blurry PLF border, which is not observed experimentally. This is in line with the recent observation that S1P is not a chemotactic factor in vivo for T cells in the LN (23) and also not for follicular B cells in vitro despite a relatively high level of expression of the receptor S1P1 (57). A similar
blurry PLF border would be induced if follicular B cells significantly responded to the T zone chemokines CCL19 and CCL21. Therefore, the simulation suggests that chemotaxis to CCL19 and CCL21 is suppressed. This is supported by in vivo experiments (6, 55), which contrasts the positive response in vitro for CCL19 and CCL21 (54, 58). Indeed, freshly isolated B cells exhibit low levels of CCR7 on the surface (46).

Lymphatic vessels

The molecular regulation of the LE remodeling is not resolved. Possible mechanisms are the lack of extracellular matrix fibers in the PLF (19, 59) induced by the FDCs (10, 60). Alternatively, the interplay of vascular endothelial growth factors provided by B cells in the PLF (22, 61) with dendritic cells (62) and macrophages (63) outside the PLF may create the observed LE pattern. Conceptually, the most simple explanation for the exclusion of LE from the PLF is a strong production of anti-angiogenic factors in the follicle that can outperform the effects of the high growth factor production by other cells, including the B cells in the PLF (22). Such a factor may be the chemokine CXCL13 which is known to be anti-angiogenic (64).

The presented model assumes that the lymphatic network formation in SLT is coupled to the dynamics of PLF. An alternative view is that the LE is formed before the PLF and determines the position and shape of the developing PLF. A direct test for the existence of the LE dynamics is to provide anti-angiogenic factors to LTα1β2−/− or similar knockouts a few hours before reconstitution by wild-type lymphocytes or bone marrow. This would suppress LE remodeling. If follicle location were preformed by LE structures, the resulting PLF would be unaltered. In contrast, the LE dynamics proposed by this study predicts that the PLFs are much smaller than usual and contain LE because the LE cannot adapt to the PLF. Alternatively, the inverse experiment could be done by blocking LTα1β2 in wild-type mice or reconstitute them with bone marrow from LTα1β2−/−. The model predicts that the disruption of the PLFs should be followed by the presence of new vessels in these areas. When using angiostatic factors during the experiment, the gaps in the vessel network due to the PLFs should be preserved when the PLFs disappear. However, if the location of the PLFs was determined by preformed gaps in the LE, the LE structure should remain unchanged, independent of the application of angiostatic factors.

The appearance of HEV in the model PLF is in contradiction to experimental findings (19, 21). We did neither consider the dynamic formation of HEV nor factors that may guide B cells away from them. Thus, the position of the first FDC may be subject to additional regulation, which is not included in the present model. However, the results of the model have been demonstrated to be robust against the separation of HEV and PLF by manually placing some initial FDC away from the blood vessels. Thus, the size regulation of the follicle does not critically depend on the exact HEV location or dynamics.

A novel mechanism of negative regulation of FDC generation

The instabilities created by the LE dynamics as well as the induced unlimited PLF growth require a mechanism that negatively regulates the induction of FDCs. Even though negative regulation may be realized by many different mechanisms, from the simulation point of view, only the source and the propagation of the corresponding signal are relevant. Either a signal has to be reduced or to be produced when FDCs are generated. In both cases, the signal overcomes a threshold at the border of the PLF. The signal is required to be diffusive in order to act on the length scale of a PLF and can be shown to require a rather fast decay in order to establish a stable follicle size (corresponding equations are in the supplementary material, Text-S2 available at International Immunology Online). Within the framework of the simulation, it is suggested that such an inhibiting signal is produced when FDCs are generated. This inhibiting signal may be secreted either by B cells located in the PLF or by FDCs.

The regulation of the follicle size under the influence of the dynamics of efferent lymphatic vessels implies that the B cell follicle consists of two zones. One zone contains B cells and the FDC network and the other one contains B cells and LE. Note that the FDC network is defined by the CXCL13 distribution. Thus, the ring devoid of FDCs may be even larger than suggested by the simulation results. From the corrosion casts, this organization cannot be determined (21) but studies with FDC markers (8, 11, 45, 65) seem to support this modeling result. The visibility of the two zones depends on the markers used to detect the FDCs. In the real follicle, the two zones will correspond to a gradual decrease of ‘FDC-ness’ towards the border of the follicle.

A theory of PLF formation and maintenance

The simulation of PLF formation and maintenance gives rise to the following vantage point: FDCs, in one way or another, replace FRCs. This process is promoted by LTα1β2, which is provided by B cells (or lymphoid tissue inducer cells, see below). The initiation of PLF formation is related to small aggregates of B cells and does not rely on the positive feedback loop between CXCL13 and LTα1β2 (8). B cells in a follicle have to desensitize their chemotactic responsiveness to T zone-derived chemokines (CCL19, CCL21) in order to establish a realistic flow of B cells through the follicle and to avoid follicle instabilities. The model predicts that a factor negatively regulating FDC generation determines the size of a PLF. This factor stabilizes PLF shape and size. It is most likely an inhibitory diffusing signal secreted from either FDCs or B cells (or lymphoid tissue inducer cells) residing in the PLF.

The current model fits best to reconstitution experiments in which LTα1β2-deficient mice are reconstituted with wild-type bone marrow that induce the generation of PLF (4, 5). The formation of FDC networks in wild-type animals occurs before a significant presence of B cells (3). Lymphoid tissue inducer cells are CXCL13 responsive and express LTα1β2. These cells are known to generate the typical PLF pattern during LN genesis prior to B cell immigration (66). The model does not resolve the difference between lymphoid tissue inducer cells and B cells. The latter take over the role of lymphoid tissue inducer cells in the presented simulations.

A restriction of the presented model is that other desensitization mechanisms for the chemotactic response of B cells are not considered due to a lack of corresponding data.
Different regulatory pathways like the transcription of the CXCR5 receptor may modify the PLF formation theory. The model for dynamic lymphatic pathways is very simple and only phenomenological. A detailed modeling of the lymphangiogenesis during PLF formation is left for future research, especially the use of a real vessel structure instead of spherical representatives. The dynamics of the adjacent T zone may influence PLF formation, although preliminary results seem not to change the conclusion drawn from the presented results. In addition, the dynamics of the HEV during PLF formation may be considered (Additional discussion speculating on the molecular pathways involved in the proposed regulatory mechanisms can be found in the supplementary material Text-S3 available at International Immunology Online).

Supplementary data
Supplementary Text-S1 Text-S2 and Text-S3 are available at International Immunology Online.

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>FDC</td>
<td>follicular dendritic cell</td>
</tr>
<tr>
<td>FRC</td>
<td>fibroblastic reticular cell</td>
</tr>
<tr>
<td>HEV</td>
<td>high endothelial venule</td>
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<tr>
<td>LE</td>
<td>lymphatic endothelium</td>
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<tr>
<td>LN</td>
<td>lymph node</td>
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<tr>
<td>LT</td>
<td>lymphotxin</td>
</tr>
<tr>
<td>PLF</td>
<td>primary lymphoid follicle</td>
</tr>
<tr>
<td>S1P</td>
<td>sphingosine-1-phosphate</td>
</tr>
<tr>
<td>S1P1</td>
<td>S1P receptor 1</td>
</tr>
<tr>
<td>SLT</td>
<td>secondary lymphoid tissue</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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References


