INVITED PAPER

The Lymph Node B Cell Immune Response: Dynamic Analysis *In-Silico*

A computer-based model helps researchers to study the ways the human body responds to the antigens that stimulate immune responses.

By Naamah Swerdlin, Irun R. Cohen, and David Harel

ABSTRACT | Lymph nodes are organs in which lymphocytes respond to antigens to generate, among other cell types, plasma cells that secrete specific antibodies and memory lymphocytes for enhanced future responses to the antigen. To achieve these ends, the lymph node (LN) has to orchestrate the meeting and interactions between the antigen and various cell types including the rare clones of B cells and T cells bearing receptors for the antigen. The process is dynamic in essence and involves chemotaxis of responding cells through various anatomical compartments of the LN and selective cell differentiation, proliferation and programmed death. Understanding the LN requires a dynamic integration of the mass of data generated by extensive experimentation. Here, we present a fully executable, bottom-up computerized model of the LN using the visual language of Statecharts and the technology of reactive animation (RA) to create a dynamic front-end. We studied the effects of amount of antigen and LN size on the emergent properties of lymphocyte dynamics, differentiation and anatomic localization. The dynamic organization of the LN visualized by RA sheds new light on how the immune system transforms antigen stimulation into a highly sensitive, yet buffered response.

KEYWORDS | Bottom-up modeling; emergent properties; lymph node; reactive animation; reactive system

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I. INTRODUCTION

The primary production of IgG antibodies to a specific antigen by the immune system is a complex process. Three different types of cells must meet and interact with an immunogenic antigen: B cells, T cells and antigen-presenting cells (APC). The interacting B cells and T cells both have to express antigen receptors specific to epitopes of the immunogenic antigen. The APC take up and process the immunogenic molecule and present epitopes to the specific responding T cells, which must provide signals essential for B-cell differentiation [56]. The responding antigen-specific B-cell clones must proliferate to expand their numbers and differentiate into plasma cells that secrete the antibodies [45] and into memory B cells that endow the system with persisting memory of the response to that antigen [44].

How does the target antigen meet with the three different types of cells in a productive interaction? Note that the frequency of naïve lymphocytes that bear receptors specific for any given antigen is estimated to be between 1 in 10 000 and 1 in 1 000 000; thus, a chance meeting between a T cell and a B cell that recognize the same antigen should occur once in 10^8 to 10^{12} random encounters [42]. In response to the numbers problem, the adaptive immune system has evolved several ways to organize the flow of cells and antigens in time and space so that the production of specific antibodies and the generation of immune memory can take place. One such organizational solution is the lymph node (LN).

The LN combines a unique anatomical arrangement of functional elements with intersecting flows of blood and lymph [9]. The LN acts as a hub in a dynamic network that brings together all the components necessary for an antibody response—B cells, T cells, and APC; the latter include mobile APC that bring antigens to the LN from the periphery

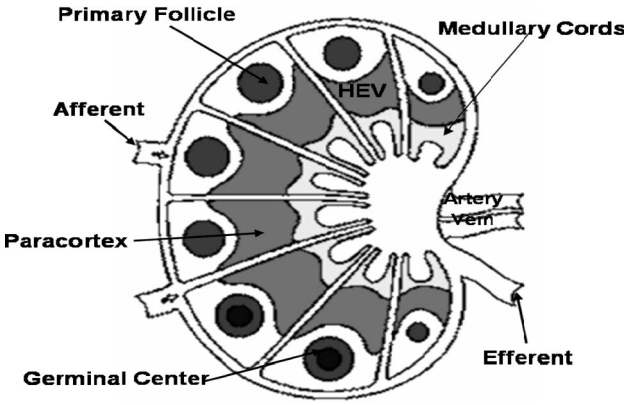

Fig. 1. *Scheme of the lymph node, illustrating its seven major regions. Afferent lymphatics: drain lymph fluid from tissues, including antigen presenting cells (APC) and antigen from infected sites to the lymph node (LN). HEV (high endothelial venules): the capillary walls where T and B cells enter the LN from the blood. Paracortex: the T cell zone. Primary Follicles (PF): where B cells are localized, includes Follicular Dendritic Cells (FDC's). Germinal Center (GC): is formed when activated B cells proliferate in the PF. Medullary Cords: where plasma cells secrete Antibodies. Efferent lymphatics: the only exit from the LN, where activated or recirculating T and B cells, as well as antibodies (Ab's) leave the LN and join the blood circulation.*

and stationary APC residing in the LN, the Follicular Dendritic Cells (FDC), that can trap and hold antigens. Fig. 1 depicts the anatomical structure of a LN. Briefly, the LN functions like this: Antigen molecules from the tissues are carried within mobile macrophages and dendritic cells to the nearest LN via the afferent lymph, while circulating B cells and T cells flow to the LN from the blood via the High Endothelial Venules (HEV). The T cells and B cells that do not encounter specific antigen in the LN return to the circulation to continue the search. An immune reaction, however, is initiated when the errant T cells and B cells do meet with a recognizable antigen in the LN. The T cells are activated by the antigen-bearing APC, and proliferate and differentiate into helper cells that are now ready to interact by way of secreted cytokines with the adjacent B cells that have also recognized the antigen. The reacting B and T cells meet within the Paracortex area of the LN, where they form what is termed a primary focus [18], in which both cells divide for a few days. The lymphocytes in the primary focus then begin dying, but some B cells differentiate into Plasma cells and migrate to the Medullary Cords to secrete antibodies that recognize the antigen [6]; other B cells migrate to the primary follicles (PF) and proliferate to form a germinal center (GC). Within the GC, the B cells undergo somatic hyper-mutation and a selection process [1], [22], [25] that results in an increased affinity of their antigen receptors and secreted antibodies for the antigen. Some of the GC B cells also differentiate into memory cells necessary for an enhanced secondary response upon future contact with the same antigen. The signaling process is complex; it involves direct cell-to-cell communication by physical interaction, indirect cell-to-cell communication mediated by cytokines, and migrations of cells to their appropriate regions mediated by chemokines [6].

Tens of thousands of papers have been published on the LN, each focusing on a particular topic; experimental biologists have studied the specific anatomy of the LN [3], the interaction between immune cells [36], chemokines involved in the process [27], cell kinetics [37], [38] and so on. This mass of data raises the problem of information overload that needs to be integrated; indeed, there is a striking lack of research that investigates the system as a whole.

One way to integrate enormous amounts of data is through mathematical modeling [4]. In the past, such studies have generally dealt with particular parts of the LN, such as the Germinal Center (GC) [24], [32] or the primary focus [34], or with certain aspects of the system, such as the kinetics of the immune cells [23] or the probability of specific differentiation processes [31].

Our goal was to build a dynamic interactive model of the LN that captures many of its various components to create a broad overview of the system working dynamically as a whole. The approach we have taken, as opposed to traditional analysis, is a bottom-up synthesis, starting with the fundamental building blocks of biological data, and bringing them together to construct a realistic and comprehensive model of the system [13]. An example of the difference between these two approaches has been examined in Kam *et al.* [21].

Representing biology dynamically is essential to achieve an understanding of realistic dynamic behavior, and computerized models such as ours not only make this possible but are also most suitable in providing the right environment to analyze the data visually and quantitatively.

In the present work, we use an object-oriented approach based on the Statecharts modeling language, which was invented for the behavioral specification of man-made reactive systems [11], [15]. Biological systems, just like many engineered systems, are complex reactive systems—interacting and responding to the environment and to other components of the system [12], [16]. Modeling reactive biological systems with the aid of Statecharts has already been proven feasible: Kam *et al.* [20] have modeled T-cell activation and Efroni *et al.* [7] have modeled T-cell development in the thymus. Here we expand the applicability of such techniques, by showing how one can model the spatial and the temporal behavior of the LN, and especially the behavior of B cells within it. States and transitions were used to simulate the LN regions and immune cell behavior—interactions, movement, receptors, signals, proliferation, differentiation, and function. In addition, we have used the reactive animation (RA) technique [8], [14], combining the Statecharts model with a Flash animation to help visualize, and interact with, the executions of our LN model by dynamically animating

the regions, the cells, their receptors, and their interactions. The animation is driven by the Rhapsody simulation in real time.

In this way, the model yielded what have been termed *emergent properties*, which arise from the bottom-up dynamic integration of many basic entities. Emergent properties, in other words, refer to higher-level behaviors generated by the simulation, and which were not explicitly programmed into the underlying data used to perform that simulation.

This approach enables us not only to see the action but also to experiment with its features *in silico*. Here, we report the dynamic cellular composition of the various anatomic compartments of a LN that hosts a cohort of reacting B cells as they respond to chemokine signals and productive interactions with T cells, antigen and FDC to generate antibody producing plasma cells and memory B cells. We studied the effects on the B cell cohort and its resulting plasma cells and memory cells of various time constants, the amount of antigen, the size of the LN, and other parameters. In this introductory study, we did not model the activation of specific T cells by antigen-bearing APC or how the LN deals with many antigens simultaneously. Nevertheless, this paper discloses a novel view of the dynamic structure and function of the lymph node that emerges bottom up from the experimental database.

II. MODEL DEVELOPMENT

The model focuses on a single, two-dimensional LN from the time of the initial entry of a subset of immune cells together with an immunogenic antigen. B cells, T cells, and FDC were modeled with regard to their behavior, movement, location, interactions, and expression of receptors and signals. Emphasis was put on B-cell behavior. A prototype of each of the cells and signal molecules was created and, during the simulation, multiple instances of the various cells and molecules were generated to represent the explicit state in time, place, differentiation and behavior of each cell. The simulation was then connected to an animation tool that enabled visualization of the on-going events of the simulation in real time and experimentation *in silico*.

The parameters used in the modeling, such as the probabilities of differentiation, receptor expression, cell location, time of interaction and so forth, were determined either directly or indirectly from experimental and theoretical studies using logic-based assumptions from analysis of published data. (A detailed description of many of the parameters used and the way they were chosen can be found in the supplementary material).

A. Describing Dynamic Behavior Using Statecharts and Rhapsody

The objects modeled in the simulation include immune cells—B, T, and FDC—, their receptors, the immunogenic antigen, chemokine signals, the anatomical regions of the LN, and others objects. A summary of this process, which was used to simplify the complexity of the biology and to aid in modeling, is shown in Fig. 2. The behavior of each of the objects was described using Statecharts. Statecharts is a modeling language designed as a system engineering tool to aid in the organization and study of complex man-made reactive systems [11]. Statecharts enables the visual specification of dynamic reactive behavior via the use of intuitive, yet mathematically rigorous, diagrams, and is based on describing discrete behavior using hierarchical structures of states and the events that cause transitions between states. The object-oriented version of the language [15] is based on an intra-object philosophy, using a Statechart to supply the full description of the internal behavior of each of the participating objects. The Rhapsody tool [15], from I-Logix, provides a working environment supporting object-oriented system development, with the Statecharts language at its heart. Rhapsody makes it possible to fashion the structure of the system using object model diagrams (OMD's), which present all the objects that participate in the system and the relationships between them; each object is supplied with its Statechart. Rhapsody is capable of automatically translating Statecharts into executable machine code (Java/C/C++), and can then generate, compile, and implement the application (for more information on the language of Statecharts and the Rhapsody tool see supplementary material).

Working with Rhapsody, we used Statecharts to describe various LN events, such as whether a B cell has recognized a specific antigen, which was determined probabilistically using biological data, whether the B cell is re-circulating or whether it has migrated to a different LN region, and so forth. (A detailed description of the Statechart of each of the participating players can be found in the supplementary material). The Statecharts language with the Rhapsody tool is a suitable modeling approach that makes it possible to build interactive models; elements are easily changeable and the emerging outcomes are revealed without the need to have prior knowledge for what it is we are in search of.

B. A Representative Example of the Statechart Modeling

When the model is executed, instances of the relevant objects are created. As the simulation advances, each of the participating objects respond to various events (such as cells or signals in their vicinity) by changing their states accordingly. To illustrate the simulation in progress, we describe here the process of the formation and behavior of the Primary Focus. Similarly detailed descriptions of other parts of the model can be found in the supplementary material.

A Primary Focus is a clonal expansion of both B and T lymphocytes, formed upon their interaction [18]. Together, they proliferate for a few days, producing over a

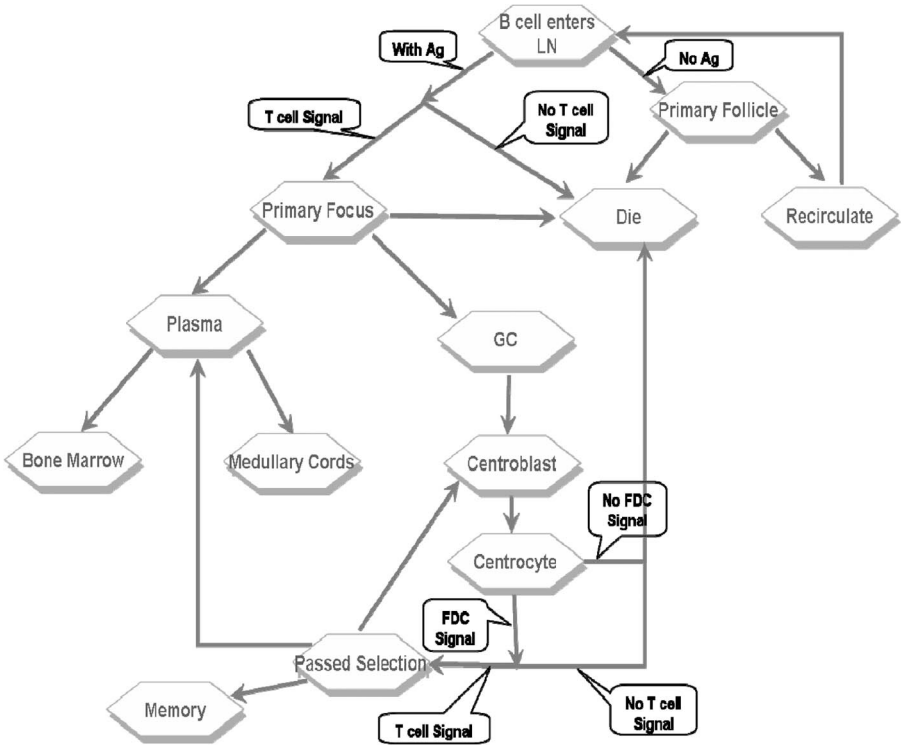

Fig. 2. ***Static flow chart of the biology illustrating the chain of events. This is a schematic drawing that was used to simplify the complexity of the biology in order to enable its dynamic modeling. Ag = antigen.***

thousand activated cells. This is in accordance with the known data, considering that cells divide at a rate of one division about every eight hours [4]. Following this process, many of the lymphocytes die. However, some of the B cells differentiate into plasma cells and migrate to the Medullary Cords where they secrete antibodies (Ab's) that leave the LN via the blood stream. Another possible fate for these B cells is to migrate to the Primary Follicles (PF), where they continue to proliferate and eventually form a GC [26], [44]. In our model this was implemented by giving B cells that come out of the primary focus a probability of 0.4 to die, 0.4 to become Plasma cells [34], and 0.2 to move to the PF to form a GC.

B cells that are destined to progress to the PF express the appropriate chemokine receptors and follow the signals to the PF region. Only after arriving at the PF and receiving the `evGC` message from the `Region` class, do they undergo the transition to the next state—`ImmuneResponse`. This state consists of two additional levels (Fig. 3); the first contains the statechart of the Centroblasts and the Centrocytes, as well as the transition between them, whereas the next level contains the subchart of each of these types of cells, describing the behavior and processes that occur within them. The initial state of B cells within a GC is a Centroblast. Centroblasts are rapidly dividing B cells (every 6 hours, [44], [55]), situated in the dark zone (DZ) of the GC, and with a reduced expression of the BCR. Their purpose is to proliferate and undergo somatic hypermutation, creating as many as a few thousand Centroblasts, each with a new, mutated BCR. The mutated B cells compete for recognizing the antigen, and the B cells with increased affinity win out and proliferate. This proliferation of mutated B cells with higher affinity for the antigen leads to an increase in the affinity of the responding plasma cells and the resulting antibodies for the antigen, making the immune response more specific. Many of the BCR mutations result in a non-functional BCR that will eventually lead to cell death. In the simulation, this was achieved by giving the Centroblasts a probability of 0.25 to die due to lack of functionality. (The extended version of this use case can be found in the supplementary material).

C. Reactive Animation (RA) Using Flash

A connection was formed between the Rhapsody simulation and a Flash animation (available from Macromedia) to create an interactive graphic-user interface to produce dynamic animations. This was done using RA, a basis for generic communication between standard reactive specification tools and standard animation tools [8], [14] through TCP/IP channels. Messages are sent from the Rhapsody to the Flash via XML objects, which are parsed by the Flash into commands that implement pre-recorded movie clips. Communication between Rhapsody and Flash

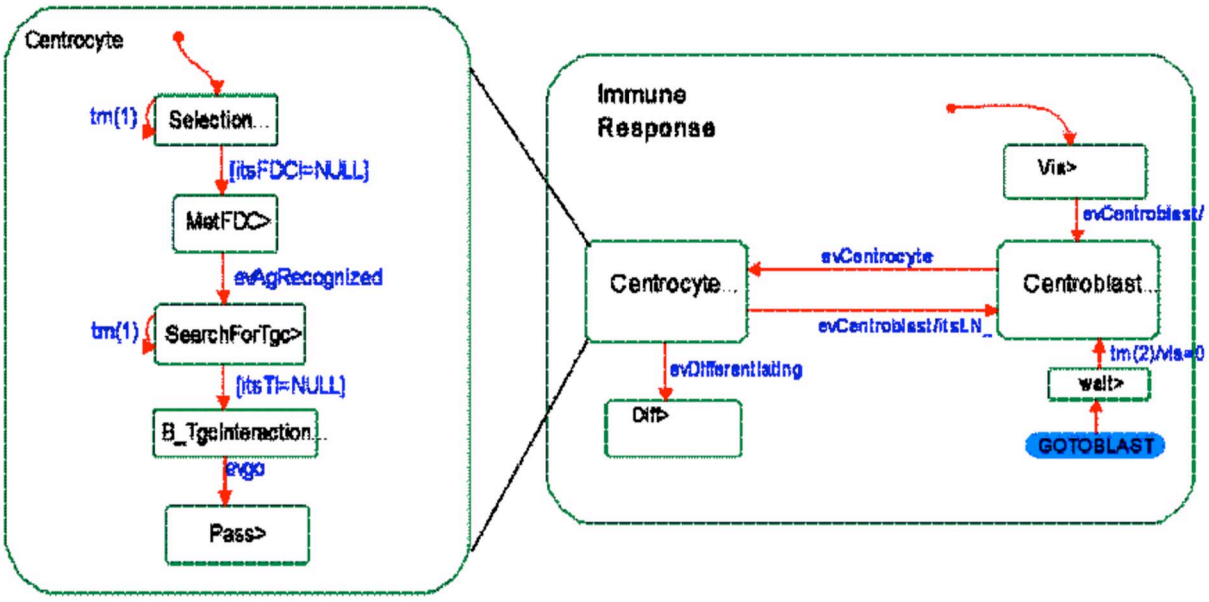

Fig. 3. *Statechart of a B-cell, the Immune Response subchart and the centrocyte substatechart.*

is carried out through a socket and occurs continuously during run time.

Several Flash movie clip motifs were constructed to represent the objects and actions that take place during the Rhapsody simulation. In this way, a moving animation is dynamically constructed in real time, producing an interactive visualization of the actual events generated by the simulation. In other words, the running Statecharts simulation itself creates the observed animation on the fly. In this way, the animation dynamically expresses aspects of the LN and the behavior of the immune cells within it, each region of the LN represented as a matrix, which includes the interacting immune cells and their receptors (Figs. 4 and 5). Cell behaviors include cell movement, cell proliferation, cell death, B-cell differentiation into Plasma or Memory cells, and antibody (Ab) secretion. The Flash animation component of the model made it possible to visualize the integrated immune response of all the different cell and molecular components described in the model bottom-up from experimental data. The fidelity of the animation to the real LN also served to validate the simulation, making sure that no non-physiological or "forbidden" actions took place. During the animation, the cells can be seen moving between regions and within regions, changing their receptors, proliferating, differentiating or dying (Figs. 4, 5 and Movie1 of supplementary material). The anatomical paths of individual cells could be tracked.

To obtain quantitative and qualitative data regarding the many elements of the model, information from the simulation was extracted and presented in graphic form. To test the roles of various parameters, input parameters were changed and the resulting output was observed and compared to standard simulation runs. Examples of modified input parameters are shown in Table 1; these

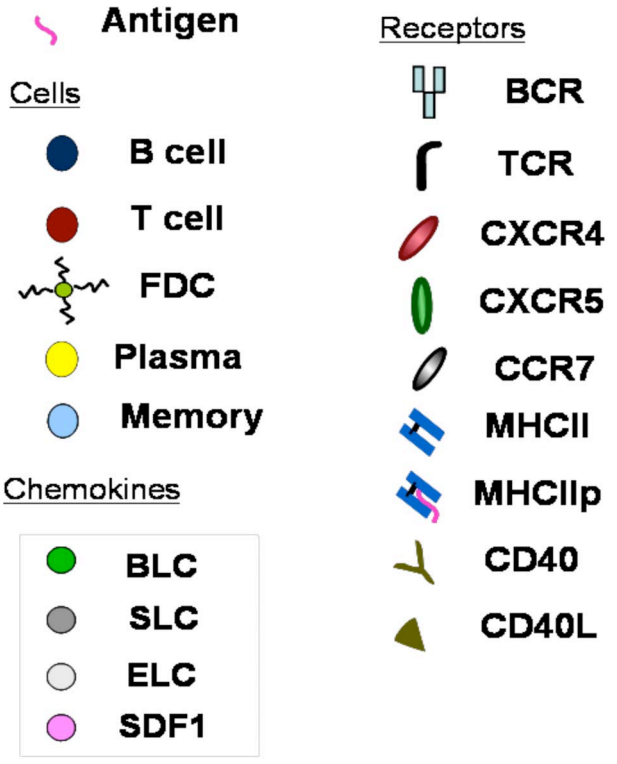

Fig. 4. *Legend for the Flash animation graphics.*

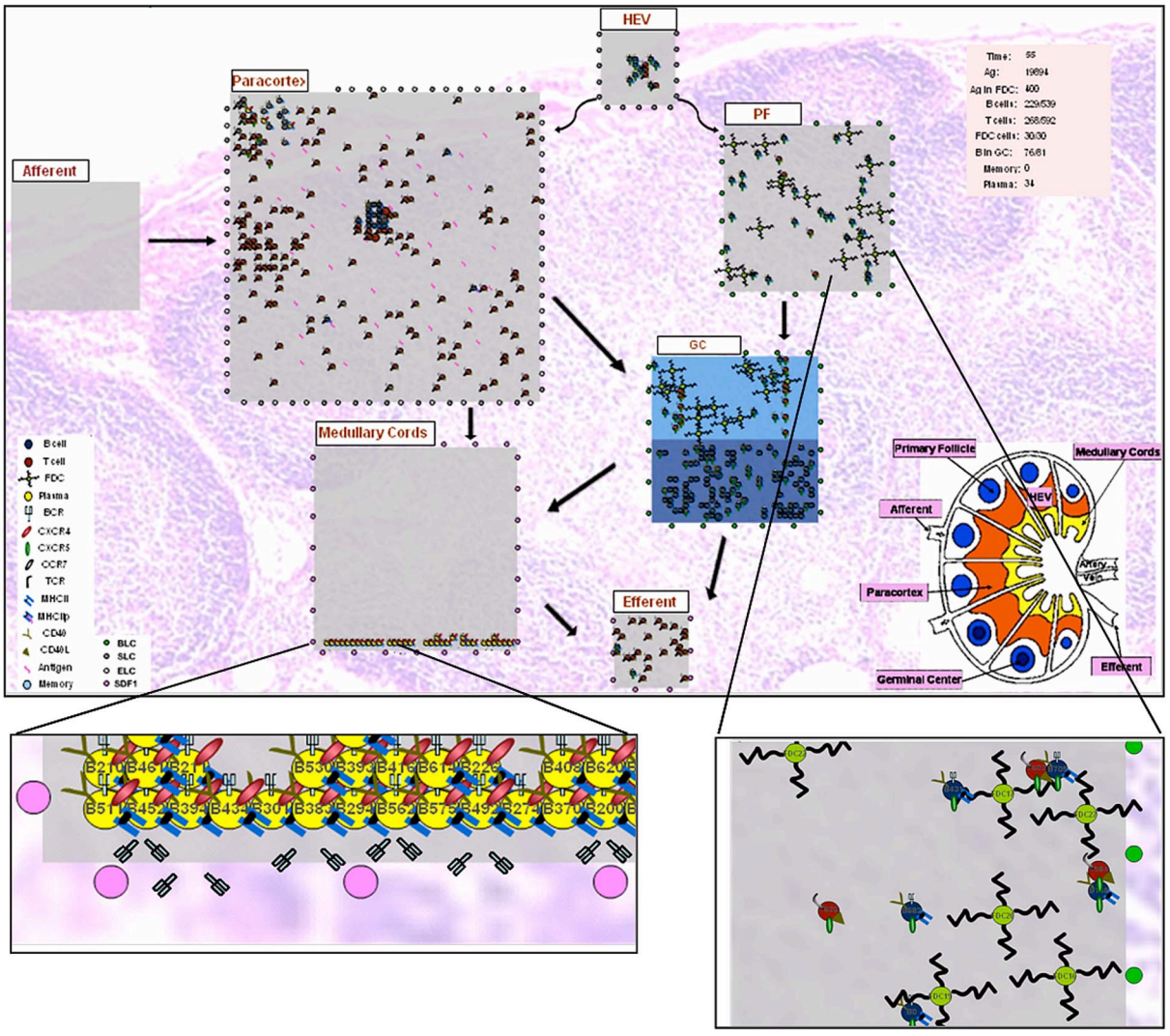

Fig. 5. ***The Flash animation interface during an execution. Each of the seven major regions of the lymph node (LN) is represented in a matrix of relative size. A legend of the different Flash objects is indicated on the bottom left, (and can be seen enlarged in Fig. 4) and a status box is situated on the top right corner, indicating, at every time point, the relevant information. Enlarged are two areas showing the specific cells and their receptors; on the right is the Primary Follicles (PF) region, which includes the B cells, Germinal Center T cells (Tgc), and Follicular Dendritic Cells (FDC's); on the left is the Medullary Cords region with the Plasma cells secreting antibodies (Ab's). This visually represents the 2-dimensional anatomy of the LN which is emerged from the cells' niches, flow, and interactions, that were later analyzed quantitatively.***

include changing the amount of initial antigen or the size of the LN, and observing the resulting effect on the LN output of B cells, Plasma cells, or Memory cells.

A great deal of data was extracted from many runs of the model; indeed, the amount of data that can be retrieved is almost unlimited. In this paper, we focused on two outcomes: an animated visualization of the running LN simulation and a statistical analysis of population dynamics. We were able to gain a clear view of the dynamics of cell localization within the LN at different time points, depending on the biological state of the cells. Cells were observed, both individually, in Rhapsody, and as populations, in Flash.

III. RESULTS

A. A Comprehensive Approach to Simulation

The approach used in this research is fundamentally different from more common approaches used in biological modeling, where a specific aspect of the system is under investigation. Here, the model was constructed from the basic building blocks of biological information and allowed to assemble on its own, in the hope of achieving a more comprehensive biological representation [5], [13]. No additional constraints were forced upon it.

This is really a bottom-up approach, which calls for beginning with small and specific details and working up

Table 1 Input Parameters

Input Parameter	Value
Initial B cells	1000 cells
Initial T cells	2000 cells
B cell specificity	1/500 cells
T cell specificity	1/1000 cells
Initial antigen amount (Ag)	S RU
Increased antigen amount (Ag>)	10S RU
Decreased antigen amount (Ag<)	S/10 RU
LN size	20000 positions
LN size>	10000 positions
LN size<	30000 positions

RU—Relative Units

towards a higher level. This is in contrast to more widely used top-down approaches, which often start with the necessary knowledge and overall description of the outcomes. The difference between these two approaches can be viewed as the analysis of some functional aspect of the whole (top-down) as opposed to a synthesis model that puts different parts together to yield the function (bottom-up). Both methods have their advantages and the ability to make certain kinds of predictions [10]. Nevertheless, our kind of modeling can also benefit from top-down analysis, since, once the different parts have been put together, it is possible to search for mechanistic gaps. This comes from the need to fine-tune the mechanism of the model in order to observe valid outcomes, and so can possibly reveal the need for assumptions that were not previously considered.

Using Statecharts in Rhapsody, a vast amount of biological data was combined into a single comprehensive model of the LN. Data were collected from various sources of biological literature, were specified into a model, and could then be executed interactively. A visual representation of the system and its behavior was then incorporated, by combining the Rhapsody model with a front end animation in Flash, leading to an overall emergent picture that is both easy to comprehend and mathematically rigorous. It also enables the user to easily manipulate data and observe resulting output. Modeling with the aforementioned tools provides the opportunity of relatively easily adding components, changing the behavior of objects, or adding further levels of complexity.

The different input parameters we used to study LN dynamics are shown in Table 1. We started the simulation with the entry into the LN of 1000 naïve B cells and 2000 naïve T cells; the frequency of antigen-specific B cells was set at 1/500 and the frequency of antigen-specific T cells at 1/1000; each cohort thus contained about 2 antigen-specific cells of each type. The time units shown in the following figs are arbitrary, but express the relative durations over which the modeled events take place. We studied the effects of modifying the amounts of antigen entering the LN using an arbitrary starting amount of S relative antigen amount units that was raised or lowered by factors of 10 to 10S or S/10 relative antigen amount units. The effects of modifying the size of the LN were also studied beginning at an arbitrary position size for 20 000 cells, and raised or lowered to 30 000 or 10 000 positions. We tested the effects of these parameters on the numbers of B cells and T cells moving through the various zones of the LN and the output of antibody-producing plasma cells and B memory cells. Note that the simulation *in silico* describes a two-dimensional slice through a three-dimensional organ *in mundo*. Thus, the numbers of cells and the positions available for them in a real LN are considerably greater; the thousands of cells we model in two dimensions probably reflect millions of cells in three dimensions.

B. B-Cell and T-Cell Population Dynamics

Lymphocyte population distribution is frequently researched as it conveys the behavior of the cells in response to antigen. In theoretical studies, this can also serve as an indication of the fidelity of the model, as it can be compared to known experimental data or other theoretical studies.

Fig. 6 illustrates the dynamics of different sub-populations of B and T cells developing in response to antigen in the LN as a function of time. The upper panel shows the transactions of a cohort of 1000 B cells that enter the LN at time 0. Only two of the entering B cells are likely to bear receptors capable of recognizing the antigen; the rest of the B cells die or leave the LN. The few antigen-specific B cells become activated by the antigen and, with the help of activated T cells, proliferate to reach a peak at 400 relative time units. The progeny of the activated B cells develop into Centroblasts and Centrocytes in the GC. The B cells in the GC reach a peak of around 1500 cells and then arrive at a plateau before decreasing sharply at about 800 relative time units.

Of the cohort of 2000 T cells entering the LN (Fig. 6; lower panel), the few reacting T cells too generate a peak of activated T cells that give rise to GC T cells, T helper cells that activate antigen specific B cells, and T effector cells that leave the LN. Note that the rise of activated T cells precedes slightly the rise of activated B cells; this is reasonable because the B cells need T-cell help for their full activation.

C. Behavior of the Model is in Agreement With Experimental Studies

Analysis of the data retrieved from the simulation revealed a close correlation with both experimental and theoretical studies. The sub-population of B cells in the GC is in agreement with models of the GC that were carried out using different methods; In [17], [32], [33], and [35] a mathematical study of the GC was carried out, the results of which revealed a close similarity to our results both qualitatively and quantitatively. The amount of cells

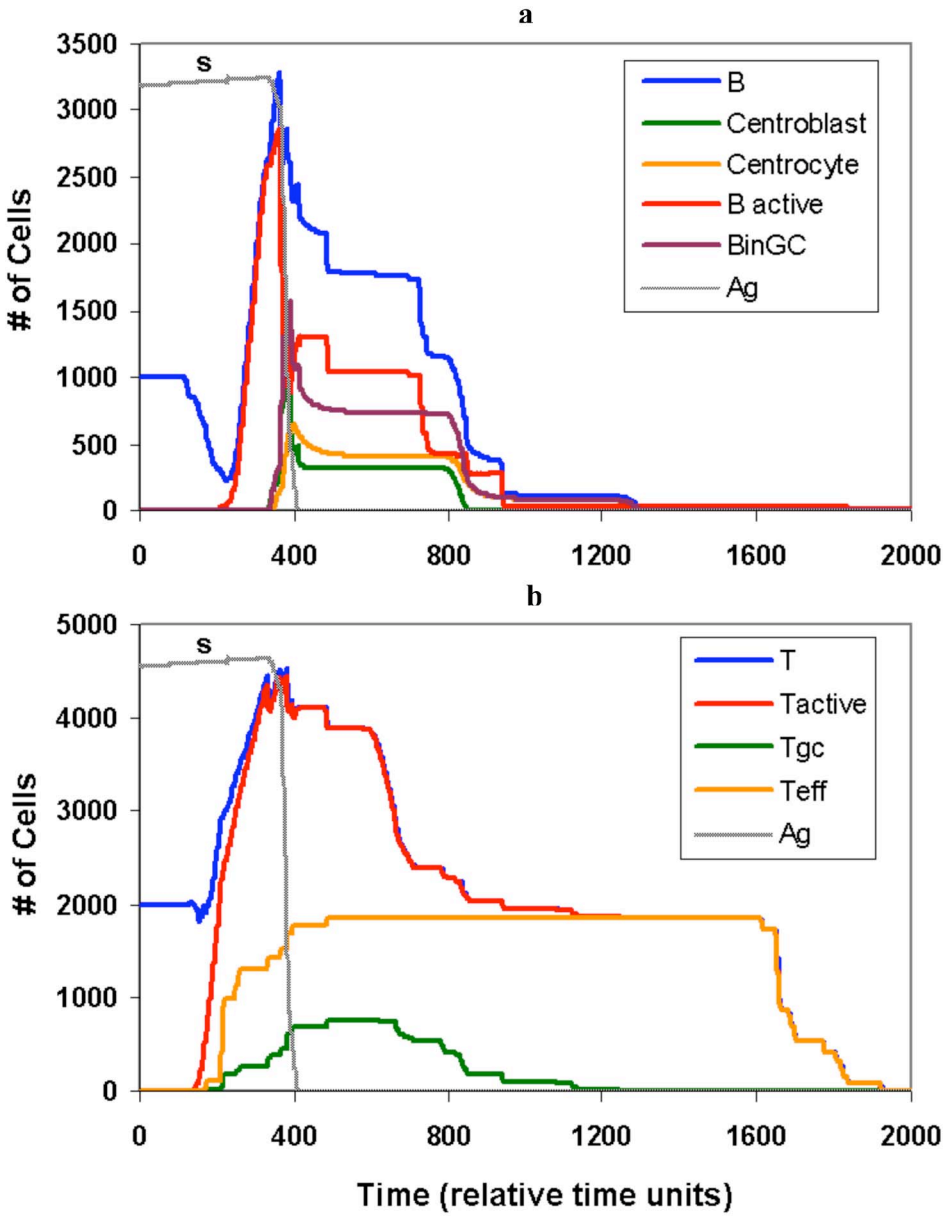

Fig. 6. *B & T cell population vs. Antigen as a function of time. s = standard antigen amount. (a) B cell and its sub-populations: Bactive, Centroblasts, and Centrocytes. (b) T cells and its sub-populations: Tactive (all active cells participating in the response) Tgc (Germinal Center T cells), and Teff (T effector cell).*

produced by the primary focus (active B cells) can also be compared to experimental data in [19] that showed that the primary focus generates up to about 2000 cells. In addition, T cell dynamics produced by the model resemble those attained by a different mathematical model [30]. The distribution of the different sub populations of B cells resembles the behavior of previous experiments, such as in [29] who claim that the Centrocytes outnumber the Centroblasts, as well as of other theoretical studies, such as in [2] where a three-dimensional model was built and qualitatively; the number of cells produced in the GC and its separate compartments are similar to those found in our model. As can be noted from our results, the incidents that take place also coincide with those found in published data; Following antigen stimulation, active B cells are the first to rise to a peak reaching an order of magnitude of thousands of cells, and producing Plasma cells within a few days from the creation of the primary focus [18]. The GC then begins, and at about day 7 it reaches a peak with thousands of cells [18], [25]. The GC finally disappears at around day 21 [18], [26] when all its B cells have either died or left the GC as Memory or Plasma cells.

D. Dynamics of Lymphocyte Occupancy in Different LN Regions

Fig. 7 shows the simulated dynamics of lymphocyte movement through the compartments of the LN as the cohorts of B cells and T cells interact, proliferate, differentiate and migrate. The lower panel of Fig. 7 shows the relative numbers of cells moving through the various compartments and the upper panel shows their occupancy as percent (%) of the cells in each compartment over time; the numbers of cells disclose dynamic changes in the size of the cell population in each compartment, and the % occupancy shows relative contribution of the cohort of cells to each compartment. The cohort of cells enters the LN by way of the HEV and the few antigen-specific reacting cells proliferate greatly in the paracortex and to a much lesser degree in the PF (see lower panel). Most of the cells that have proliferated in the paracortex die there, and a relative few of the reacting cells then move transiently through the DZ and then to the LZ of the GC, where they persist for a more extended time. This transition is probably due to the proliferation of Centroblasts, which then turn into Centrocytes and leave the DZ for the LZ [28].

The dynamics of % occupancy show us that the antigen-specific reacting cells occupy most of the paracortex and the DZ for a relatively short time peak; the LZ of the GC contains a significant number of reacting cells for a much longer time. Thus we can see that various LN compartments are fashioned by the dynamics of the immune response. The simulation brings together the spatial and temporal dynamics of the system showing how each cell, according to its specific state, migrates through the different regions of the LN. Note that the simulation follows single cohorts of B cells and T cells as they temporarily occupy greater or lesser amounts of the available space in each LN compartment. The various LN compartments, however, are never empty of cells; they continue to be filled with other cohorts both before and after the dynamic passage of the particular study cohort. Thus the proportion of the antigen specific cells is different at every compartment of the LN—the HEV is almost fully occupied by non-specific cells, whereas the DZ of the GC is transiently, but almost fully occupied by antigen-specific cells as they march through the LN. This information regarding the fine LN anatomy of cell dynamics can be obtained at present only through dynamic simulation.

E. Experimentation *in Silico*

RA allows *in silico* experimentation; one may test the outcome of any thought experiment by observing the effects of the manipulation on the outcome of the animated simulation. Fig. 8, for example, shows the effects on the LN of eliminating a chemokine receptor—CXCR5; the knock-out results in a LN with no migration of cells to the PF region and no GC formation.

F. B Cell and Plasma Cell Populations Respond Differently to Varying Amounts of Antigen

Fig. 9(a) shows the numbers of activated B cells developing in a LN over time in response to three different amounts of antigen: a "standard" amount of S relative antigen amount units (Ag-S); ten-fold less antigen (S/10; Ag <); and ten-fold more antigen than the standard (10S; Ag >). Note that the 10-fold increase in antigen (Ag >) leads to a 6-fold increase in the number of activated B cells in the LN. Surprisingly, the 10-fold decrease in antigen (Ag <) hardly affects the number of activated B cells in the LN compared to the standard amount of antigen (Ag-S). Thus there appears to be non-linear relationship between the amount of antigen and the numbers of developing B cells.

Fig. 9(b) shows the numbers of plasma cells developing from the activated B cells. Plasma cells are differentiated B cells that secrete antibodies, so the relative number of plasma cells correlates with the relative amounts of antibody produced in the responding LN. Note that numbers of B cells and plasma cells differ dynamically in response to varying the amount of antigen. In contrast to the 6-fold increase in B cells to the high amount of antigen, the numbers of plasma cells changed relatively little (from 550 to about 800 cells) over a range of two orders of magnitude in the amount of antigen (from S/10 to 10S units). This conservation of plasma cell number in the face of variable amounts of antigen was not pre-programmed by us; the phenomenon emerged from the simulation itself. One might conclude that the LN operates dynamically as a machine designed to respond well to relatively low amounts of antigen, but with a response that is buffered against a large increase in the amount of antigen in terms of the plasma cells it generates. The LN is sensitive to antigen, but is resistant to over production of plasma cells. The buffering of the response to "over-stimulation" is an emergent property of the simulation that could be tested productively by experimentalists.

Fig. 9(c) shows the numbers of activated B cells developing in a LN over time in response to three different LN size values: a "standard" sized LN that can accommodate 20 000 cells (LN-S); a decreased sized LN with 10 000 available positions (LN-size <); and an increased sized LN with 30 000 available positions (LN-size >). The results stress the importance of the size of the LN; When the LN is too large, there were cases when no response occurred and the antigen was not eliminated. This is due to the fact that the specific activated B and T cells must come into contact with each other in order to become activated; when the LN is too large the cells cannot find each other. When LN size is small, the response occurs more frequently as the specific lymphocytes easily find each other. However, when the size is too small there might not be enough space for proper cell proliferation, consequently resulting in a weaker response (less immune cells). This also illustrates the dependency between the area of interaction of the LN regions and the number of cells.

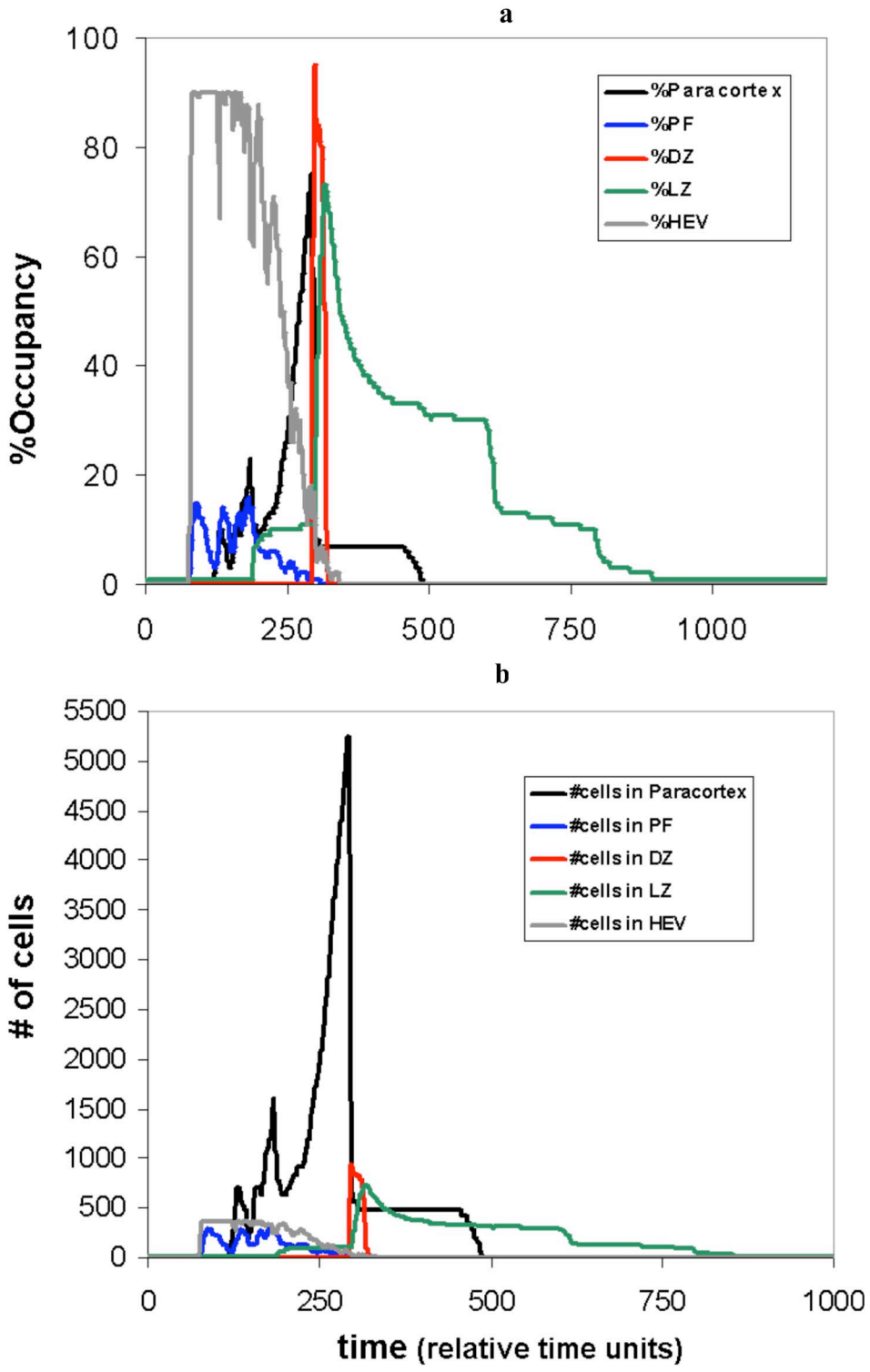

Fig. 7. ***Occupancy in each region. (a) The occupancy percentage of the immune cells of each region is extracted from the simulation at every time point. Shown are these results for the Paracortex, PF (Primary Follicles), DZ (dark zone), LZ (light zone), and HEV (high endothelial venules) taken from a standard run. (b) Number of cells in each region throughout time using the same data as for (a).***

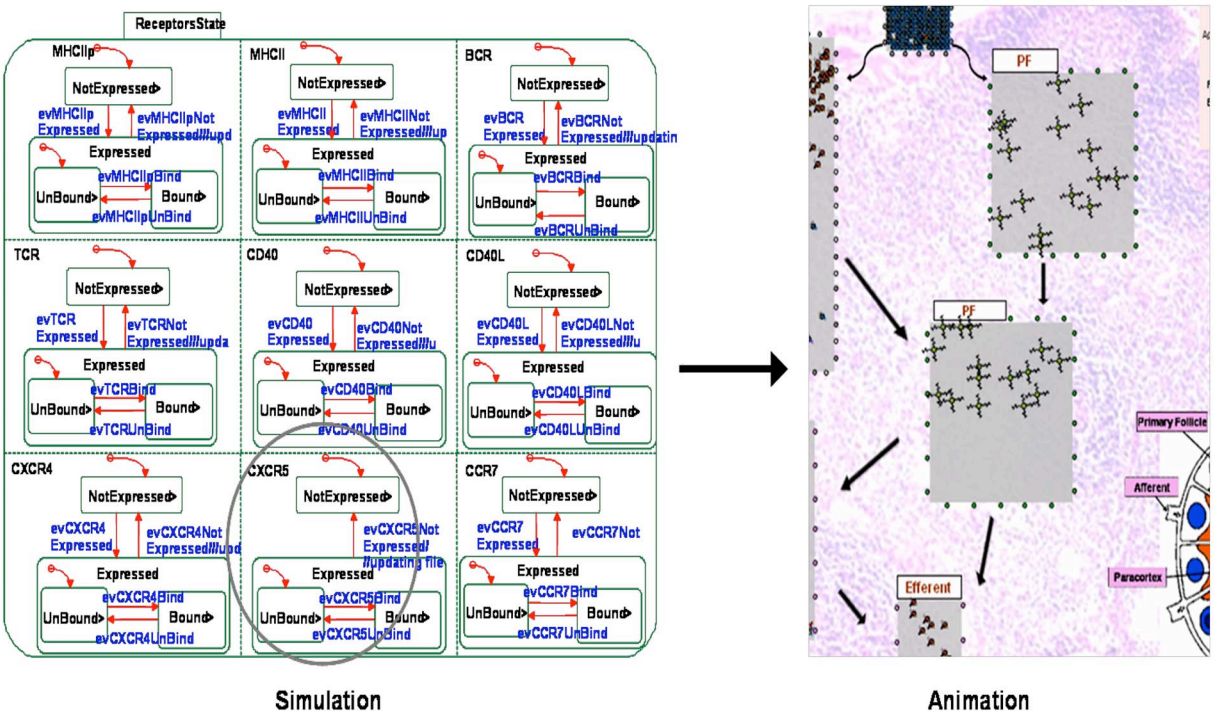

Fig. 8. ***The arrow that allows receptor CXCR5 to be expressed in the simulation (left) has been removed and as a result B and T cells cannot express this receptor. This receptor is necessary for cell migration as it responds to the chemokine signal, BLC, which is secreted from the Primary Follicles (PF) regions and attracts cells to these regions. In the animation snapshot (right) this can be observed by the fact that no B or T cells are present in these areas.***

G. Numbers of Memory Cells Vary With the Amount of Antigen and LN Size

The output of a LN immune response includes memory cells, as well as antibody-producing plasma cells. Memory cells are vital for an enhanced secondary response when the immune system experiences a second contact in the future with the same antigen. Fig. 10 is a representation of the amounts of memory cells that leave the LN as the result of a primary immune response; the effects of modifying the initial amount of antigen and the size of the LN are shown. Under "standard" conditions of S relative antigen amount units of antigen and a LN that can accommodate 20 000 cells, about 8 memory cells are generated from the one or two nave antigen-responsive cells that initially entered the LN. The number of memory cells increases more than 3-fold in response to a 10-fold increase in the initial amount of antigen (10S relative antigen amount units); the numbers of memory cells are much reduced when the amount of antigen is decreased (S/10 relative antigen amount units). Thus it would appear that varying the amount of antigen has a greater effect on the generation of memory cells than it has on the generation of plasma cells (see Fig. 9). This emergent property of the simulation constitutes a testable prediction.

The size of the LN also influences the output of memory cells; increasing the numbers of available positions to 30 000 (a larger LN) reduces the output of memory cells, and decreasing the positions to 10 000 (a smaller LN) increases the output of memory cells. A smaller LN makes it more likely that the few initial antigen-specific B and T cells can find each other, and a larger LN reduces the likelihood of cellular interactions in a given amount of time. These effects of modifying LN size exemplify the kinds of *in silico* experimentation that can be done in this system.

IV. DISCUSSION

The study described here exemplifies how an integrated, dynamic and multi-scale synthesis of a functioning organ can be fashioned informatically from disparate experimental data collected piece-by-piece. This type of bottom-up modeling provides a better understanding of the emerging dynamic events that take place within the LN as a result of the combined behavior of the separate entities. Fig. 11 summarizes the main steps of the study, starting from the biological data through to the visual representation of the LN and its components.

Statechart modeling together with RA embodies two key features: it serves to translate computer simulation into realistic animation and it enables the user to carry out experiments on the fly *in silico* and view the resulting

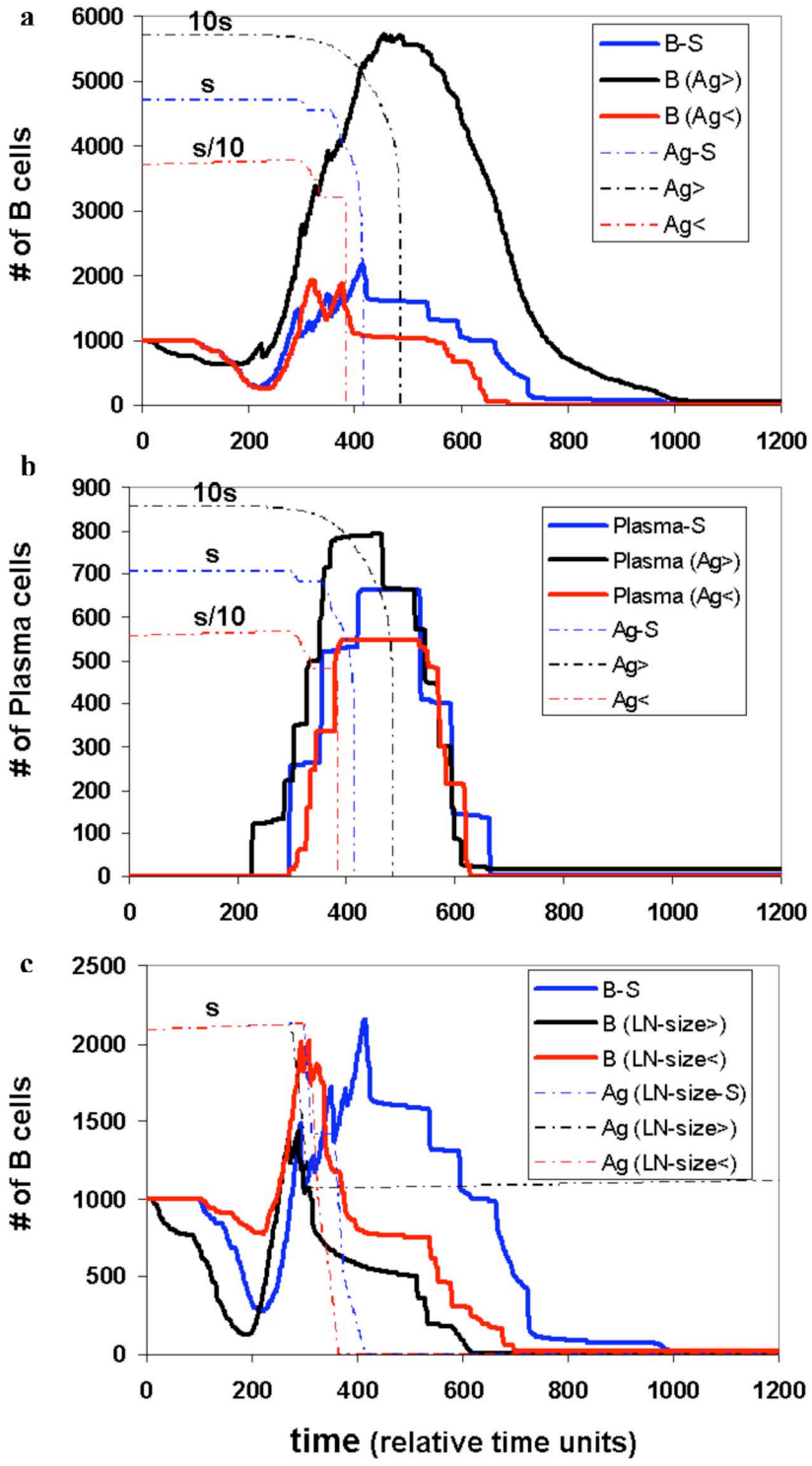

Fig. 9. *B cell vs. antigen as a function of time under different circumstances. Each line in the graphs represents the average of ~3 runs with the same indicated input. Broken lines are the antigen amount (with the relative amount written on top of each line), solid lines are the B cell amount, same color represents same run. S = standard, > = increased amount, < = decreased amount (a) B cell vs. changed antigen. (b) Plasma cells vs. changed antigen. (c) B cell vs. changed lymph node (LN) size.*

outcomes. The value of *in silico* experimentation is obvious; one can observe the effects on the whole system and its component parts of modifying any variable that comes to mind: knocking it in or out, changing its quantity, adjusting interactions and existence in space and time, etc. One can play with the system of interest without restraint.

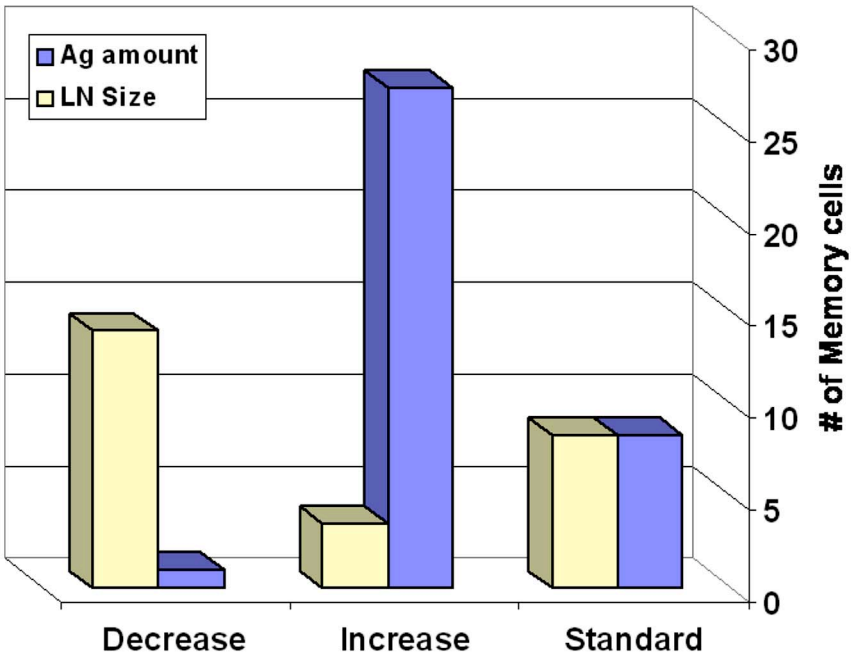

Fig. 10. *Memory cells under different circumstances. The number of memory cells created in each of the indicated runs.*

Of course, the value of unfettered experimentation *in silico* is that it leads to novel experimentation *in mundo*; by using RA, one can get help in deciding just what experiments are likely to be productive. Visualization is a key factor in human understanding; visualization, especially when familiar images appear realistically animated, provides a cue for creative thinking. Indeed, animation can reveal to the observer's eye and mind the emergent properties of a complex system hidden in the static data. Dynamic animation can trigger ideas for new experiments.

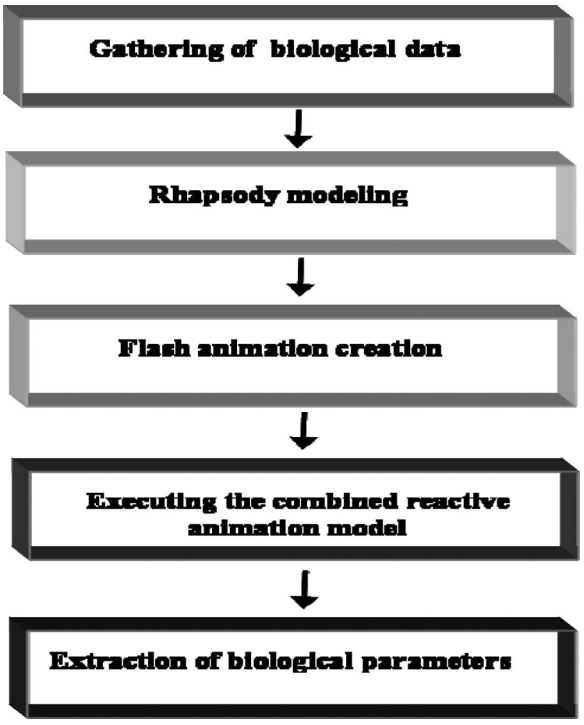

Fig. 11. *Work protocol.*

Here we used the language of Statecharts and RA to study a LN and the lymphocytes within it. We were able to transform static experimental data into dynamical behavior: cell migration through anatomical compartments, cell interactions, cell proliferation and differentiation, receptor expression and responsiveness to signals. Statistical analysis, dynamic reconstruction, *in silico* experimentation, and visual animation allowed us to see the quantitative march through the LN and its subdivisions of a cohort of reacting B cells and T cells. The generation of antibody-producing plasma cells and memory cells and the dynamic occupancy of LN compartments emerged from the study. Evidently, this interactive approach can provide a view of LN physiology that would otherwise be inaccessible to the mind's eye. Most importantly, these emergent properties of the LN were generated bottom-up from the data, rather than top-down from expert opinion. While extending the scope and depth of LN physiology beyond what can be done experimentally [4], the model was validated by its consistency with conclusions derived from both experimental and theoretical studies.

Most importantly, we were able to observe outcomes of LN modeling that raise several new questions for experimentation: First, anatomical sub-compartments of

the reacting LN may be created by the migration dynamics of the responding cells; the cells occupy compartments that they create as they modify their receptors for chemokines. Second, A wide range of amounts of an antigen may have a relatively small affect on the numbers of antibody-producing plasma cells but a relatively greater affect on the numbers of memory cells produced by the response; the LN is designed to be sensitive and not to over-respond, but yet to generate a memory for future use that reflects the initial amount of antigen. Also, the size of the LN appears to be important; a relatively small naïve LN may be better poised to organize productive cell interactions in response to a new antigen, while a large LN that can accommodate too many cells cannot ensure an optimal immune response to the new antigen.

Further modeling work in LN research would include fine-tuning of the existing model to improve its accuracy and the addition of deeper molecular levels of description. The present model features two dimensions in space plus a dimension in time; extending the model to four dimensions (three in space plus time) would bring it yet closer to reality and allow for more precise quantitative comparisons with a real LN. Combining this modeling work with experimental work would test the validity of the observations and further assist in predicting biological outcomes. Finally, realistic modeling might help elucidate the pathophysiology of clinical LN abnormalities in chronic inflammation, neoplasia, and immune deficiency states. ■

APPENDIX I
SUPPLEMENTARY MATERIALS

A. Methodology

1) The Statecharts Language: Statecharts is a modeling language proposed as a system engineering tool to aid in the design of complex reactive systems [11]. Behavior in Statecharts is described using states, and events that cause transitions between states (Figs. 12 and 13).

Orthogonal/concurrent state components are also allowed, such that the system or part of it may be in several different states simultaneously, often in accordance with the different stages of the simulation (see later Fig. 15).

The hierarchical nature of the language means that states, including orthogonal components, may be nested to any level, and may incorporate level-rich transitions. Thus behavior can be described in a rich multiple level way. The semantics of the Statecharts language specifies dynamic behavior, and enables full executability and code generation.

2) The Rhapsody Tool: The Rhapsody tool from I-Logix (recently acquired by Telelogic) provides a working environment supporting object-oriented system development, with the Statecharts language at its heart [15]. In Rhapsody, one specifies the structure of the system using object model diagrams (OMD's), and then supplies each object with a statechart. Rhapsody is then capable of automatically translating the model into executable code (in, e.g., Java, C or C++). Rhapsody can then generate, compile, and implement the application in either Tracing mode (transmitting messages on the status of the model during run time in a script file) or Animation mode, where one can easily follow the simulation's progress in an animated version of the objects' statecharts (Fig. 13).

During the animation mode, it is possible to run the simulation one step at a time, pause the run, observe the attributes for each instance and their current values, and interfere with the simulation by inserting input events.

3) Object Model Diagrams: OMD's describe the structure of the system. They present all the objects that participate in the system and the relationships between them. The different types of connections between two objects include associations, whole-part and inheritance. These relations can be unidirectional or bidirectional and comprise of a multiplicity component, such that one object can be associated with 1, 2, or many (indicated by a $*$) instances of another object at any given time [Fig. 12(a)].

4) The Flash Tool: The Flash tool from Macromedia makes it possible to create an interactive graphical

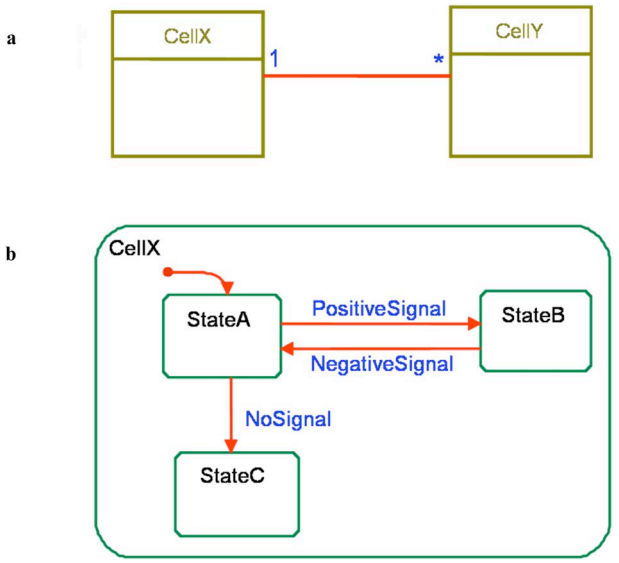

Fig. 12. ***A very simple example of Statechart modeling. (a) The Object Model Diagram (OMD) represents the static relationship between the objects (brown boxes) of the system. (b) The Statechart represents the specific behavior of each object.***

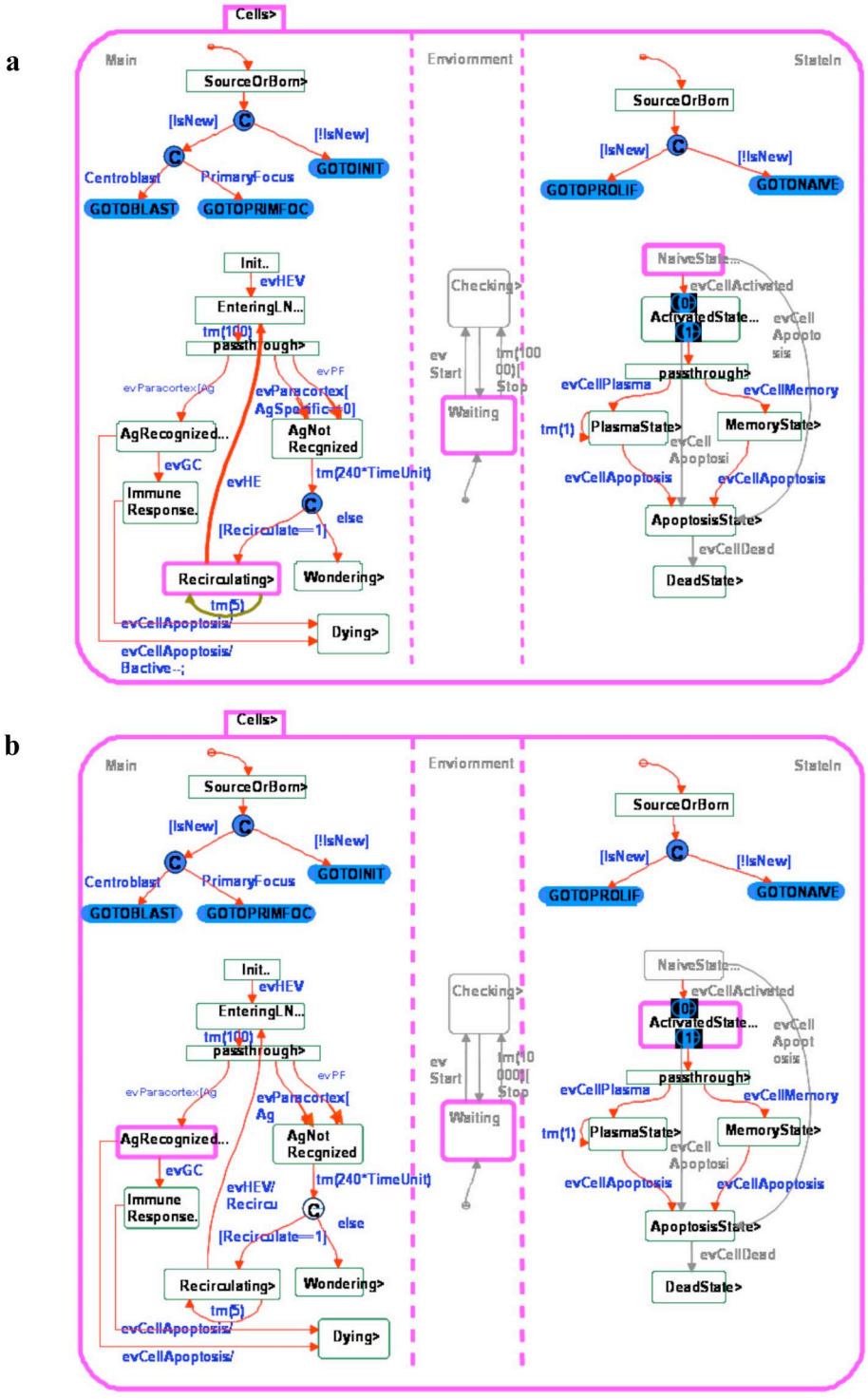

Fig. 13. ***Animated Statecharts. An example of the animation mode in run time. (a) Shows the current state of B[0]'s behavior (in pink), while (b) shows the current state of B[1]'s behavior in the same run. B[0] is a navive cell, currently in the*** `Recirculating` ***state, while B[1] is an activated cell, currently in the*** `AgRecognized` ***state.***

interface, producing dynamic animations. In our case this was done using a large collection of (often very short) individual movie clips constructed with the aid of classes, scripts and graphical objects.

B. Model Development

1) The Object Model Diagram: The object model specification of the LN model (Fig. 14) consists of three separate

diagrams that describe the relations between the various participating players: `B cells`, `T cells`, `FDC's`, `Receptor`, `Signal`, `Antigen`, `LN_Controller`, the manager of the system, Input, which passes information to the controller at the start of the simulation but does not participate in the rest of the process, and the `FlashSocket`, which passes and receives information to and from the `LN_Controller` throughout the simulation.

2) The Statecharts: The dynamic behavior of all the aforementioned objects was described using statecharts. The entire model consists of many charts and subcharts but only some will be described here. Describing biological processes using states and transitions is very intuitive; since a cell goes through different stages during its lifetime, these can easily be described using a statechart where each state represents one of these stages. Signals and interactions may be captured by transitions that take the cell from one state to another.

The B-cell class: A single B cell progresses throughout time, changing its state, its type, and its location [42], [54]. Consequently, the main statechart of the B cell has been divided into three orthogonal components (Fig. 15): a Main chart reflecting its progression throughout time, an Environment chart representing the cell's constant awareness of its surrounding, giving it the ability to respond to a certain signal, and the `StateIn` chart representing its current type.

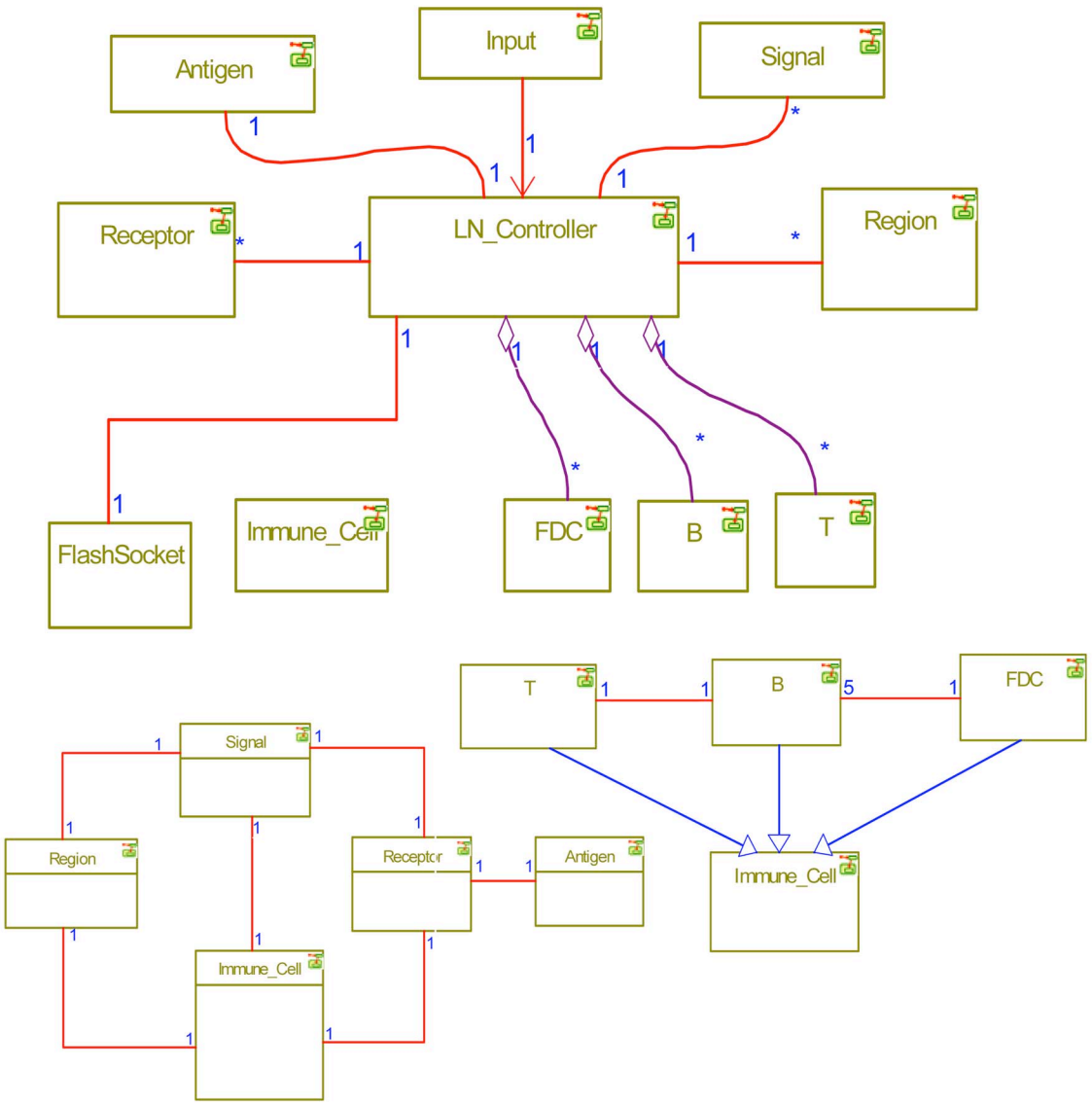

Fig. 14. ***OMD's of the LN model. The structure of all the objects that take part in the simulation, and the relationships between them.***

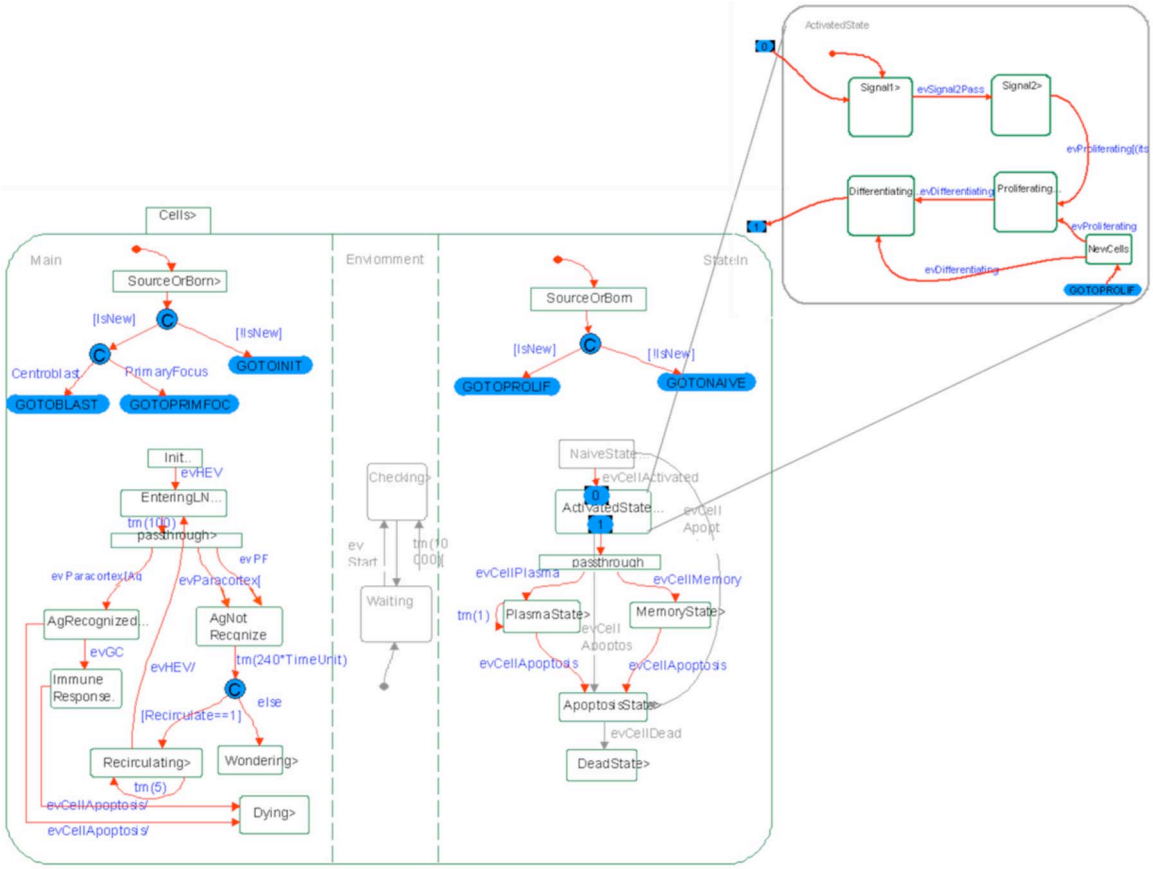

Fig. 15. ***Statechart of a B-cell. Broken lines indicate orthogonal (concurrent) states, grey lines leading to the ActivatedState on the right illustrate a zoomed-in subchart.***

The main chart: Once a B cell has entered the LN it either continues directly to the B cell zone (the Primary Follicle), or, if it has recognized an antigen, it is arrested in the T cell zone (Paracortex) [6]. Each B cell is given a probability to be specific for the antigen, such that approximately two randomly chosen B cells within the entire initial B cell population are to be specific for the antigen. Although the number of initial B cells in the simulation is smaller than in reality, as the simulation reflects a 2-dimensional LN, this value of specificity is in accordance with the literature; B cell specificity is 1 in 10 000 to 1 in 1 000 000 (out of a few million B cells that enter the peripheral lymphoid organs every day) [42].

The next step is different for antigen-specific B cells and for those cells that did not recognize the antigen. Those that are not specific have the option of either recirculating between the LN and the blood, or dying [42]. The selected route is chosen randomly with a probability of 0.5 either way. However, a cell can only recirculate a finite number of times in its lifetime; without antigen recognition its fate, within a few days, is death [50]. We have limited this number of circulations to five. The more interesting path is that of an antigen-specific B cell. When a B cell has encountered its antigen on its B Cell Receptor (BCR), it will process it and present a peptide of it on its MHCII molecule; the complex between the antigen peptide and the MHCII molecule can be recognized by a T cell, which makes it possible for the B cell to receive T-cell help. Since the B cell is arrested in the Paracortex—the T cell zone—it has an increased chance of encountering a T cell. An activated T cell that recognizes the specific antigen peptide is now capable of rescuing the specific B cell from Apoptosis (programmed cell death) [40]. This process can be observed in the `AgRecognized` subchart (not shown) where a B cell is in a `Waiting` state until it receives a T cell signal event from a nearby activated T cell, in which case it passes to the `PrimaryFocus` state.

A Primary Focus is a clonal expansion of both B and T lymphocytes, formed upon their interaction [18]. Together, they proliferate for a few days, producing over a thousand activated cells. This is in accordance with the known data, considering that cells divide at a rate of one division about every eight hours [4]. Following this process, many of the lymphocytes die. However, some of the B cells differentiate into plasma cells and migrate

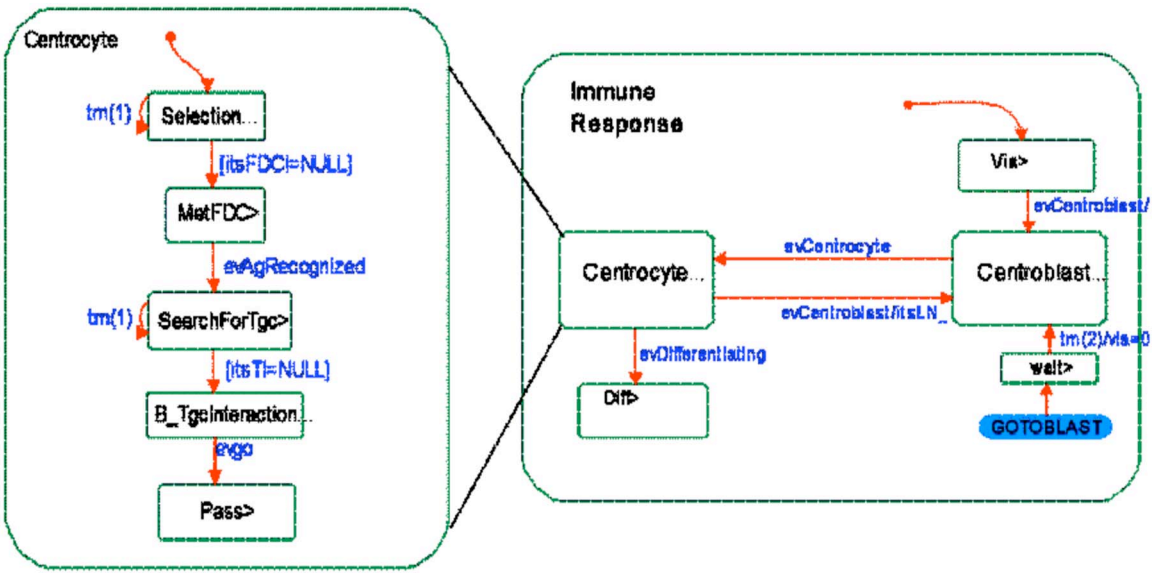

Fig. 16. ***Statechart of a B-cell, the immune response subchart and the centrocyte substatechart.***

to the Medullary Cords where they secrete antibodies (Ab's) that leave the LN via the blood stream. Another possible fate for these B cells is to migrate to the Primary Follicles (PF) where they continue to proliferate and eventually form a GC [26], [44]. In the model this was implemented by giving B cells that come out of the primary focus a probability of 0.4 to die, 0.4 to become Plasma cells [34], and 0.2 to move to the PF to form a GC.

B cells that are destined to progress to the PF express the appropriate chemokine receptors and follow the signals to the PF region. Only after arriving at the PF and receiving the `evGC` message from the `Region` class, they undergo the transition to the next state—the `ImmuneResponse` state. This state consists of two additional levels (Fig. 16); the first contains the statechart of the Centroblasts and the Centrocytes, as well as the transition between them, whereas the next level contains the subchart of each of these types of cells, describing the behavior and processes that occur within them. The initial state of B cells within a GC is a Centroblast. Centroblasts are rapidly dividing B cells (every 6 hours, [44], [55]), situated in the dark zone (DZ) of the GC, and with a reduced expression of the BCR. Their purpose is to proliferate and undergo somatic hypermutation, creating as many as a few thousand Centroblasts, each with a new, mutated BCR. The mutated B cells compete for recognizing the antigen, and the B cells with increased affinity win out and proliferate. This proliferation of mutated B cells with higher affinity for the antigen leads to an increase in the affinity of the responding plasma cells and the resulting antibodies for the antigen, making the immune response more specific. Many of the BCR mutations result in a non-functional BCR that will eventually lead to cell death. In the simulation, this was achieved by giving the Centroblasts a probability of 0.25 to die due to lack of functionality.

After a certain number of divisions, the Centroblast differentiates into a Centrocyte, expressing the BCR, and moving on to the light zone (LZ) [26] of the GC to undergo selection. The regulation of Centroblast proliferation and differentiation to Centrocytes is unclear [32]; it may occur as a result of competition for space [24], [28] or signals, or that simply, after a certain set amount of divisions [23] they become Centrocytes. Different research studies have arrived at different conclusions; some speculate that only one division takes place before differentiation to a Centrocyte [17], while some claim otherwise [24]. Thus, a 0.5 probability was given for Centroblasts to continue to proliferate (after their first division), leaving a 0.25 chance of differentiating to Centrocytes.

The selection process of the Centrocytes involves binding to, and recognizing a native antigen on a FDC via its improved BCR, and receiving a T-cell signal from helper T cells that are in the GC (Tgc's) [53]. This process is illustrated in the subchart of the `Centrocyte` (Fig. 16, left).

The initial state of the Centrocyte is to look for an antigen presented by FDC within the LZ. Only once it has encountered such a complex via a link to an FDC, it continues to the following state—the `MetFDC` state. In this state, it has the option of either specifically recognizing the antigen, or not, in which case it dies. Out of the B cells that have remained functional following the hypermutation process, most will not have improved their affinity to the antigen [52]. Therefore, the chance for recognition is given the value of 1/3 [17]. Only after receiving an `evAgRecognized` message, the cell can

progress to the next state of searching for a nearby Tgc. Once it has encountered a Tgc, it passes on to the next state to allow for the B-Tgc interaction to take place and for all the necessary signals to be received.

Once all this has taken place, the Centrocyte has successfully passed selection and can move on to the last state of this chart—the `Pass` state.

The possible pathways of a Centrocyte that has passed the selection process are either to recycle back into the DZ and start the process over again as a Centroblast [43] attempting to further improve the affinity of the BCR for the antigen, or to differentiate to a Plasma or a Memory cell [6]. Several studies have tried to come up with the probability value for this recycling process [46], [48]; the most probable value was shown to be 0.7, although this issue is still under controversy [31] where some researchers claim that recycling occurs very little, if at all [33]. Hence, a 0.5 probability value was given for recycling, and 0.5 for differentiating to either Plasma or Memory cell.

The StateIn chart: The `StateIn` chart progresses simultaneously with the `Main` chart. It deals with the current biological state of the cell. If it is an initial source cell it begins at the `NaveState`. Once a cell is activated, i.e., it is specific for the antigen, it passes on to the `ActivatedState`. The subchart of the `ActivatedState` contains four states (Fig. 15, top right); `Signal1`, indicating that the cell has recognized its specific antigen, `Signal2`, indicating that the cell has also received necessary signals from an active and specific T cell, `Proliferating`, which takes place under the condition that all necessary signals have been received and `Differentiating`.

The proliferation process itself involves the creation of an additional instance of the same object, with precisely the same states and parameter values as its mother cell. With the help of the diagram connectors that enable to jump from one state to another without direct connection between the states, it is possible to allow the newborn cells to immediately enter the appropriate states. Proliferation is allowed only under the condition that there is free space around the proliferating cell.

The last state of the `ActivatedState` is the `Differentiating` state, which is the state B cells move to after receiving a differentiating message and changing their type to either Plasma or Memory cell. If it is differentiating from the GC, it will have a 2/3 chance of becoming a Plasma cell, and a 1/3 chance of becoming a Memory cell, values which reflect the literature [35], [51].

In the `PlasmaState`, the cell's function is to secrete Ab's, which bind the antigen with high affinity [45]. Plasma cells secrete around 2000 Ab's every second for a few days [49]. In the model, every time step, a secretion function is called from the `PlasmaState`, which causes a decrease in the antigen amount. The B cells that differentiate into Memory cells receive an `evCellMemory` trigger and move to the `MemoryState` and in parallel leave the LN. Memory cells are long-lived cells with a high affinity to the antigen, which are immediately activated by a second encounter with the same antigen.

The final event that can happen to a B cell is an apoptosis-driven death. This is the last state for all the B cells that die, irrespective of whether this happens as a result of not receiving T cell signals or of finishing their role as plasma cells after a few days. As death can occur at any stage, there is a link to the `Apoptosis` state from every state within the `StateIn` chart. In addition there is also a natural chance of dying for any cell at any stage, and this is implemented by giving a probability value of 0.01 for a cell to die in each of the different stages. When the cell is dies, it proceeds to the `DeadState`, where any links to other objects are released, its location within the region is freed, and it is barred from performing any action.

The environment chart: Lymphocytes are highly responsive to their surroundings, and are attracted to certain chemokine signals, in agreement to the chemokine receptors they are expressing [6]. The `Environment` orthogonal statechart component is responsible for constantly sensing the environment for signals and responding accordingly. It rotates between two states—a `Checking` state, which calls a function termed `SignalsToRegions`, and a `Waiting` state, which allows time for the response. The `SignalsToRegions` function checks all the expressed receptors of the cell, together with the signals it can sense from its surrounding and gives the cell a command as to which area to follow.

The receptor and signal classes: These two classes [Fig. 17(a), (b)] are associated with each cell, giving it the ability to regulate its receptors, and signals sensed.

Each class consists of all the possible receptors (BCR, TCR, MHCII, MHCIIp, CD40, CD40L, CXCR4, CXCR5, and CCR7) and signals (BLC, ELC, SLC, SDF1, IL2, IL4, IL5) within the frame of the simulation. Each one is in an orthogonal state, a structure which easily enables the expression of any combination of receptors or signals concurrently. Each of these receptors or signals can be in a `NotExpressed`, `Expressed`, or `Bound` state, while the appropriate triggers cause the transitions between these states. Tables 2 and 3 summarize the various receptors expressed by the immune cells at different time points, the signals they follow, and their area of expression.

The region class: The `Region` class is also linked to every cell instance, and has a very significant role in the model. It contains the seven major regions of the LN [Fig. 17(c)] and gives a precise indication of each individual cell's whereabouts at each time point.

Transitions exist between the different regions that have a passageway between them. Each region represents a matrix, within which each cell occupies a single point; whenever a cell enters a region, it is first allocated a free location within that region and whenever the cell exits that region, its location is vacated. Movement of the cells is also implemented within the region's states; a cell is allowed to

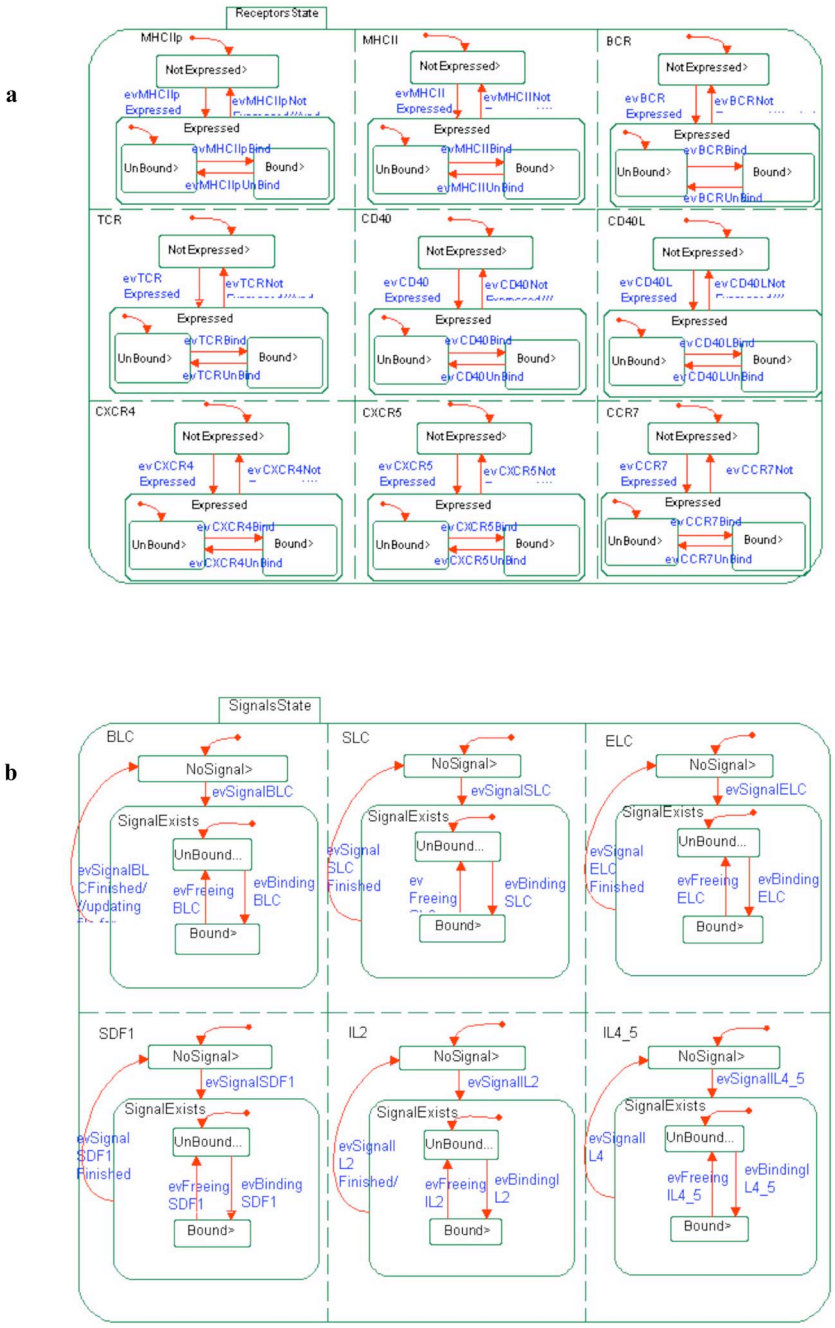

Fig. 17. ***Various statecharts from the LN model. (a) The*** `ReceptorsState` ***statechart, representing the receptors of the immune cells. (b) The*** `SignalsState` ***statechart, representing the signals of the immune cells.***

move only one step (one point) in any direction at each time step under the condition that that space is free. The direction of movement is chosen randomly if there is no chemokine signal, as in this case the motion of B and T cell is known to be random [47]. Furthermore, these region states express the relevant chemokines (Table 2) by sending the appropriate events to the `Signal` class of the cell, in which case the cell's movement is influenced by the chemokines for which it has receptors.

The T cell, cntigen, and FDC classes: The T cell, like the B cell, also has a probability value of antigen specificity. Only those T cells that recognize the antigen participate in the response and can become either helper T cells (Th) to help activate B cells, effector T cells (Teff) that leave the LN to the infected site, or Tgc's to provide the necessary signals to B cells in the GC [6], [36], [39].

The FDC statechart is a much simpler one, as the FDC's part in the immune response is to stay in the PF (and the

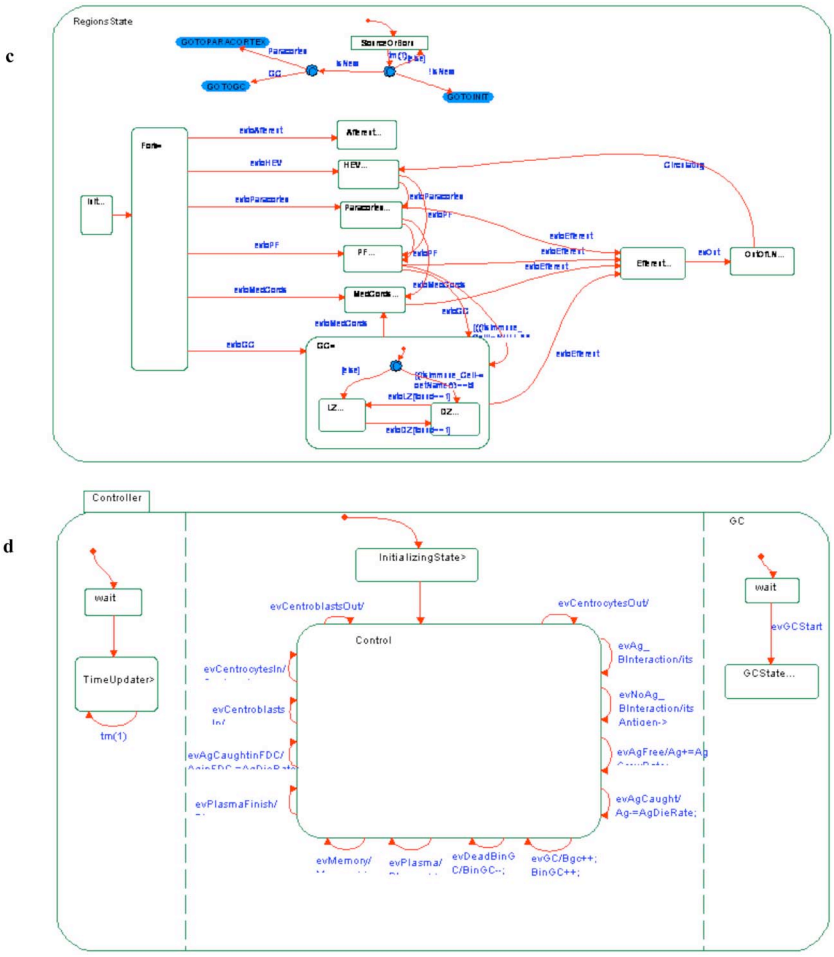

Fig. 17. ***(Continued.) Various statecharts from the LN model. (c) The*** `RegionsState` ***statechart, representing the regions of the lymph node (LN). (d) The*** `Controller` ***statechart, representing the LN controller that handles global elements of the model.***

GC, when it is formed) and merely to hold the antigen in its native form for the B cells to encounter [44]. Since each FDC can bind 3 to 7 B cells at a time [41], in the model they are permitted to bind to 5 B cells. The FDC has a vital role in the creation of Memory cells, and they are also thought to secrete signals essential for B cell survival [53], but not much else is known about their behavior [44].

The behavior of the antigen in the model is also quite simple. The antigen is represented as a numerical amount which has the ability to either decrease if it has being recognized by a B cell or an Ab, increase if time passes and it has not been captured, or terminate the response if all antigen has been eliminated. Once the antigen has been removed, the mission of the immune response can be said to have been accomplished, nevertheless, the simulation continues to show us continued cell behavior.

The input and the FlashSocket class: The `Input` class does not represent any object participating in the immune response, nevertheless it has a crucial role in initializing the process. It provides the `LN_Controller` with all the initial values for various parameters such as cell amounts, initial antigen amount, antigen specificity probabilities, size of the LN, time unit, etc. The user can choose to either provide this information through a file or to use the default values. It is then ready to initiate the simulation, starting with the `LN_Controller` that successively initiates other objects of the model.

The `FlashSocket` class has no statechart attached to it. It is a virtual class that takes care of the connection to the Flash tool, which will be discussed in the following section.

Table 2 Chemokine Receptors, Their Matching Chemokines and Their Area of Expression

Receptor	Cemokine	Expression
CXCR4	CXCL12/SDF1	Medullary cords, HEV, BM
CXCR5	CXCL13/BLC	Stromal cells in the follicles
CCR7	CCL19/ELC	T zone stromal cells, DC
CCR7	CCL21/SLC	T zone stromal cells, HEV's

Table 3 Receptor Expression and Interactions of Cells at Certain Time Points

LN time	Cell	Receptors	Interactions with immune cells
Naïve (prior to antigen exposure)	B cells	BCR, MHCII, CD40, CCR7, CXCR5	Activated T cells
	T cells	TCR, CCR7	Activated DC
Activated (after antigen exposure)	B cells	CCR7, CXCR4	FDC, Tgc
	T cells	CD40L, CXCR5	B cells
Following activation	T cells	Secretion of IL-2, IL-4,5	

3) Spatial Location and Movement: Each of the regions is represented as a grid, and each cell is identified with a position on that grid. Cell movement is random to any of the adjacent free spaces around the current position of the cell.

4) Cell-Cell Interactions: As each cell takes on a specific location at each time point, in order to undergo interaction with another cell (e.g., B-T interaction, B-FDC interaction) the two cells must be adjacent to each other, in either direction. Relative time is then allowed for the interaction to take place and the appropriate actions take place (such as receptor expression, differentiation, proliferation etc).

5) Time: As the actual time of the processes that take place during an immune response is too long to be modeled directly, a simulated time unit (TU) was created, in the interest of keeping the relative times between the different processes correct. Rhapsody makes it possible for one to simulate using time conditions (tm), where 1 tm = 1 ms real time. In this way the TU can be changed to any amount of tm's, while the time relations stay the same. Several processes, for which time is an important factor, were examined; a typical lymphocyte circulation cycle takes ~12–24 hours [44]; normal proliferation takes ~8–12 hours [4], [42]; primary focus proliferation continues for a few days [18], etc. In the model, we attempted to take such processes to be of corresponding time values, although adjustments were made to allow for technical obstacles.

APPENDIX II
SUPPLEMENTARY MOVIE 1: REACTIVE ANIMATION

The Flash animation displays, in real time, the on-going events of the lymph node (LN) simulation, which is run by Rhapsody. Shown is a sample run that is initiated by the Rhapsody simulation, which passes the information to the Flash animation that visualizes all the elements of the system on the one screen. Apparent are the main regions of the LN (represented as matrices) and the chemokines they secrete, as well as a subset of lymphocytes (B and T cells) as they enter the LN and travel to different areas in accordance with the commands of the simulation. Also visible are the cells' expressed receptors, their interactions, differentiation states, proliferation, death, and their tracked individual paths.

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