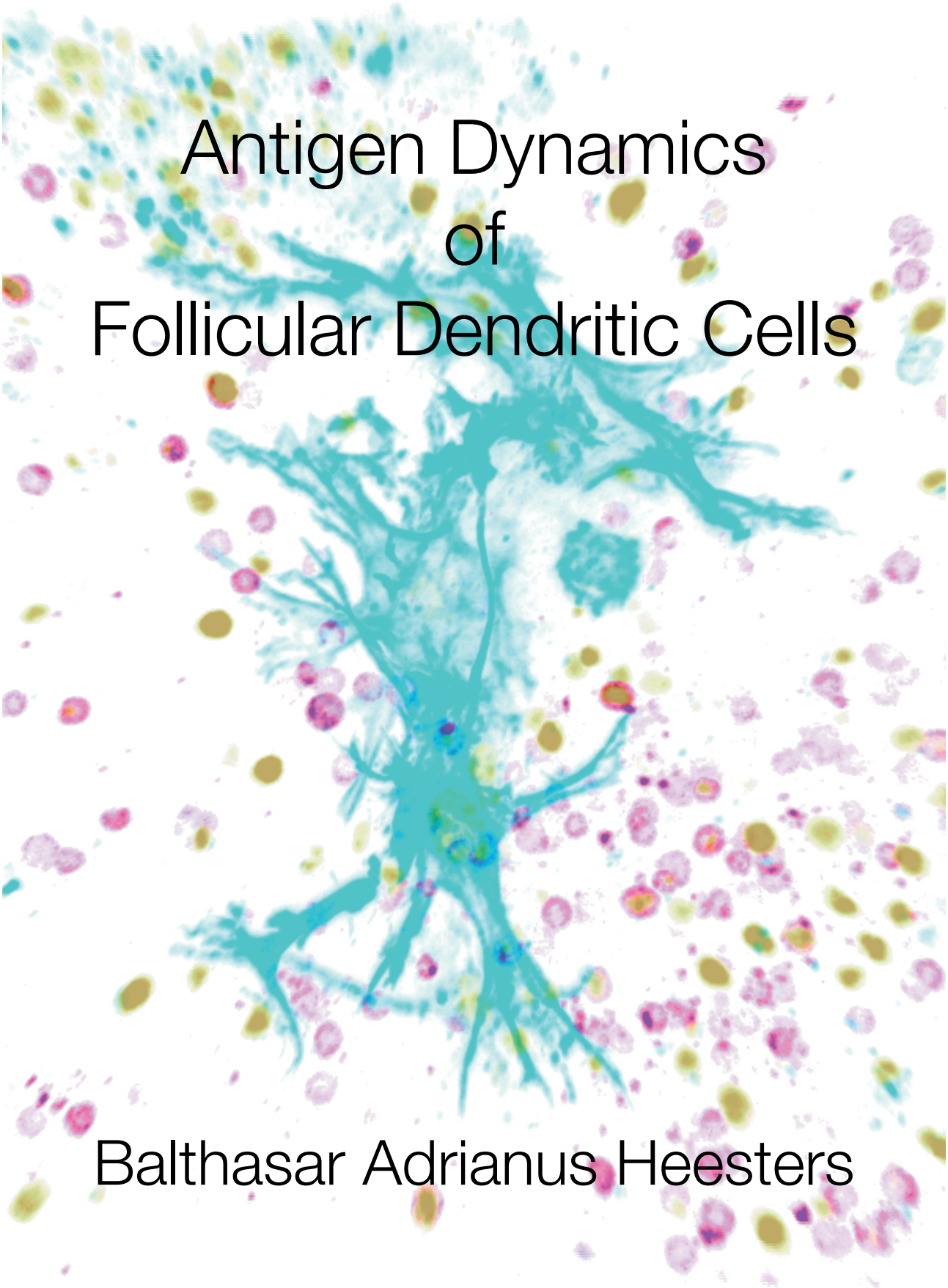
Antigen Dynamics of Follicular Dendritic Cells



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Antigen Dynamics of Follicular Dendritic Cells

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Thesis Utrecht University

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Purified follicular dendritic cells were cultured in an INvert 3D gel matrix pyramid and surounded by B cells, also casted in a fibrinogen gel.

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Antigen Dynamics of Follicular Dendritic Cells

Antigeen Dynamiek van Folliculaire Dendritische Cellen
(met een samenvatting in het Nederlands)

Proefschrift

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Prof. dr. M.C. Carroll

Contents

Chapter 1

Follicular Dendritic Cells: Dynamic Antigen Libraries

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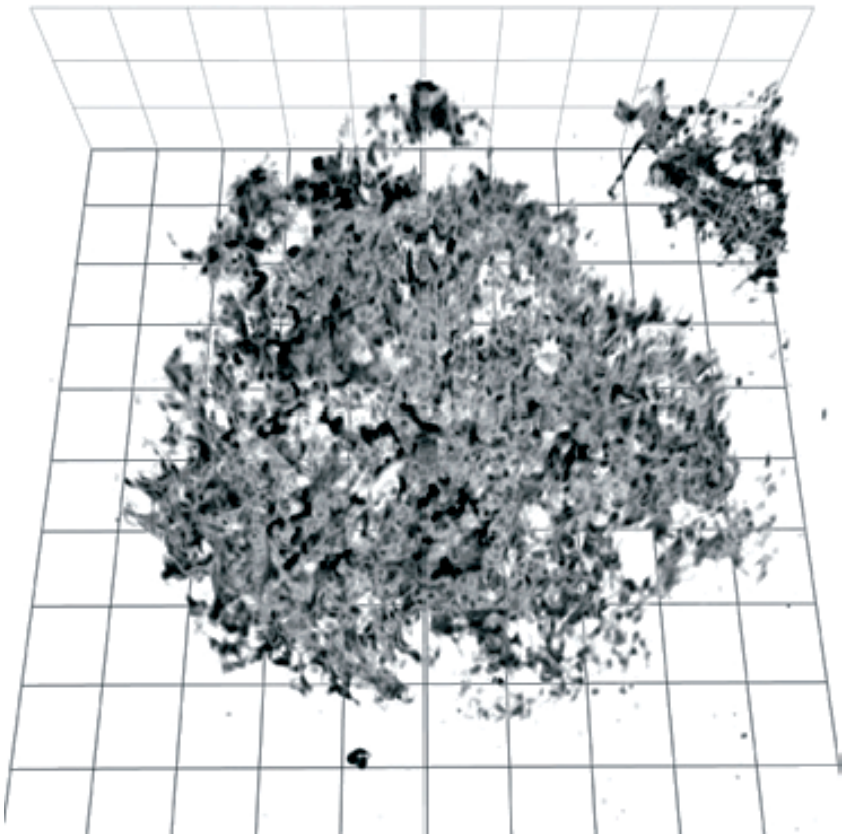


The image shows a three-dimensional image of the entire FDC network of a B cell follicle in the lymph node. FDCs were labelled in vivo (stained using the 8C12 antibody, which binds complement receptor 1) and then the lymph node was harvested, optically cleared and imaged. The image highlights the complexity of the FDC network.

Abstract

Follicular dendritic cells (FDCs) are essential for high-affinity antibody production and for the development of B cell memory. Historically, FDCs have been characterized as 'accessory' cells that passively support germinal center (GC) responses. However, recent observations suggest that FDCs actively shape humoral immunity. In this Review, we discuss recent findings concerning the antigen acquisition and retention functions of FDCs, and relevant implications for protective immunity. Furthermore, we describe the roles of FDCs within GCs in secondary lymphoid organs and discuss FDC development within this dynamic environment. Finally, we discuss how a better understanding of FDCs could facilitate the design of next-generation vaccines.

Introduction

Follicular dendritic cells (FDCs) are a unique population of cells that is essential for efficient germinal center (GC) formation and for the production of high-affinity antibodies[1] (Figure 1 a). They are centrally located within B cell follicles in secondary lymphoid organs and, as shown by Aguzzi and colleagues in an elegant study[2], they develop from perivascular precursors of stromal cell origin that are seeded throughout the body. FDC maturation requires lymphotoxin and tumor necrosis factor (TNF) signaling through B cells, and the disruption of these pathways leads to the loss of FDCs[3–5]. In the spleen and lymph nodes, FDCs are just one stromal cell type within a network of stromal cells (Figure 1 b, c). Although incompletely defined, the interplay between these different stromal cell populations may have substantial effects on the generation of protective immunity.

First identified in 1965 as 'antigen-retaining reticular cells', FDCs are now known to support GC responses through a variety of functions[6] (Figure 2 (time-line)). FDCs maintain an organized follicular structure by producing CXC-chemokine ligand 13 (CXCL13), which signals via CXC-chemokine receptor 5 (CXCR5) to attract B cells and specific subsets of T cells to the follicles[7]. Interestingly, during selective ablation of FDCs, follicles lose their typical round shape and become disorganized 'bands' of cells. These B cell bands retain a CXCL13-expressing stromal cell population, which indicates that FDCs are not the only stromal source of this chemokine[8]. FDCs also express an array of adhesion molecules that are thought to stabilize their interactions with cognate GC B cells, and they promote B cell survival in GCs through the production of interleukin-6 (IL-6) and B cell-activating factor (BAFF; also known as TNFSF13B)[9,10].

FDCs have the unique ability to retain intact antigen for extended periods. Indeed, this is required for GC maintenance, robust B cell somatic hypermutation (SHM) and the promotion of long-term immune memory[11]. Activated B cells that participate in a GC reaction interact with antigen on the surface of FDCs in order to receive survival signals and undergo affinity maturation, which leads to the formation of memory B

cell populations[12,13]. Cyster and colleagues[14] have excellently reviewed the multiple functions of FDCs in the GC reaction. In short, activated B cells migrate to the T cell–B cell border of the follicle where they present antigen to T helper cells and receive co-stimulation. Selected B cells then migrate to the center of the follicle, where they start a cycle of proliferation and hypermutation in the dark zone before undergoing antigen-driven selection by FDCs in the light zone. After selection by the FDC, the B cell can re-enter the GC or, with the help of T follicular helper (TFH) cells, can exit the GC as a memory B cell or as a plasma cell (Figure 3).

FDCs express high levels of complement receptor 1 (CR1; also known as CD35) and CR2 (also known as CD21; B.A.H. and M.C.C., unpublished observations), which are essential for antigen retention. In the absence of complement component C3 or following the deletion of the Cr2 locus (which encodes CR1 and CR2 in mice) FDCs are unable to retain antigen and GCs are reduced[15]. Although affinity maturation can occur

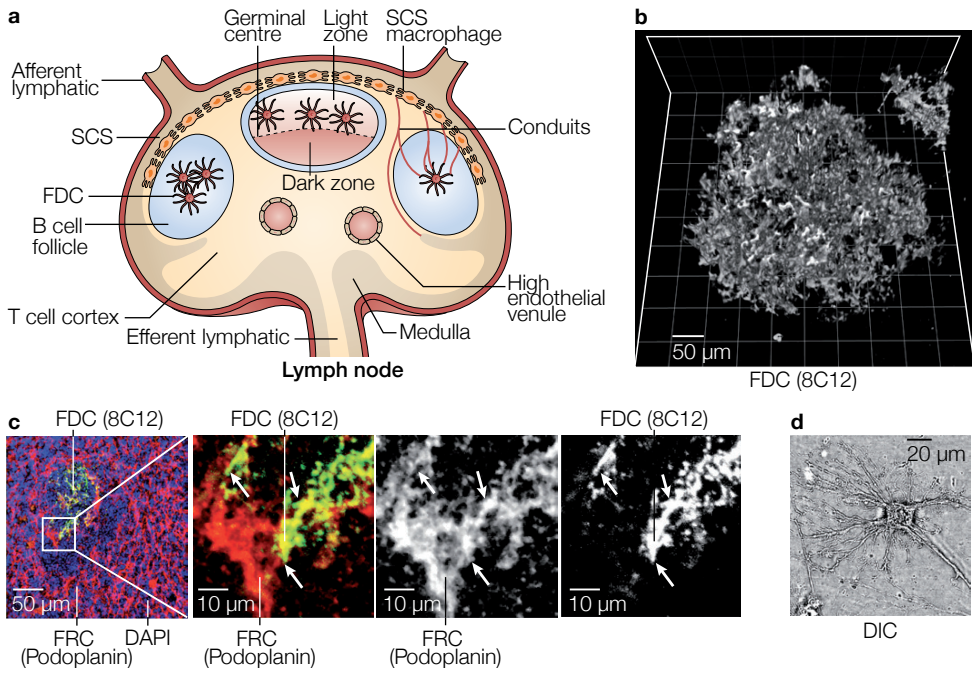


Figure 1 | Basic overview of lymph node architecture and FDC morphology

a | Schematic overview of the lymph node anatomy indicating the positions of key cell populations. Follicular dendritic cells (FDCs) are found within B cell follicles and in the light zones of germinal centers. **b |** The image shows a three-dimensional image of the entire FDC network of a B cell follicle in the lymph node. FDCs were labelled in vivo (stained using the 8C12 antibody, which binds complement receptor 1) and then the lymph node was harvested, optically cleared and imaged. The image highlights the complexity of the FDC network. **c |** Confocal microscopy of FDCs (green; stained using the 8C12 antibody) that were imaged as part of the wider stromal network (stromal cells expressing podoplanin (also known as gp38) are labelled in red). FDCs (indicated by white arrows) are clearly incorporated into the stromal network. **d |** The image highlights the cellular structure of an isolated FDC. Single FDCs were isolated and imaged ex vivo by differential interference contrast (DIC) microscopy. DAPI, 4',6-diamidino-2-phenylindole; FRC, fibroblastic reticular cell; SCS, sub-capsular sinus.

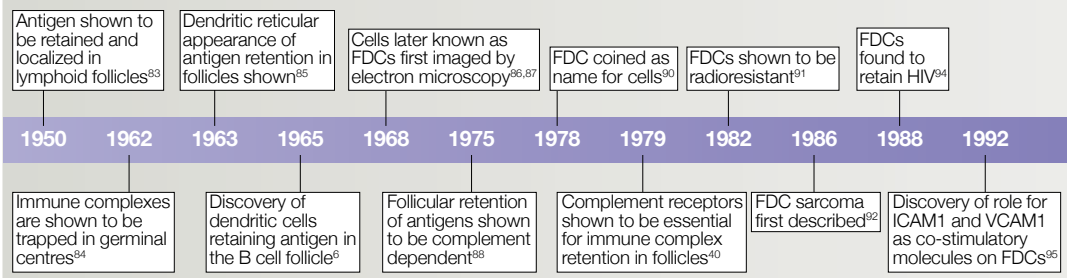
outside of GCs, the depletion of FDCs, the disruption of antigen binding, or the ablation of CR1 or CR2 on FDCs results in the loss of GC maintenance and severely impairs SHM responses[8,15,16]. To preserve antigen for long time periods, FDCs trap immune complexes in recycling endosomal compartments, thereby protecting the antigen from degradation[17]. This observation suggests a mechanism by which FDCs may control antigen availability and regulate the humoral immune response.

In this Review, we detail recent developments in FDC biology and discuss how FDC interactions with other stromal cells may affect humoral immunity. We also explain how the regulation of antigen cycling by FDCs may be beneficial to the host by promoting the generation of high-affinity antibodies but may be disadvantageous during HIV infection.

Development and maintenance of FDCs

Within the lymph node environment, there is a heterogeneous population of stromal cells that are characterized, in part, by their differential expression of CD31 (also known as PECAM1) and podoplanin (also known as gp38 in mice and gp36 in humans)[18] (Figure 1 c). The five major stromal cell types that can be distinguished on the basis of their morphology and function are fibroblastic reticular cells (FRCs), marginal reticular cells (MRCs), lymphatic endothelial cells (LECs), blood endothelial cells (BECs) and FDCs. The descriptions of FRCs, MRCs, LECs and BECs, and their contributions to immunity have recently been reviewed in detail[18]. This Review focuses on the development and maintenance of FDCs, and on their interactions with other components of the stroma. FDCs mature from ubiquitous vascular mural cells that are seeded throughout the body. This becomes evident when one takes a closer look at human FDC sarcoma, in which there is a rare deregulation of FDC proliferation[19]. This very uncommon neoplasm can occur anywhere in the human body, which is in line with recent studies that have identified FDC precursor localization in the mouse[2,20]. Lineage-tracing experiments have shown that splenic FDCs, FRCs and MRCs originate from precursors that express homeobox protein NKX2-5 and insulin gene enhancer protein ISL1 (ISL1; also known

Figure 2 | **Timeline of major discoveries in the FDC field***



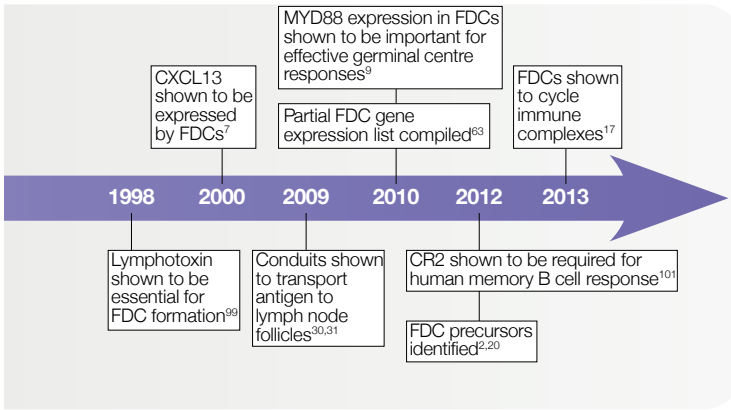
CR2, complement receptor 2; CXCL13, CXC-chemokine ligand 13; FDC, follicular dendritic cell; ICAM1, intercellular adhesion molecule 1; VCAM1, vascular cell adhesion molecule 1.
*Major discoveries in the FDC field are listed in chronological order[2,6,7,9,10,17,30,31,40,63,83-101].

as islet 1); however, lymph node stromal cells are derived from other precursors that are as yet unknown. When precursor cells were implanted under the kidney capsule of mice, normal lymphoid structures developed, which indicates that the implanted cell population contained lymphoid tissue organizer cells, as well as stromal precursors for FRCs, MRCs and FDCs[20]. In contrast to in vitro FDC cultures that are derived from lymph nodes (Figure 1 d), cultures that are derived from the spleen do not form B cell clusters (B.A.H. and R.C.M., unpublished observations). These observations suggest a developmental and functional distinction between lymph node-derived and spleen-derived FDCs.

FDCs develop as part of a stromal network

During prenatal development, retinoic acid (converted from vitamin A) triggers lymphoid tissue organizer cells to produce CXCL13, which attracts early lymphoid tissue inducer cells (LTi cells) to sites of lymph node formation[21]. In response to lymphotoxin-β receptor (LTβR; also known as TNFRSF3) signaling in LTi cells, lymphoid tissue organizer cells express various adhesion molecules and chemokines that promote the clustering of haematopoietic cells, which leads to the expansion and differentiation of the stromal network[22–28]. MRCs are among the first subset of stromal cells to appear[29]. They are present in the lymph nodes at birth and are morphologically similar to lymphoid tissue organizer cells[29]. MRCs differentiate in response to lymphotoxin that is produced by LTi cells and subsequently localize just below the lymph node sub-capsular sinus (SCS). Here, they produce CXCL13, further supporting stromal organization and the trafficking of leukocytes into the developing lymph node[29–31].

Although the exact sequence of events is not clear, FRCs and MRCs are thought to develop during the same period and to require similar developmental signals[29]. At birth, FRCs are found throughout the parenchyma, where they ensheath a dense conduit network that they generate through the secretion of extracellular matrix components[32–34]. During the first week of postnatal development, the lymphocyte compartment within the lymph node is almost entirely composed of T cells, and this corresponds with the appearance of conduits[31]. Incubation of CD4+ T cells with lymph node-derived stromal cell lines induces the production of an extracellular matrix in vitro, which suggests that this influx of T cells into the early lymph node leads to conduit formation through interactions between T cells and FRCs[35]. In the absence of B cells during initial lymph node formation, conduit networks throughout the paracortex are dense and have an extensive branching pattern[36]. However, once B cell follicles are fully formed, the conduits are sparser and branching is reduced within the B cell area[36]. In adult μMT mice, which lack mature B cells, the lymph node paracortex



contains an abundant web of conduits that is surrounded by ERTR7+ FRCs[31]. Following the adoptive transfer of wild-type B cells into μMT mice, the conduit network around aggregating donor B cells in the paracortex is progressively remodelled[31]. Although the mechanism for this remodeling is not well understood, it is thought that the induction of FDC maturation by incoming B cells that are forming primary follicles has a central role. Indeed, close interaction between FRC conduits and FDCs is observed within mature B cell follicles (Figure 4 a). Additionally, the FDC population is absent in irradiated wild-type mice that have been reconstituted with LTβ-deficient bone marrow and this allows FRCs to return to the follicles[37]. This suggests that FRCs are displaced from B cell follicles by developing FDCs. Alternatively, FDCs might differentiate from a local population of stromal cells that can also differentiate into FRC-like cells. Although the method of communication has not yet been identified, these studies demonstrate that FDCs interact with other stromal cells during development for the proper localization of each population within the lymph node environment.

Antigen acquisition and processing by FDCs

In mice, FDCs are the only cells that are known to be capable of retaining intact opsonized antigen for long periods of time[11]. It is generally accepted that FDCs can retain antigen for up to 12 months, although experimental confirmation is lacking[11,14,38,39]. However, on the basis of extrapolation from decay rates, FDCs are estimated to retain antigen for years after its introduction[40]. Nevertheless, it is not yet clear how antigen is transported to FDCs and by what mechanisms it is retained by these cells.

Trafficking of antigen

Lymph entering the node through the afferent lymphatics is channeled through the SCS into the medulla[41]. FRC conduits access afferent lymph and traverse B cell follicles, where they intersect FDCs. Alternatively, FRC conduits continue into the cortex, where they terminate at high endothelial vessels or the medulla. In the lymph node, the delivery of lymph-borne antigens to FDCs occurs via multiple pathways and the exact route that is taken is determined by the size of antigen and whether it is opsonized with complement. Lymph-borne antigens that are smaller than 70 kDa (approximately 5.5 nm) flow directly to the FDCs via the conduit network[30,31]. By contrast, large complexes are captured and transported across the SCS by SCS macrophages, which have been referred to as the 'guardians of the lymph node'[42–44]. Complexes that are opsonized with complement are shuttled to naive B cells in the underlying follicles, where they are then delivered to the FDCs[17,42,45] (Figure 4 b). Alternatively, intact bacteria, viruses and other large antigens are captured and processed in the medulla by SCS macrophages or $CD11c^+$ lymph node-resident DCs[30,46]. Whether antigen is transferred to naive B cells by resident DCs or by medullary macrophages is not clear. However, it is probable that DCs are capable of directly transporting antigen to the FDCs (Figure 4 c). This suggests that the conduit network functions as a safety filter for FDCs by protecting them from unprocessed pathogens but also enabling the sampling of small antigens in the lymph. Immune complexes that arrive in the afferent lymph are taken up by SCS macrophages

via Fc receptors and CR3. Strikingly, the C3d-coated immune complexes are transferred from the apical to the basolateral surface of the SCS macrophages, and these complexes are subsequently transferred to CR2-expressing naive B cells in the underlying follicle[43]. This CR2-dependent transfer of immune complexes raises the question of how C3d can simultaneously bind two different receptors — that is, CR3 on SCS macrophages and CR2 on naive B cells. Recent crystallography studies have solved the crystal structures of C3d bound to CR2 and of C3d bound to CR3[47–49]. It was noted that CR3 and CR2 bind to distinct sites on C3d and, in combination with the stable in vitro formation of CR3–C3d–CR2 complexes; this suggests a molecular mechanism for antigen transfer. Alignment of the crystal structures of the CR2–C3d complex with the CR3–C3d complex identifies the distinct molecular sites at which C3d contacts these receptors .

The 'hand-off' of antigen (which is present in C3d-coated immune complexes) from B cells to FDCs is unidirectional, although the underlying mechanism is unclear[17]. Treatment of FDCs with the actin inhibitor cytochalasin D blocks the uptake of immune complexes[17], which shows that the FDC mechanism that is involved in antigen transfer is actin-dependent. C3d-coated immune complexes form an aggregate or 'patch' on the B cell surface[17,45], which suggests a spontaneous aggregation of the receptor–ligand complexes. Live-cell imaging of the transfer process in vitro identifies a rapid dispersal of the C3d-coated immune complexes on the FDC surface upon contact with the naive B cell[17]. The unidirectionality of transfer may be explained by an active 'pulling force' of the CR2 receptor, which is anchored to the actin cytoskeleton, and the higher density of CR2 receptor expression by FDCs relative to naive B cells (B.A.H., unpublished observations). The naive B cells can only bind a small proportion of the

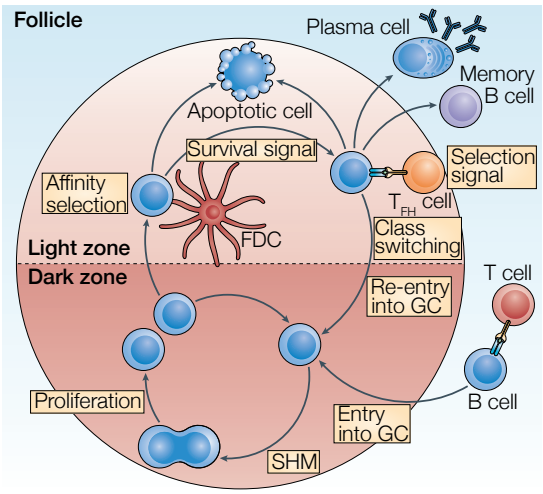


Figure 3 | The germinal center reaction
Simplified schematic of affinity maturation in the germinal center (GC). At the T cell–B cell border of the lymph node, B cells present antigen to T helper cells and receive co-stimulatory signals. The selected cells enter the dark zone of the GC and undergo somatic hypermutation (SHM) by upregulating components of the SHM machinery, including activation-induced deaminase (AID). After one cycle (or possibly more cycles) of proliferation and SHM, the B cells migrate to the light zone. In the light zone, the mutated BCRs that are the product of SHM are now exposed to antigens that are incorporated into immune complexes on the follicular dendritic cells (FDCs). If the affinity of the BCR is very low, the B cell will not receive survival signals and will undergo apoptosis. The remaining B cells need to compete for limited T cell help, which favours the higher affinity B cells and forces the others to undergo apoptosis. Surviving B cells can then undergo one of three fates: they can re-enter the dark zone and undergo further proliferation and SHM, they can exit the GC as plasma cells or they can exit as memory B cells. Re-entry will allow for further affinity maturation. It is thought that FDCs might influence affinity maturation by regulating the amount of antigen on their surface, however, due to technical limitations this has not been shown experimentally. TFH cell, T follicular helper cell.

C3d molecules that are present in the immune complex but, as FDCs are much larger cells, they will probably bind the majority of the available C3d molecules. By pulling the immune complexes inside and thus rapidly sequestering them, the CR2 receptors on the FDC are able to strip the antigen complexes from the B cell. Thus, the combined effect of C3d-coated immune complex aggregates on B cells, the high density of CR2 on FDCs and the active pulling force of the CR2 receptors on FDCs bring about a unidirectional transfer of C3d-coated immune complexes to the FDC.

Cycling of antigen

Early electron microscopy studies identified immune complexes on the FDC surface, which helped to explain how the antigen gains access to GC B cells. However, this did not explain how antigen could be retained by FDCs for extended periods of time. A solution to this apparent paradox was recently proposed, with the finding that FDCs cycle CR2-bound C3d-coated immune complexes in non-degradative endosomal compartments[17]. This pathway enables the FDC to protect the antigen from degradation and keep it available for B cells in its native form (Figure 4 a). How long antigen can cycle and be retained is unknown, however we have found that stable immune complexes containing intact opsonized antigen can still be observed 3 months after antigen injection (B.A.H., unpublished observations).

Lymph flow through conduits alters stromal cell function

Under homeostatic conditions, there is a constant flow of lymph fluid through the lymph nodes. However, studies in sheep have suggested that a substantial increase in the rate of lymph flow occurs as an early response to injury or infection[50–52]. As stromal cells are in close proximity to the sinuses and conduits of the lymph node, they may be affected by the increase in fluid pressure and shear stress that occurs in association with increased lymph flow. For example, exposure to low fluid flow rates in vitro enhanced the production of CC-chemokine ligand 21 (CCL21) by FRCs[53]. By contrast, increased flow rates in this model substantially decreased CCL21 production by FDCs[53–57]. Interestingly, this suggests that the rate of lymph flow may regulate stromal cell function and have an immunosuppressive effect under inflammatory conditions. In addition to FRCs, FDCs directly interact with the conduit network in B cell follicles (Figure 4 a), which suggests that they may also be sensitive to changes in the rate of lymph fluid flow.

If a high rate of lymph fluid flow regulates stromal cell functions, why would this be advantageous under inflammatory conditions? One possibility is that fluid flow may help to 'tune down' immunity to secondary infections. For example, Mueller et al.[58] observed in mice that following initial splenic infection with lymphocytic choriomeningitis virus (LCMV), the immune response to a secondary infection was suppressed. Importantly, the Armstrong strain of LCMV that was used for these studies does not infect stromal cells, unlike LCMV clone 13[59]. A similar observation was made following the infection of mice with other pathogens or the administration of virus-like particles, which confirmed that the effect was not specific to LCMV infection[58]. Intriguingly, the local production of the homeostatic chemokines CCL21 and CXCL13 was dramatically reduced by day 3 following infection, which correlated with the impaired migration of circulating

lymphocytes and DCs into the appropriate splenic compartments[58]. The interpretation of these findings was that a local reduction in the levels of homeostatic chemokines might bias the immune response towards responding to the primary infection and limit competition from incoming lymphocytes and DCs for space and resources in the lymph node. Based on these observations, we propose that an increase in lymph pressure and shear stress during inflammation may alter antigen cycling by FDCs, favoring the presentation of pre-existing antigens and limiting the uptake of antigen from a secondary infection. In this model, as well as regulating FDC function, the increased lymph fluid flow could also enhance B cell responses to the primary infecting agent.

FDC antigen cycling could also be affected by the content of the lymph, as the dendritic processes that are extended by FDCs are in intimate contact with the FRC conduits and these cells are therefore likely to be affected by factors other than the

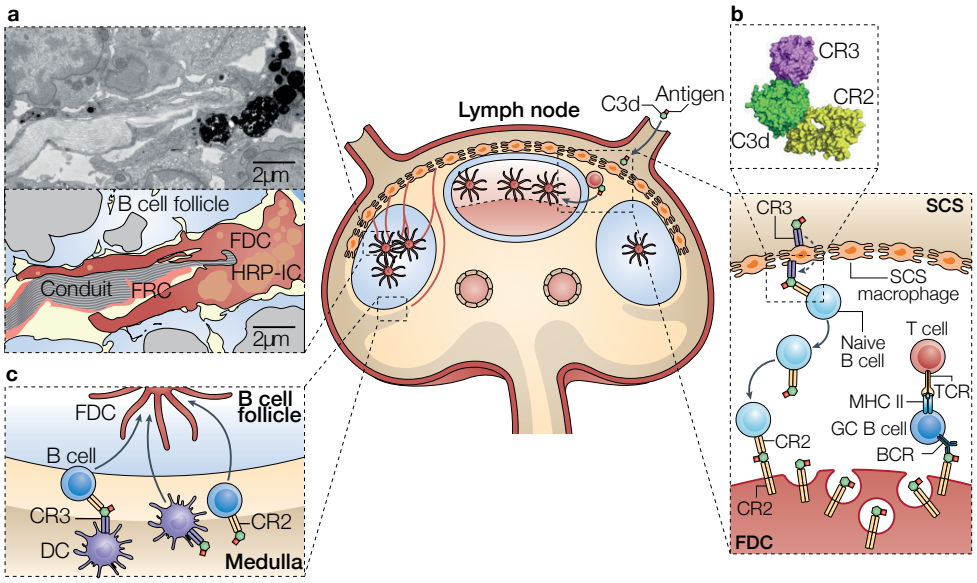


Figure 4 | Antigen acquisition by FDCs

a | Electronmicroscopy image of a follicular dendritic cell (FDC) loaded with horseradish peroxidase-labelled immune complexes (HRP-ICs) showing that the FDCs wrap around a conduit. For clarification purposes, a schematic is provided. **b |** Sub-capsular sinus (SCS) macrophages bind opsonized antigen in the SCS via complement receptor 3 (CR3), which is expressed on their apical surface, and their cargo is transferred to the basolateral surface of the cell, where it is handed over to naive CR2+ B cells in the underlying follicle. The top panel shows the position of C3d in the overlaid crystal structures of the CR2–C3d complex and the CR3–C3d complex, clearly highlighting the opposite binding sites on C3d. The bottom panel shows a B cell transporting an immune complex to the FDC, which internalizes it through CR2 in an actin-dependent manner. The immune complex is shuttled to a non-degradative vesicle that periodically cycles to the FDC surface. At the surface of the FDC, the immune complex is exposed to the B cell receptors (BCRs) of B cells that have exited from germinal centers (GCs). If these B cells receive T cell help, they become activated and differentiate into antibody-producing plasma cells or memory B cells. **c |** Alternative pathways for the acquisition of immune complexes by FDCs. Dendritic cells (DCs) in the lymph node can capture, process and subsequently handover antigens to B cells for transport to the FDC (left). DCs can capture, process and directly deliver immune complexes to FDCs (middle). B cells can capture antigens in the medulla and directly transport them to FDCs (right). FRC, fibroblastic reticular cell; TCR, T cell receptor.

shear flow of lymph. This would provide a rapid response to the breakdown products of infectious agents or cytokines and chemokines that are released in response to infection. It is known that the availability of antigen regulates GC persistence and promotes SHM, which is crucial for the generation of broadly neutralizing antibodies. Therefore, by regulating the availability of antigen, FDCs could affect the extent of SHM and, potentially, the generation of broadly neutralizing antibodies. Various pathogens, such as HIV, require multiple rounds of SHM (as many as 200) to elicit an effective broadly neutralizing antibody response[60]. As selection for antigen is necessary and as GC B cells are not thought to migrate among follicles but to remain within a specific site to promote clonal expansion, the longer retention of native antigen by FDCs is important in the successful generation of broadly neutralizing antibodies[61].

FDCs in pathogenesis

Function of Toll-like receptors on FDCs

FDCs express an array of Toll-like receptors (TLRs) but the role of these receptors on FDCs is not clear[9,62,63]. One possibility is that FDCs may use TLRs to respond to the products of viral and bacterial degradation in the periphery that flow into lymph node conduits. Bone marrow chimeric mice that lack TLR-4 expression on stromal cells (including FDCs) have lower levels of SHM and high-affinity antibody production, which suggests that TLR-4 signaling in FDCs is crucial for robust antibody responses[9]. Additionally, TLR-2 and TLR-4 stimulation of FDCs in the gut induces the production of transforming growth factor-β (TGFβ) and BAFF, which leads to IgA class switching in B cells[63]. In these two studies TLR signaling in FDCs induced the upregulation of chemokine and adhesion molecule expression, which indicates that TLR ligands may regulate FDC function[9,63]. In an elegant study, Pulendran and colleagues[64] showed that the immunization of mice with nanoparticles that contained antigen and ligands of

Box 1 | FDCs as a reservoir for HIV

What is the benefit for HIV to be taken up by follicular dendritic cells (FDCs) and why does this not promote the clonal selection of B cells that are capable of producing broadly neutralizing antibodies specific for the virus? As has been suggested in the case of HIV uptake by CD11c+ DCs, the concentration of HIV virions on FDCs could facilitate the infection of CD4+ T cells, particularly T follicular helper (T_{FH}) cells[102,103]. The T_{FH} cell compartment contains the highest frequency of HIV-infected cells, as compared with other CD4+ T cell subsets[104]. As T_{FH} cells differentiate and proliferate in close proximity to FDCs within the light zone of germinal centers (GCs), FDCs may serve as a major reservoir for HIV. Indeed, HIV proviral DNA is found in human CD4+ T cells cultured with FDCs that are isolated from infected mice[105,106]. Low-level viraemia can be detected in patients who are undergoing antiretroviral therapy and it is speculated that latent infection of CD4+ T cells may be responsible[104]. The novel finding that FDCs internalize and cycle foreign antigens within the non-degradative endosomal compartment suggests that they may act in a similar manner by binding C3d-opsonized HIV virions. Thus, FDCs may provide a safe haven to infectious virus, allowing it to escape antiretroviral therapies and thus contributing to latent viraemia (Figure 5). It will be important to determine whether human FDCs cycle immune complexes in a similar manner to FDCs in mice and, if so, whether FDCs that are isolated from patients with HIV retain infectious virus.

TLR-4 and TLR-7 induced sustained GCs that persisted for more than 1.5 years. They also showed that the presence of these TLR ligands enhanced B cell affinity maturation. As FDCs are crucial for GC B cell survival and SHM, it is possible that TLR ligands enhance these responses not only through their effects on DCs and B cells but also by directly affecting FDC function. Thus, following infection, TLR ligands in the lymph may rapidly activate FDCs and this could markedly affect the early events in GC formation.

Prions and HIV

Although the primary contribution of FDCs to host defense against infection is promoting humoral immunity to protein antigens, pathogens may interact with FDCs directly. For example, prions are taken up by FDCs (as reviewed recently by Aguzzi and colleagues[65]). Prions are proteinaceous infectious particles that, when converted from their physiological form (PrPC) to a pathological configuration (PrPSc), cause transmissible spongiform encephalopathy (TSE) in mammals[66,67]. FDCs express high levels of PrPC and they are thought to be an important site for PrPC to PrPSc conversion[68]. Experiments using reciprocal bone marrow chimeras have suggested that PrPC expression in stromal cells, but not in haematopoietic cells, is required for PrPSc replication in the spleen[69,70]. Following intra-peritoneal inoculation, infectious prions accumulate in FDCs before infiltrating the central nervous system (CNS). Furthermore, the ablation of FDCs (through the depletion of B cells or the treatment of mice with LTβR–Ig) prevents the accumulation of prions in the spleen and slows the neuroinvasion

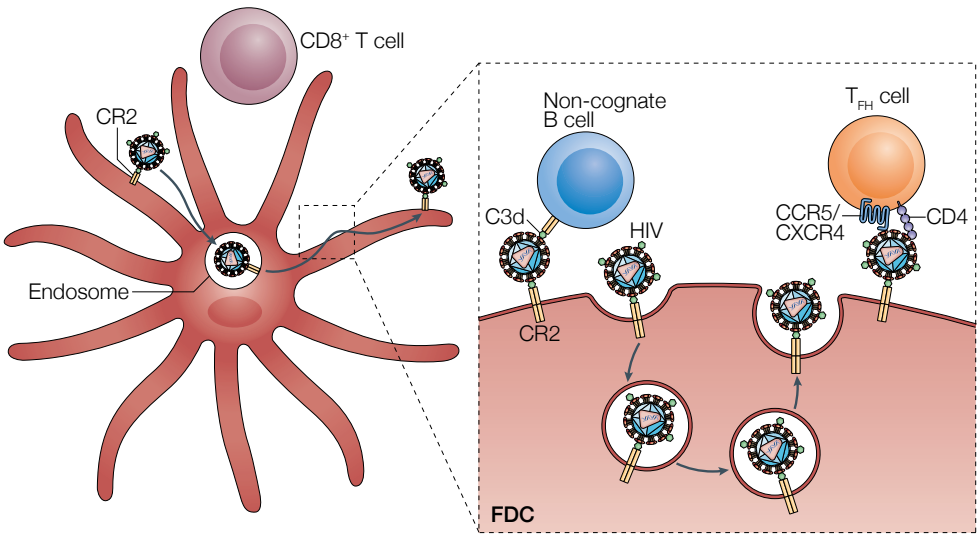


Figure 5 | FDCs function as HIV reservoirs

Schematic of a mechanism for the capture and subsequent cycling of opsonized infective HIV by complement receptor 2 (CR2) on the follicular dendritic cell (FDC). As long as the HIV virion remains in the protective recycling endosome of the FDC (in which it cannot be degraded), it remains hidden from CD8+ T cell surveillance. Upon emerging from the endosome at the cell surface, HIV can infect surrounding T follicular helper (TFH) cells using CD4 and either CXC-chemokine receptor 4 (CXCR4) or CC-chemokine receptor 5 (CCR5) as a co-receptor. The question mark indicates the inability of CD8+ T cells to detect HIV on FDCs.

process[71–74]. Although these studies suggest a crucial role for FDCs during the sub-clinical stages of TSE, the mechanism by which prions travel from FDCs to the CNS is not well understood. The distances between FDCs and splenic nerves are reduced in mice that are deficient in CXCR5, and this correlates with accelerated neuroinvasion of prions and with clinical manifestations of scrapie[75]. However, the mechanism by which prions travel through the splenic nerves into the spinal cord is not known.

In a similar manner to prions, HIV is thought to directly interact with FDCs (Figure 5). HIV evades host immunity by exploiting the very mechanisms that the immune system uses for host defense. Although few studies have directly examined FDC function during HIV infection, indirect evidence suggests that FDCs actively support viral survival and dissemination[76]. HIV is capable of independently fixing complement through complement factor I and, paradoxically, this enhances HIV infectivity in vitro[77–79]. Fragments of C3 bound to gp160 allow HIV to adhere to the complement receptors CR1 and CR2, which are expressed at high levels by FDCs[80]. Additionally, non-neutralizing antibodies specific for HIV, which are found in most subjects with HIV, may contribute to the trafficking of virions to FDCs through FcR-mediated binding, whereby the virus maintains the ability to infect surrounding cells for many months[81,82] (Box 1).

Box 2 | FDCs in autoimmunity

Given the importance of follicular dendritic cells (FDCs) in promoting humoral immune responses to foreign antigens, it seems reasonable to assume that they also contribute to the pathogenesis of autoimmunity. For example, a hallmark of B cell-mediated autoimmune diseases is the localization of ectopic germinal centers (GCs) within inflamed tissues[107]. Morphologically, ectopic (or tertiary) GCs are similar to those that form in the draining lymph nodes in response to immunization or infection. However, it is not clear whether FDCs are actually required for the maintenance of these autoreactive GCs.

An early study identified a requirement for FDCs for the development of disease in the K/BxN arthritis model, where FDCs were found to be important both for the retention of self antigen-containing immune complexes and for the recruitment of

T follicular helper (TFH) cells[108]. Additional evidence that self-reactive B cells survey FDCs for cognate self antigen was provided by Rickert et al.[109]. Using a conditional model in which a membrane form of a self antigen was expressed on FDCs, they found that developing self-reactive B cells were, for the most part, efficiently eliminated109.

FDCs may also participate in regulating autoimmunity by secreting the phosphatidylserine-specific opsonin milk fat globule epidermal growth factor 8 (MFGE8; also known as lactadherin)[110]. Opsonization of dying GC B cells by MFGE8 is crucial for their clearance by neighboring tingible-body macrophages. MFGE8-deficient mice show excessive accumulation of apoptotic B cells and develop a lupus-like autoimmunity[111].

Future studies should help to further clarify the importance of FDCs in autoimmunity and may facilitate the development of new therapies to inhibit the pathological FDC activities in the context of chronic autoimmune disease.

Perspective

The long-term retention of antigens by FDCs is required for the maintenance of GCs and for the efficient production of high-affinity antibodies. Indeed, FDCs can be considered as the ‘catalysts’ of the GC reaction and, as with every catalyst-driven reaction, the

lower the concentration of the substrate (in this case, the antigen), the more important the catalyst is for ensuring the successful generation of the product (that is, high-affinity antibodies). Concentrating antigen on FDCs becomes more important when antigen availability is limiting, which is often the case under physiological conditions. The discovery that FDCs continuously cycle the antigens that they have taken up within non-degradative endosomal compartments explains not only how antigen is retained for extensive periods but also how it is made available to cognate B cells. Future experiments should help to further define how FDCs participate in actively shaping humoral immunity by regulating antigen availability.

As FDCs express an array of TLRs, it will be of interest to determine whether TLR signaling in FDCs affects the cycling of antigen and, in turn, the process of affinity maturation and the production of broadly neutralizing antibodies. As pathogens such as HIV may be protected within endosomal compartments, reducing the internalization of virions by FDCs may be an important step in generating protective immunity to these infections. Finally, a better understanding of the mechanisms that regulate antigen availability on FDCs could be useful for developing therapies for antibody-mediated autoimmune diseases (Box 2).

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Chapter 2

II

Endocytosis and Recycling of Immune Complexes by Follicular Dendritic Cells Enhances B Cell Antigen Binding and Activation

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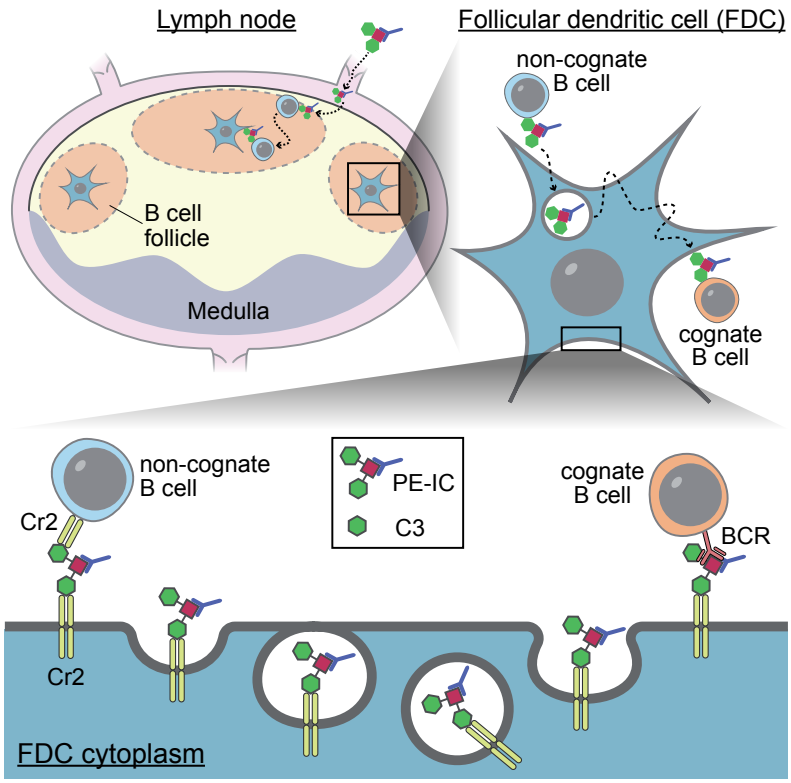


Abstract

Stromal-derived follicular dendritic cells (FDCs) are a major reservoir for antigen that are essential for formation of germinal centers, the site where memory and effector B cells differentiate. A longstanding question is how FDCs retain antigen in its native form for extended periods and how they display it to specific B cells. Here we found that FDCs acquired complement-coated immune complexes (ICs) from non-cognate B cells via complement receptors 1 and 2 (CD35 and CD21, respectively) and rapidly internalized them by an actin-dependent pathway. ICs were retained intact within a non-degradative cycling compartment and were displayed periodically on the cell surface where they were accessible to antigen-specific B cells. This would explain how antigens are protected from damage and retained over long periods of time, while remaining accessible for B cells.

Introduction

Follicular dendritic cells (FDCs) are centrally located within B cell follicles of secondary lymphoid tissues, including the spleen and lymph nodes (LNs), where they are the major source of B cell attractant (CXCL-13)[1,2]. They are also a source of survival factors such as B cell activating factor (BAFF) and cytokines such as IL-6 and IL-10 that modulate the differentiation of B cells and T follicular helper cells within an active germinal center (GC)[3,4]. FDCs are stromal derived and are identified by their extensive dendritic morphology and cell surface markers such as CD21, CD35, FDC-M1 (Mfge8), FDC M2 (complement C4), BP-3, complement C3, and FcgR[5-9]. In a recent elegant study, Aguzzi and colleagues identified the source of FDCs as platelet-derived growth factor receptor beta-positive perivascular cells that are located throughout the host and this would explain their capacity to develop at ectopic sites[10]. B cell surface lymphotoxin a and b and TNFa signal FDC precursors to develop into mature FDCs[11-15].

More than 40 years ago, FDCs were recognized to retain antigen within B cell follicles for extensive periods where it is required for maintenance of GCs[16-18]. Within GCs, activated B cells that undergo somatic hypermutation and class switch recombination require antigen for survival signals, to enhance affinity maturation, and for the formation of memory and effector B cells[19,20]. Although affinity maturation can occur in the absence of GCs in lymphotoxin deficient mice, elimination of FDCs by ablation or blockade of lymphotoxin signaling, antigen, or complement receptor CD21 and CD35 results in a rapid elimination of GCs[21-24]. In mice, complement receptor 1 (CD35) and complement receptor 2 (CD21) are both encoded by the *Cr2* locus; because both are coexpressed on FDCs and B cells, CD21 and CD35 was referred to as Cr2.

Antigen acquisition from FDCs in vivo by cognate B cells was recently visualized by multi-photon intra-vital imaging[25]. How antigens are retained in a native state and made readily accessible to cognate B cells over long periods has remained an enigma. Based on electron microscopy studies, it was proposed that immune complex (IC) is retained

on the surface of FDCs in two forms: filiform and beaded structures termed immune complex bodies or ICCOSOMES. Early in a GC response, it is held that the latter are released and taken up by B cells for presentation to T cells but this model doesn't explain how antigens are sequestered by FDCs without degradation[26-28].

Recent studies have identified a novel pathway by which LN-resident sub-capsular sinus macrophages (SSMs) capture lymph-borne ICs and shuttle them to non-cognate B cells in the underlying follicles[29,30]. Both the initial capture of ICs from the lymph by SSMs and the uptake by non-cognate B cells is dependent on complement receptors (Cr), i.e., CD11b (Cr3) and CD21 (Cr2), respectively. For example, by using bone marrow chimeras in which WT mice are reconstituted with Cr2-deficient bone marrow, Phan et al. show that substantially fewer ICs are taken up by the Cr2-deficient B cells relative to control WT chimeras and overall deposition of ICs on FDCs is reduced in the Cr2-deficient chimeras[29,30]. Therefore, although other pathways such as conduits are capable of delivering antigen to FDCs, non-cognate B cells represent one major pathway[31,32].

To study the cell biology of antigen acquisition and retention in living cells, we used a combination of flow cytometry and in vivo and ex vivo imaging of FDCs. With multi-photon intra-vital imaging, direct transfer of complement-coated ICs from non-cognate B cells to FDCs was observed. Unexpectedly, we found that FDCs rapidly internalize intact ICs into a non-degradative, cycling compartment. Notably, internalized ICs undergo several rounds of surface recycling. The cycling of ICs bound to FDC complement receptors helps explain the directional transfer of ICs from non-cognate B cells to FDCs and it explains how ICs are efficiently displayed on the FDC surface for extensive periods in order to be accessible to cognate B cells.

Results

FDCs Acquire Immune Complexes from Non-cognate B Cells

In WT mice, FDCs become decorated with ICs within 8 hr after immunization, suggesting that non-cognate B cells deliver and hand-off the ICs to the FDCs. However, actual transfer of ICs from the B cell to the FDC has not been visualized in vivo so it is possible that an intermediate cell type is involved or that the ICs are released and later acquired by the FDCs. Moreover, because both the capture by B cells and transfer to FDCs is dependent in large part on Cr2 recognition of the complement C3d-coated ICs, this raises a question about the directional transfer of ICs, i.e., from B cells to FDCs[30,32]. Thus, how are the ICs removed from the B cell by the FDC when Cr2 binds IC on both cell types? To address this question, we first wanted to test in vivo whether non-cognate B cells directly transfer ICs to FDCs. As a model system, mice received 5 x 10^6 enriched GFP^+ non-cognate B cells and were passively immunized with rabbit anti-phycoerythrin. Twenty-four hours later, anesthetized mice were injected in the footpad with 8 mg of phycoerythrin (PE) and the popliteal LN was imaged by multi-photon intra-vital microscopy (MP-IVM). As reported by Phan et al., non-cognate B cells were

identified bearing clusters of immune complexes (PEICs) (Figure 1 a; Movie S1)[29,30]. Tracking of individual donor B cells within LN follicles, we visualized direct transfer of a cluster of PEICs from the B cell to the FDC (Figure 1 a; Movie S1). Thus, within minutes of contact between the B cell and the FDC, the complexes were efficiently transferred to the FDCs.

To examine transfer of antigen from the B cell to the FDC in more detail, we established an ex vivo model based on our in vivo observation of non-cognate B cell transfer of PEICs directly to the FDC. Therefore, rabbit IgG anti-PE was mixed with PE antigen to form PEICs and then fresh mouse serum was added to the complexes to activate and

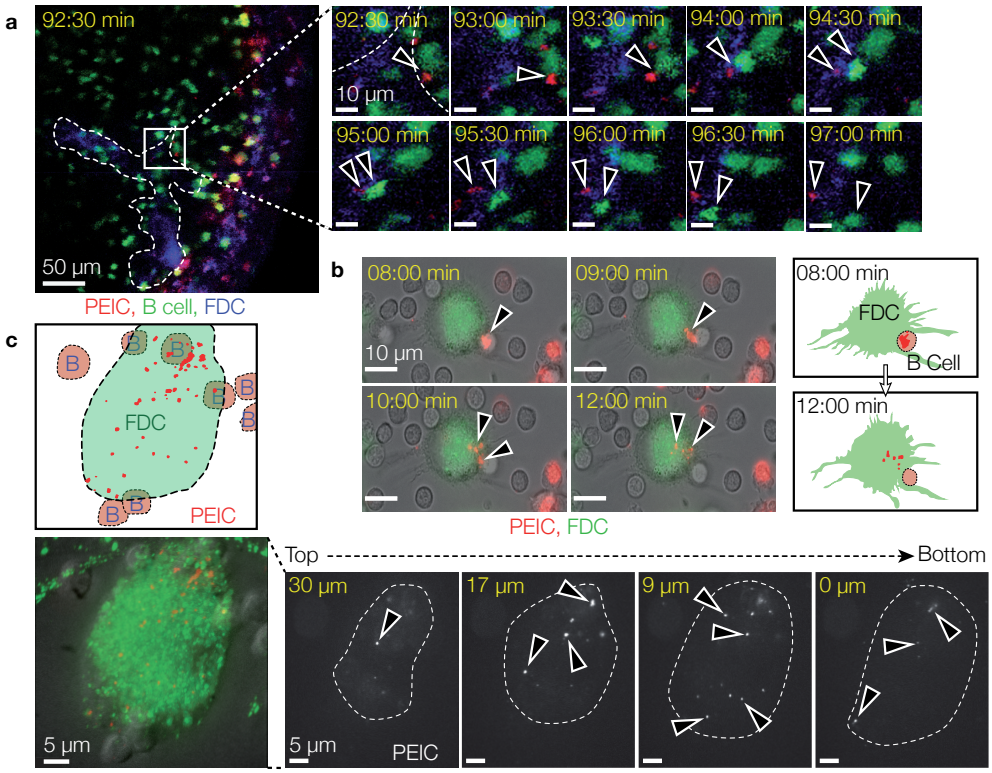


Figure 1 | Acquisition of ICs by FDCs

a | Left: snap shot taken from multiphoton-intravital imaging of the popliteal LN in a mouse passively immunized with PE antibody and adoptively transferred with WT fluorescent labeled B cells (green). LN was imaged from 60 to 120 min after PE (red) injection into the footpad showing B cells transfer PEICs onto FDCs (blue). Right: series of higher-magnification images over 5 min showing transfer of PEICs (red) bound to a B cell (green) to an FDC (blue) (arrowheads). **b |** Live-cell imaging of cultured FDCs labeled with lipophilic DiO dye (green) and incubated with PEIC-bound non-cognate B cells. Images were generated every 60 s. Images show transfer of C3-opsonized PEICs clustered on the B cell to the FDC and subsequent fragmentation on the FDC surface. Schematic representation of transfer of PEIC clusters from B cells onto FDCs and their fragmentation upon contact with FDCs is shown at right. **c |** Left: spinning disk confocal image of PEICs (red) within FDCs (green). Right: representative Z-stack series projection through 30 mm at approximate 0.4 mm intervals identifies PEICs (gray and arrowheads) within FDCs (outlined). See also **Figures S1** and **S2** and **Movies S1, S2,** and **S3.**

bind complement C3. On culture with non-cognate B cells, the complement-coated PEICs formed clusters on the B cell surface similar to those observed in vivo (Figure S1 a). Inspection of the patches of gold-labeled PEICs on non-cognate B cells loaded in vitro by electron microscopy revealed that the patches consist of multiple complexes (Figure S1 b). Moreover, staining of the loaded B cells with anti-PE and analysis by flow cytometry confirmed that the majority of the complexes were retained on the outside of the B cell (Figure S1 c). Therefore, for the experiments with ex vivo cultures of FDCs, non-cognate B cells were loaded with complexes in vitro.

To follow transfer of PEICs from the B cells to the FDCs in living cells, non-cognate B cells loaded with complement-coated PEICs were mixed with FDCs in ex vivo cultures and analyzed by live cell fluorescence spinning disk confocal microscopy. FDCs were harvested according to the procedures reported by Tew and colleagues and cultured on collagen-coated plates for 5–10 days where they regained their dendritic morphology[33]. Isolated cells were >90% mature FDCs based on morphology, cell surface markers (Cr2, FDC-M1, FcgRIIb), and binding of ICs. Real-time images demonstrate that on contact between donor B cells with FDCs, the clustered PEICs were rapidly transferred (Figure 1 b; Movie S2). The large cluster of PEICs on the B cell seemed to dissociate into much smaller particles upon transfer to the FDCs (Figure 1 b; Movie S2). In the absence of FDCs, approximately 90% of non-cognate B cells retained PEICs after 4 hr of incubation, showing that transfer from the B cells to FDCs is specific. Consistent with these observations, the frequency of PEIC-positive B cells decreased in a time-dependent manner when incubated with FDCs (Figure S2 a) whereas FDCs acquired PEICs in a reciprocal manner (Figure S2 b). Efficient uptake of PEICs by FDCs was dependent on their expression of Cr2 but not FcgRIIb as predicted from results in vivo (Figure S2 b)[32,34]. Thus, PEIC-bearing donor B cells rapidly transfer the complexes to FDCs in real time after cell contact and the efficiency of transfer is dependent on FDC expression of Cr2.

FDCs Rapidly Internalize Immune Complexes

Antigen was tracked on and within FDCs in the ex vivo model by means of live-cell fluorescence spinning disk confocal microscopy. Analysis of optical sections (spaced approximately < 0.4 mm) identified PEICs both on the FDC surface and distributed within the FDCs during the first 30 min after delivery (Figure 1 c; Movie S3). The relatively rapid internalization of PEICs by FDCs after contact with B cells suggests a possible mechanism to explain the unidirectional movement of complexes from the FDC to the B cell. For example, a mechanism by which Cr2 is periodically internalized could provide a one-way transfer of complement-coated complexes from the loaded non-cognate B cell to the FDC. To test whether uptake and internalization of PEICs requires a dynamic actin cytoskeleton, FDCs were pretreated for 30 min with 5 mM cytochalasin D (Cyt D), which blocks actin polymerization[35]. Treatment of cells with 5 mM Cyt D for 30 min blocks cellular processes dependent on actin polymerization such as phagocytosis in a reversible manner[36]. To confirm that 5 mM Cyt D was not toxic in our ex vivo model, FDCs were pretreated with Cyt D prior to culture with fluorescent-labeled transferrin ligand (Tf). Comparison of the treated with untreated FDCs demonstrated that the actin inhibitor did not impair uptake of Tf as expected (data not shown)[37].

To circumvent any effects of the Cyt D on the non-cognate B cells loaded with PEICs, Cyt D was removed from the media before culture for an additional 30 min with donor B cells. Subsequently, the FDCs were washed, fixed, and then stained with anti-FDC-M1 and Cr2 (Figure 2 a). Confocal imaging of FDCs cultured in media without Cyt D identified significant uptake of the PEICs as expected based on total mean fluorescent intensity (MFI) (Figure 2 b, c). By contrast, FDCs pretreated with Cyt D acquired significantly less antigen from donor B cells, i.e., mean PE intensity 528 ± 56 versus 338 ± 33 ($p < 0.002$) (Figures 2 b, c). Treatment with Cyt D did not completely block uptake, as shown by the fact that FDCs began to recover once Cyt D was removed from the media. The reduction in PEIC uptake by FDCs is probably not due to effects of the Cyt D treatment on Cr2 expression because the amount of cell surface Cr2 was similar on FDCs treated with either PBS or Cyt D (Figure 2 d). The results suggest a pathway by which capture of PEICs by FDCs requires actin-dependent internalization of the receptorligand complex that effectively removes the complex from the B cell surface. Thus, directional transfer of immune complexes on cell contact may be mediated by the active internalization of the FDC Cr2 protein.

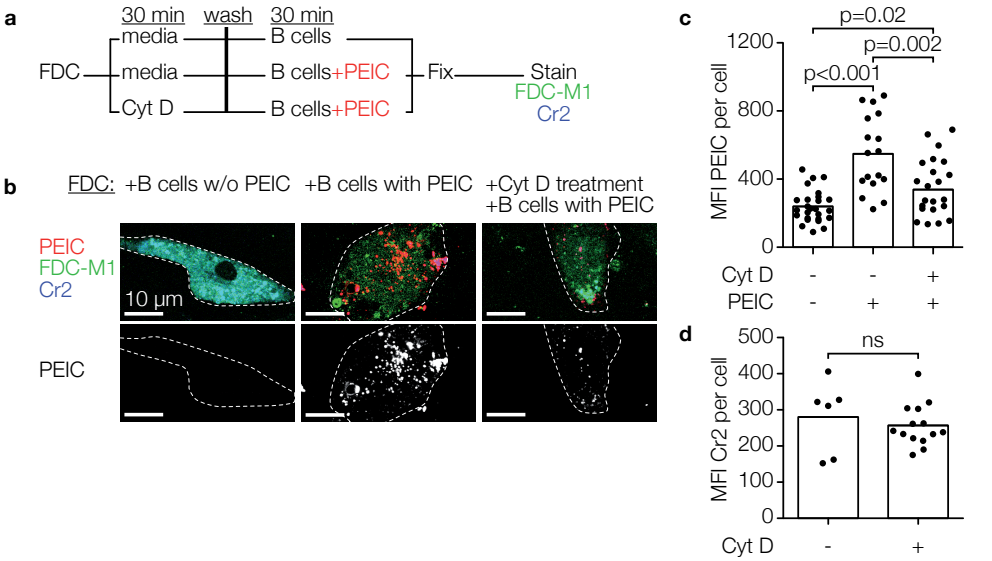


Figure 2 | Antigen Uptake by FDCs Is Actin-dependent

a | FDCs were treated with 5 mMCytochalasin D (Cyt D) or media for 30 min, washed, and then incubated with C3-opsonized PE-IC-loaded non-cognate B cells for another 30 min. **b |** Representative confocal images of fixed cells show that PE-IC (red) uptake by FDCs from B cells is diminished in Cyt D-treated FDCs. FDCs were identified by FDC-M1 (green) and Cr2 (blue) staining. **c |** Quantification of confocal images by Volocity software showing PE mean fluorescent intensity per cell (PE intensity/number of voxels) for each group. **d |** Cr2 MFI is not altered by Cyt D treatment. Cumulative data of FDC culture from three mice over two independent experiments (minimum of 15 cells per group). Each symbol represents a single cell; horizontal bar is mean value. p values calculated by Student's t test; ns, not significant.

Internalized ICs Return to FDC Surface

The finding that PEICs are internalized upon transfer suggests that they may be returned to the cell surface to be acquired by cognate B cells as identified in vivo[1-3,23,25].

To test this possibility, two separate approaches were used, i.e., imaging of the cell surface ICs by antibody staining after a mild acid wash and flow cytometry to detect acquisition of surface antigen by cognate B cells after a mild acid wash. FDCs were loaded with PEICs, then washed with a mild acid solution to remove surface-bound ICs, and then either fixed and stained with anti-PE or cultured in media to allow recovery (Figure 3 a). Staining of acid-washed and fixed FDCs with anti-PE confirmed removal of detectable surface antigen (Figure 3 a). By contrast, acid-washed cells allowed to recover in media for 30 min before fixation and antibody staining revealed PE on the cell surface (Figure 3 a). Staining of the recovered cells with antibody to complement C3 identified colocalization with the PEIC supporting that the ICs are returned to the cell surface intact (Figure 3 a). Quantification of these results show negligible staining for surface PEICs on acid-washed and fixed FDCs relative to FDCs allowed to recover prior to staining with PE antibody (Figure 3 b, left). Removal of surface bound C3 by the acid treatment was less efficient than PEICs (Figure 3 b, right). Thus, based on imaging, endocytosed PEICs are returned to the cell surface where they are accessible to antibody binding. Furthermore, PEICs colocalize with C3, suggesting that the ICs are retained intact during cycling.

As a second approach to evaluate whether endocytosed PEICs return to the cell surface and to determine whether antigen is functionally intact, FDCs, loaded with PEICs (control) or turkey egg lysozyme (TEL) coupled to PE (TEL-PEIC) and acid washed, as described above, were cultured with cognate B cells and uptake of antigen was assayed by flow cytometry (Figure 3 c). FDCs were incubated with MD4 B cells specific for hen and turkey lysozyme for 2 or 4 hr to allow for their acquisition of antigen[38]. After exposure to acid-stripped FDCs, the B cells were harvested and prepared for analysis by flow cytometry. As expected, the TEL-specific MD4 B cells cultured with FDCs loaded with PEICs were negative for uptake of antigen, demonstrating that antigen uptake is specific (Figure 3 d). Notably, cognate B cells incubated 2 or 4 hr with FDCs loaded with TEL-PEICs efficiently acquired PE relative to the control FDCs. By contrast, FACS analysis of MD4 B cells cultured with acid-stripped FDCs that were fixed with 1% PFA showed negligible uptake of the labeled antigen (Figure 3 d). Thus, based on flow cytometry analysis, endocytosed PEICs return to the surface of FDCs after acid stripping where it is accessible for acquisition by cognate B cells.

To determine if uptake of cognate antigen in the ex vivo model results in B cell activation, MD4 B cells were cultured as above for 2 hr with FDCs loaded with PEICs or FDCs loaded with TEL-PEICs. B cells were harvested and assayed by flow cytometry after overnight culture. As expected, culture of MD4 B cells with FDCs loaded with nonspecific antigen (PEICs) resulted in negligible activation. By contrast, MD4 B cells exposed to FDCs loaded with specific antigen (TEL-PEICs) were activated and expressed the B cell activation marker CD86 (Figure 3 e). The combined results demonstrate that internalized antigen returns to the FDC surface where its acquisition by cognate B cells can lead to activation.

It is possible that cognate B cells pick up antigen from dying FDCs in the ex vivo cultures and that cell surface display of antigen is not an active process. To address this

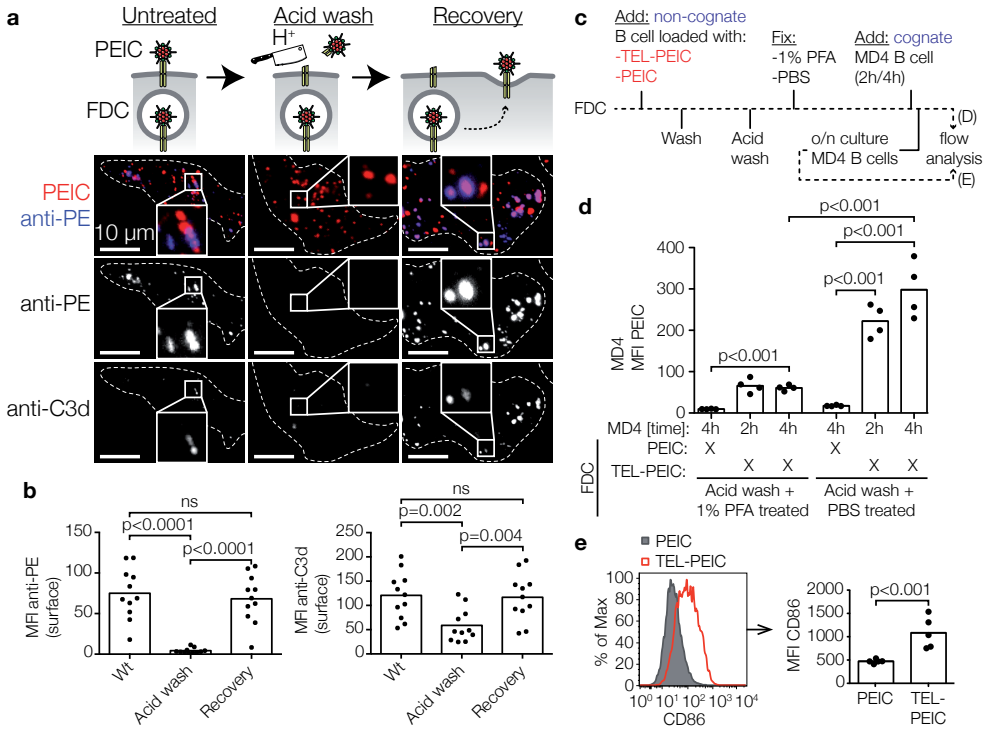


Figure 3 | Antigen Acquired by FDCs Resurfaces

a | Schematic diagram of FDC loading and acid wash procedure and recovery. PE-ICs appearing on the surface of the FDC after acid wash must come from inside the cell. FDCs were loaded with C3-opsonized PE-ICs, then either (1) fixed, (2) acid washed and fixed, or (3) acid washed, recovered for 30 min, and then fixed. Cells were stained with PE and C3d antibody. **b |** Quantification of the results shows efficient stripping of C3-opsonized PE-ICs by acid wash and recovery of PE-ICs on the surface after 30 min. C3d remains fixed on PE-ICs during resurfacing. **c |** Schematic representation of the experiment. FDCs were loaded with TEL (turkey egg lysozyme)-PE-ICs or PE-ICs by non-cognate B cells in culture. FDCs were acid washed to remove cell surface antigen and fixed with 1% PFA or left in media alone to recover. MD4 Ig transgenic B cells (specific for TEL) were mixed with FDCs for 2 or 4 hr in culture, then harvested, stained with B220, and analyzed by flow cytometry. **d |** Results indicate efficient uptake of TEL-PE-ICs by cognate MD4 B cells when cultured with live FDCs but negligible uptake of PE-ICs (without TEL) or TEL-PEICs from FDCs after acid wash and fixation. **e |** MD4 B cells were cultured for an additional 20 hr before staining with anti-CD86 to assay for B cell activation. Results indicate that cognate B cells express CD86 when exposed to specific antigen on FDCs but negligible expression when cultured with FDCs loaded with nonspecific PE-ICs. p values calculated by Student's t test; ns, not significant.

question and to determine whether the return of antigen to the FDC surface is actin-dependent, B1-8 B cells specific for NP hapten; 4-hydroxy-3-nitrophenyl acetyl, were cultured with FDCs loaded with NP-PEICs[39]. To block actin-dependent cycling, FDCs were first treated with 1 or 5 mM Cyt D for 30 min and then washed to remove Cyt D before mixing with cognate B cells (Figure 4 a). Cyt D is known to interfere with B cell receptor internalization, so it was important to remove the actin inhibitor before adding cognate B cells. The B1-8 NP-specific B cells were distinguished from non-transgenic B cells carried over from the knockin B1-8 mice based on the IgM^a allo-marker of the immunoglobulin heavy chain (IgH) knockin allele (Figure 4 b). Flow cytometry analysis of NP-specific B cells cultured with antigen-loaded FDCs identified capture of a similar

range of antigen as described above with the TEL-PE and MD4 B cells (Figure 4 b, c). Although 1 mM Cyt D had limited effect on uptake of cognate antigen, treatment of the FDCs with 5 mM Cyt D resulted in a significant reduction in uptake by the IgM^{a+} B1-8 B cells relative to the media-alone control, i.e., 12% ± 1% versus 21% ± 0.3%, respectively (Figure 4 c). As expected, the IgM^{b+} non-cognate B cells take up negligible amounts of NP-PEICs (Figure 4 b). It is noted that the effects of 5 mM Cyt D treatment on the FDCs probably represent an underestimate because the FDCs begin to recover from the actin inhibitor during culture with the cognate B cells.

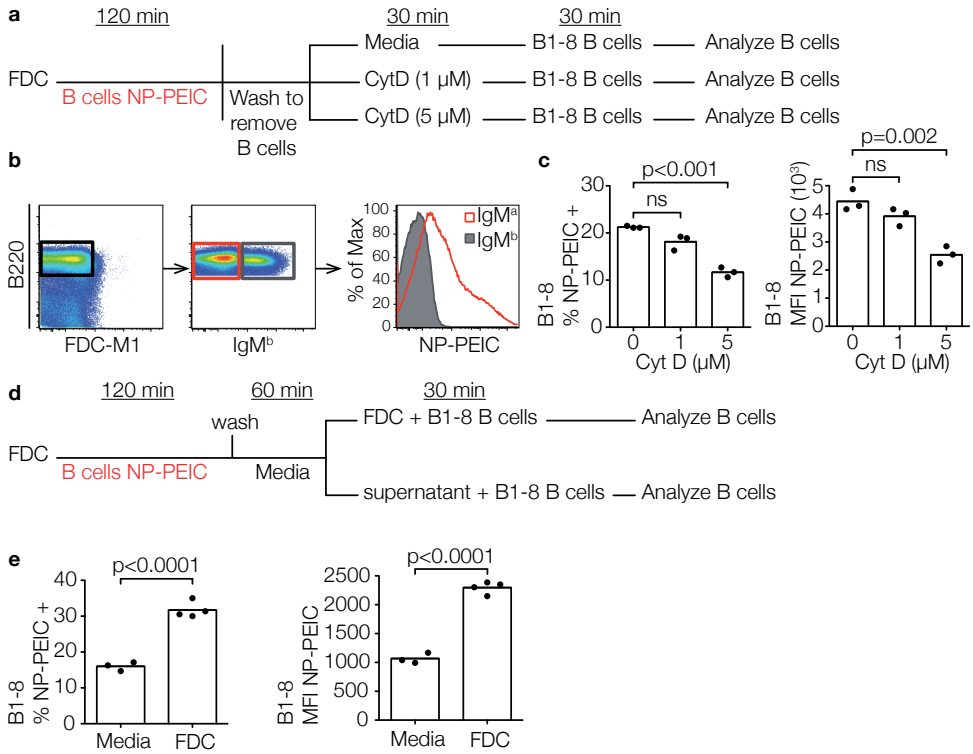


Figure 4 | FDCs Display of Antigen for Acquisition by Cognate B Cells Is Actin-dependent

a | Non-cognate B cells bearing clusters of NP haptenated PE-ICs were incubated with FDC culture for 2 hr. Non-cognate B cells were removed by washing and pulsed FDCs were treated with media alone or with 1 or 5 mM Cyt D for 30 min. Cyt D was removed and the FDCs were incubated with a mix of WT (IgMb) and NP-specific B1-8 (IgMa) B cells for 30 min before harvest and analysis by flow cytometry. **b |** Gating scheme for NP-PE-ICs bound by B1-8 B cells (B220+, IgMa+). Histogram shows that uptake is specific for B1-8 (IgMa) but not WT (IgMb) B cells as expected. **c |** Percent PE-positive B1-8 B cells (left) and the MFI of NP-PE-IC bound by B1-8 B cells (right). Results indicate that display of specific antigen on FDCs is actin dependent. Results shown are from three separate experiments with three mice each. **d |** FDCs were loaded with NP-PE-ICs as described above and cultured for 60 min before removal of the FDCs. Subsequently, B1-8 B cells were mixed with FDC supernatant or directly with FDCs in fresh media for 30 min. **e |** B1-8 cells were harvested and analyzed by flow cytometry as in **b** and **c**. Results show that efficient acquisition of antigen by B1-8 B cells requires intact FDCs. Results represent three separate FDC cultures derived from two sets of mice. p values calculated by Student's t test; ns, not significant.

An alternative possibility for B cell acquisition of antigen in the ex vivo cultures is that antigen is released by viable FDCs into the media before it is taken up by cognate B cells. To test this possibility, NP-specific B cells were cultured for 30 min either with media taken from a 2 hr culture of FDCs loaded with NP-PEICs or with the FDCs taken from the culture (Figure 4 d). NP-specific B cells efficiently acquired antigen when mixed directly with FDCs as expected; however, substantially less antigen was taken up when the cognate B cells were incubated with the FDC culture media (Figure 4 e). Importantly, acquisition of antigen by cognate B cells is dependent on presence of loaded FDCs because supernatant from FDC culture alone is not an efficient source. The combined results support a dynamic process by which internalized ICs are returned to the FDC surface by a process that is actin-dependent.

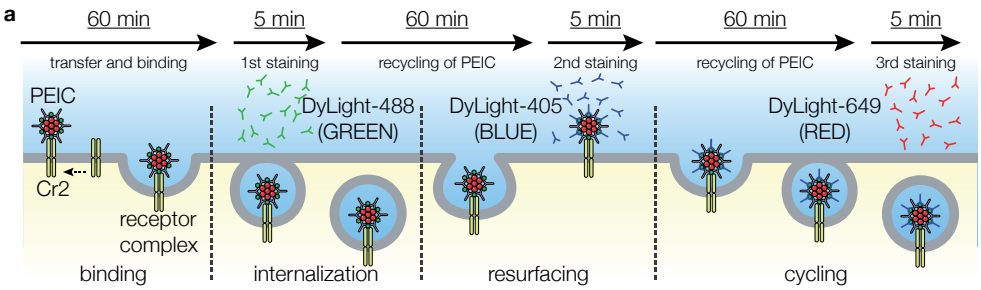
Immune Complexes Periodically Cycle to FDC Surface

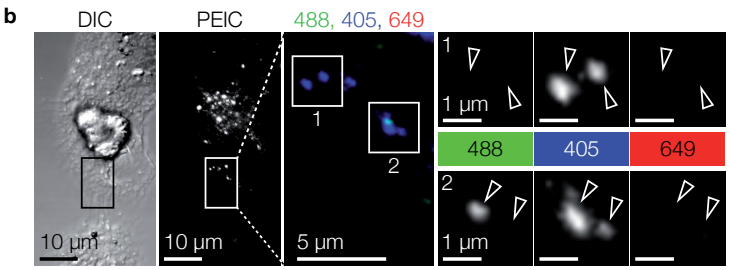
Our results with the ex vivo cultures show that FDCs capture C3-coated PEICs from non-cognate B cells and rapidly internalize them into endosomal vesicles, some of which return to the cell surface intact. A process in which ICs cycle continuously from inside to outside of the FDCs would provide a novel pathway to retain antigen for extended periods without degradation. To examine whether PEICs are included within a cycling pathway, FDCs were loaded with C3-coated PEICs as described above and stained sequentially with donkey anti-rabbit IgG labeled in three different colors: DyLight 488 (green), DyLight 405 (blue), or DyLight 649 (red) (Figure 5 a). After final staining, FDCs were washed and fixed for confocal imaging. To confirm that the secondary donkey antibody stably binds to rabbit Ig IC, non-cognate B cells loaded with PEICs were stained with either donkey anti-Ig labeled in DyLight 405 or 649 or both, then B cells were cultured at 37°C for 60 min. Subsequently, the cell supernatants were added to B cells bearing PEICs to determine whether the donkey antibody label could transfer between PEICs. Analysis of the stained cells by flow cytometry demonstrates negligible antibody transfer (Figures S3 a, b).

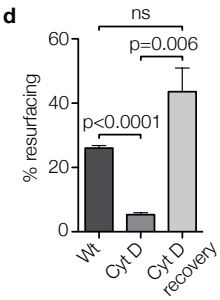
Sequential staining of FDCs with the three secondary antibodies results in four “states” of the PEICs based on the different color combinations: surface, internalization, resurfacing, or cycling (Figure 5 b, c). For example, a high-magnification image of a PE^+ particle visualized in each channel of fluorescence identifies no fluorescence in the green or red channels but is positive for blue (Figure 5 b, inset 1). Thus, the particle is scored as cycling because it was unavailable for the first antibody, stained with the second (blue), but was inside again for the third antibody. In a second example, the PE^+ particle was identified as internalized because it was positive for the first two antibodies (green and blue) but not the third (red) (Figure 5 b, inset 2). Overall, 700 PE^+ particles were analyzed in each separate color channel and the results were quantitated for the resurfacing and cycling (Figure 5 c). For example, 94 (13.4%) PE^+ particles were identified as cycling and 182 (40%) PE^+ particles were surfacing (Figure 5 c). As expected, fixation of cells after initial loading with PEIC blocked cycling of PEICs (results not shown).

To test whether the cycling of antigen to the cell surface was actin-dependent, FDCs were first loaded with PEICs and then subsequently treated with 5 mM Cyt D to block

actin polymerization prior to antibody staining. As a control, Cyt D was washed out (Cyt D recovery) before staining. Results demonstrate that treatment of loaded FDCs with Cyt D before exposure to donkey anti-rabbit Ig significantly impairs resurfacing of PEICs relative to the recovery control, i.e., 5.3% versus 43.6% ($p = 0.006$) and cycling, i.e., 1.1% versus 9.9% ($p < 0.001$) of antigen (Figure 5 c, d). Thus, rabbit IgG-PE complexes transferred from non-cognate B cells are not degraded appreciably over







c

	PE								
	Surface	Internalization			Resurfacing			Cycling	
	488 405 649		488	488 405		488 649	405 649	405	total
Wild type	134	112	104	74	44	71	67	94	700
percentage	19.1%	41.4%			26.0%			13.4%	99.9%
Cyt D	204	293	13	5	14	6	9	6	550
percentage	37.1%	56.5%			5.3%			1.1%	100.0%
Cyt D recovery	64	43	22	17	38	52	47	31	314
percentage	20.4%	26.1%			43.6%			9.9%	100.0%

Figure 5 | Cycling of ICs Is Actin-dependent

a | Schematic representation of the experimental set-up for antibody cell surface staining: C3-opsonized PE-ICs is transferred to FDCs for 60 min before removal of B cells. FDCs are incubated with DyLight488 labeled donkey anti-rabbit IgG (green) for 5 min to detect surface PE-ICs. FDCs are washed, ICs are allowed to cycle, and the process is repeated with two additional labeled donkey anti-rabbit IgGs, i.e., DyLight405 (blue) and DyLight649 (red). This results in several color combinations that indicate cycling or resurfacing. **b |** Representative confocal image of FDCs with cycling PE-ICs. PE channel (gray value) was used to identify objects and then objects were scored for green (DyLight488, first staining), blue (DyLight405, second staining), or red (DyLight649, third staining). If the PE-IC object was blue only, it was scored as cycling (inset 1). If the object was blue and green, it was scored as internalization (inset 2).
c | Table of all possible color combinations, their interpretation, and the raw cumulative data. This setup will result in an underestimation of the actual number of cycling PE-ICs. **d |** Bar graphs showing percent PE-IC resurfacing (top) and percent PE-IC cycling (bottom). See also Figure S3. p values calculated by Student's t test; ns, not significant; data are represented as mean ± SEM.

time but a fraction remains intact and periodically cycles to the cell surface via an actin-dependent mechanism.

Long-Term Retention of Immune Complexes within Cycling Endosomes

To compare results from ex vivo cultures with capture of antigen by FDCs in vivo, mice previously treated with rabbit anti-PE Ig were injected subcutaneously with PE antigen (Figure 6 a). LNs were harvested at days 5 or 10 for analysis by immunohistochemistry (IHC) and extraction of FDCs for culture ex vivo and characterization. IHC analysis of LN cryosections of immunized mice at both time points confirmed deposits of PEICs on FDCs as expected (Figure 6 b and not shown). To prepare ex vivo cultures, FDCs harvested at day 5 from immunized mice were cultured on collagen-coated slides for an additional 6 days. Slides were stained for Cr2 and analyzed by confocal microscopy to confirm retention of PE at day 11 after immunization. Quantitation of individual FDCs isolated from the immunized mice relative to nonimmune controls identifies a significant MFI of PE^+ staining per cell as expected (Figure 6 c). Notably, the pattern of PE retained by ex vivo cultured FDCs is similar to that of the IHC images taken at day 10 after immunization in that both show retention of PE antigen (Figure 6 b, c). Thus, PEICs acquired by FDCs in vivo are retained by the FDCs for at least 6 additional days in culture.

To determine whether PEICs loaded on FDCs in vivo continued to undergo cycling for an extended period, FDCs were harvested at day 10 after immunization. Cells were allowed to recover in culture for an additional 6 days before acid wash and staining cell surface ICs with anti-PE. Image analysis confirmed removal of surface PEICs by acid wash as expected (Figure 6 d, e). By contrast, when FDCs were allowed to recover after acid wash and then stained, PE was detected on the surface (Figures 6 d, e). C3 staining was also detected on the surface of the recovered FDCs, indicating that the complement-coated PEICs are intact and cycle 16 days after loading (Figure 6 d, e). Overall, these results validate the findings with cultures of FDCs loaded with ICs in vitro and provide an explanation for how GC B cells may sample antigens in vivo over an extended period.

Discussion

Although it is well established that FDCs retain antigen over extended periods and that this retention is important for both primary and secondary B cell responses, how antigen is initially taken up and sequestered by FDCs without degradation and how it is subsequently made available to B cells has remained unexplained[1-3,23,25]. In the current study, we resolve this problem by identifying a set of steps by which non-cognate B cells, loaded with ICs either in vivo or in vitro, deliver antigen in the form of large aggregates to FDCs, where it is stripped from the B cells and rapidly internalized as smaller particles within a cycling compartment. Thus, we propose a cell-to-cell transfer followed by a cycling pathway in a non-degradative compartment that provides an efficient mechanism for FDC capture, internalization, long-term retention, and

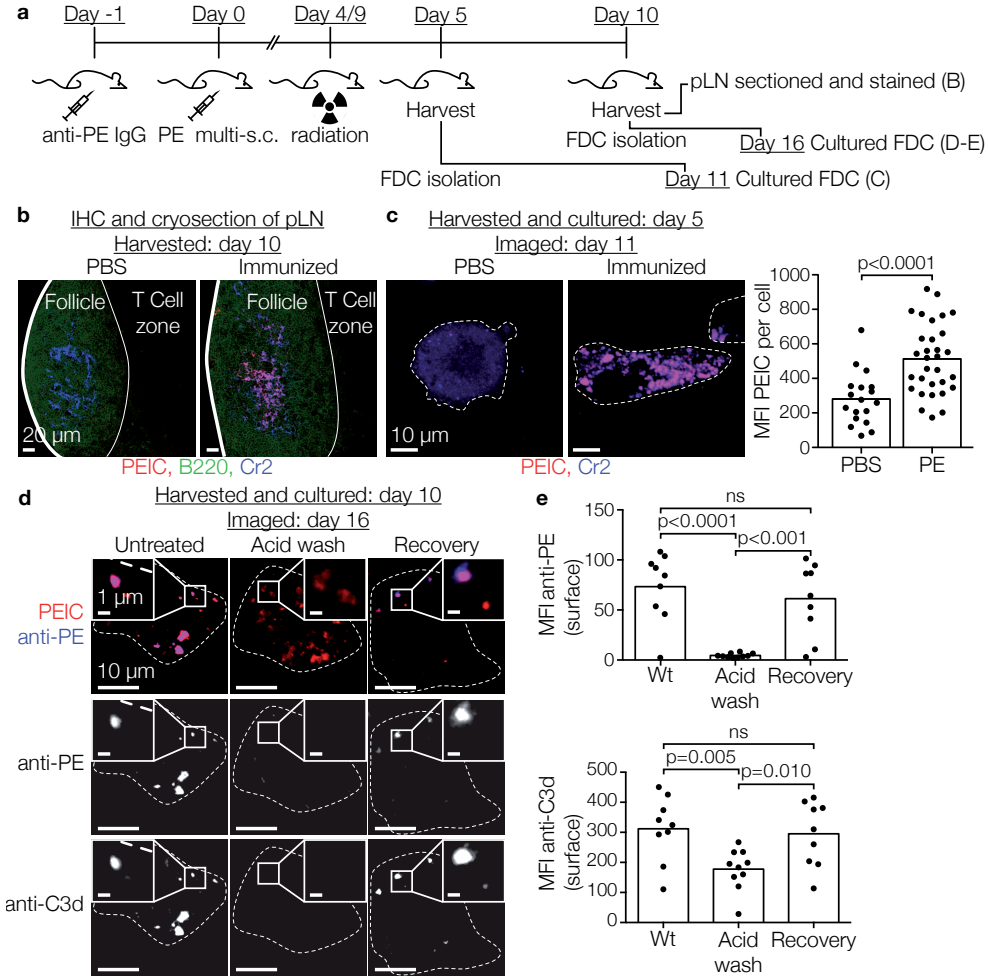


Figure 6 | Antigen Acquired by FDCs In Vivo Is Periodically Cycled to the Cell Surface

a | Schematic representation: mice were passively immunized with rabbit anti-PE on day 1, injected with PE on day 0, then irradiated on day 4 or 9 and FDCs isolated on day 5 or 10. Alternatively, draining popliteal LNs (pLN) were harvested and cryo-preserved on day 10. **b |** Sections of pLN show PE (red) localizing with Cr2 (blue) that marks the FDC within the B cell (B220, green) follicle at day 10 after immunization. **c |** Representative confocal images of FDCs harvested at day 5 after immunization with PE (red) and cultured in vitro until day 11. Cells were stained with antibody to Cr2 (blue). Quantification of images shows mean fluorescent intensity of PE per cell for FDCs isolated from immunized and PBS control mice. Each symbol represents a single cell. **d |** Representative confocal images of FDCs isolated from immune mice at day 10 and cultured until day 16. FDCs were either (1) fixed, (2) acid washed and fixed, or (3) acid washed, recovered for 30 min, and then fixed. Cells were stained with PE and C3d antibody. **e |** Quantification shows efficient acid wash and recovery of PE-ICs (top) and C3 (bottom) on the surface after 30 min. C3d remains fixed on PE-ICs during resurfacing. Results indicate that C3-PE-ICs recycle at day 16 after loading in vivo. p values calculated by Student's t test; ns, not significant.

presentation of native antigen to cognate B cells.

Earlier studies identified the novel pathway by which C3 coated ICs are trapped by sub-capsular sinus macrophages and shuttled to non-cognate B cells in the underlying B cell follicles. Subsequently, the B cells transport the ICs to the FDCs. The pathway was shown to be dependent on B cell expression of Cr2 because substantially less antigen was deposited on FDCs in chimeric mice bearing Cr2-deficient B cells[29,32]. Moreover, disruption of the pathway led to a reduction in affinity maturation of GC B cells[30]. Our observation when we used MP-IVM in anesthetized mice immunized with PE demonstrates direct transfer of the complement-coated PEICs from the B cells to FDCs. Although the overall efficiency of the process cannot be determined from the present study, it does demonstrate that non-cognate B cells hand-off antigen complexes directly. It seems most probable that other cell types such as resident dendritic cells may also participate in delivery of ICs to FDCs as proposed for transport of particulate antigens such as influenza virus[40].

FDC is the only cell type known to store antigen intact and accessible to cognate B cells for extended periods[3]. Macrophage and dendritic cells efficiently phagocytose ICs through immunoglobulin Fc receptors but the type of receptor determines the antigen fate [41]. For example, uptake of ICs by activating FcgR, i.e., FcgRI, FcgRI III, and FcgR IV, results in internalization and degradation. By contrast, uptake of ICs via the inhibitory FcgRIIB results in a transient return of the complexes to the cell surface for possible engagement by B cells[42]. However, unlike the pathway we describe for FDCs, ICs internalized by inhibitory FcgR are eventually degraded within 48 hr based on studies in vitro. Murine FDCs and B cells also express FcgRIIB but its expression is not required for uptake either in vivo or in our in vitro model[8,29]. However, given the findings of Bergtold and colleagues, it is possible that the Fc inhibitory receptor may be important in the cycling of the ICs once they are internalized via Cr2; this will be an important question to address in future studies[42].

Earlier results, based on electron microscopy studies, proposed that FDCs retained and periodically released antigen to B cells in the form of immune complex-coated bodies or ICCOSOMES, where it can be directly acquired by cognate B cells[1-3,23,25,26,28]. Our results are consistent with the earlier findings of antigen exposure on the FDC cell surface but add that the presentation of antigen is dynamic. Thus, we identify an active process involving cycles of surface presentation. The timing of these events is such that snapshots obtained by EM are unable to resolve how antigen is temporally displayed. Moreover, our results that uptake of antigen from FDC culture supernatant alone or disruption of cycling endosomes by actin inhibition or fixation leads to inefficient acquisition by cognate B cells support a direct contact model; however, we cannot rule out a role for release of immune complexes as an alternative source of antigen. It is possible that multiple pathways exist in which FDCs provide antigens to B cells. For example, in an inflammatory environment where there are ongoing GCs, the activation status of the FDCs may influence the display of antigen.

Our finding of periodic cycling of intact ICs by FDCs suggests a mechanism to explain

the observed retention of antigen over long periods. Another important factor in long-term retention of antigen is the intrinsic turnover of FDCs. It is unclear whether FDCs actively divide and retain antigen in daughter cells or if they are periodically replaced by precursor cells such as those recently described as platelet-derived growth factor receptor beta-positive perivascular cells[10]. Given the relative dense network of FDCs within the follicles, it is possible that after uptake and internalization, ICs are relayed to neighboring FDCs and this pathway could help explain the highly efficient retention. In future studies, it will be important to track the long-term fate of FDCs and how the immune status alters the local retention of antigen.

In addition to their known role within secondary lymphatic tissues, FDCs are found in tertiary lymphatic tissues that form at sites of chronic inflammation or infection where they are thought to be important in maintaining B cell follicles and germinal centers[10]. Because these ectopic sites form outside of normal lymphatic tissues, it is not clear whether antigen is delivered to the FDCs by one of the known routes, i.e., non-cognate B cells and FRC conduits or possibly dendritic cells[30-32,40,42,43]. Moreover, because the ectopic sites are often transient relative to known lymphoid structures, it will be important to learn whether the fate of ICs is regulated differently. For example, are ICs retained primarily in a non-degradative, cycling endosomal compartment or are ICs more actively shuttled to the lysosomes, limiting the period of antigen display?

Overall, the combined results resolve a long-standing question in humoral immunity and identify a pathway by which FDCs take up complement-coated ICs from non-cognate B cells via Cr2 and internalize them into a cycling endocytic pathway. For the most part, antigen is retained intact within a non-degradative compartment that periodically cycles to the cell surface, exposing it to cognate B cells.

Experimental procedures

Mice

C57BL/B6 background CD45.1, CD45.2, MD4, B1-8, Cr2-deficient, and EGFP mice were purchased from Jackson Laboratories and maintained in specificpathogen-free facilities at Boston Children's Hospital Program in Cellular and Molecular Medicine (PCMM), Harvard Medical School. Institutional Animal Care and Use Committees (IACUC) at Harvard Medical School and PCMM approved animal experimental protocols.

Immune Complex Generation

B-Phycoerythrin (PE) (Anaspec) was used as a model Ag. ICs were generated by mixing 10 mg of PE, 5 mg of rabbit anti-PE IgG (Rockland), and 10 ml freshly isolated C57BL/B6 serum (as a source of complement) in GVB++ buffer (Complement Tech) for 30 min at 37°C. 1 x 10^7 splenocytes from a C57BL/B6 mouse were then incubated with the immune complex mix for 30 min at 37°C to generate IC-bound B cells.

FDC Isolation and Ex Vivo Culture

FDC isolation and culture procedures were modified from that described[33]. In short, mice were irradiated with 1,200 rads by a ^{137}Cs irradiator located at the Immune Disease Institute at Harvard Medical School, one day prior to FDC isolation. LNs (brachial, axillary, inguinal, popliteal, cervical, lumbar, sacral, and mesenteric) were harvested from irradiated mice and digested with 0.26 U Liberase DH and 0.2 mg/ml DNase I (Roche). FDCs were enriched with MACS separation column (Miltenyi Biotec) according to manufacturer's protocol, with 0.7 mg FDC-M1 antibody (in-house) and 5 mg/ml biotinylated anti-rat kappa antibody (clone MRK-1, BD Biosciences). Positively selected FDCs were resuspended in FDC media (DMEM supplemented with 10% FBS, 20 mM HEPES buffer, 0.2 mM MEM nonessential amino acids, 2 mM L-glutamine, and 1 mg/ml gentamicin) and plated on collagen-coated (Rat tail derived collagen; Roche) glass coverslips (#1.5, 25 mm diameter, Warner Instruments) at a density of 5×10^5 FDCs per coverslip. 5–7 day cultured FDCs were used for different assays.

B Cell Isolation

Splenocytes from C57BL/B6, B1-8, and MD4 mice were enriched for B cells by negative selection via biotinylated anti-CD43 (S7 clone; BD Biosciences) followed by streptavidin micro beads (Miltenyi Biotech). Cells were separated by MACS columns (Miltenyi Biotech) according to manufacturer's protocol. Purity of the cells used for assays was greater than 90% as assessed by flow cytometry.

FDC and B Cell Assays

For different assays involving IC-bound B cells and FDCs, 2×10^6 B cells per 5×10^5 cultured FDCs were used. In experiments where the role of actin was analyzed, FDCs were either left untreated or treated with Cytochalasin D (Sigma-Aldrich).

Flow Cytometry

B cells were washed from FDC cultures after the assay and stained in a 96-well U-bottom plate. Anti-B220-PerCP and IgM^b-FITC antibodies were purchased from Biolegend. FDC-M1-Alexa 633 was made in-house. FACSCanto II (BD Bioscience) was used to acquire the samples and FlowJo software (Tristar) and Prism (Graphpad Software) were used for analysis of the acquired data.

Live Cell Labeling

B cells were labeled with the CellTracker dye CMFDA (Invitrogen) at final concentration of 2.5 mM for 15 min, followed by extensive washing, and used for adoptive transfer. For spinning disk epifluorescent or confocal microscopy, B cells and FDCs were labeled with DiI or DiO Vybrant Cell-labeling solution (Molecular Probes).

In Vivo Imaging

Mice were passively immunized with 1 mg of rabbit anti-PE IgG and pretreated by intravenous injection with fluorescent anti-Cr2 to label the FDCs in vivo. Purified, fluorescent labeled B cells were adoptively transferred as described. After 24 hr, 10 mg of PE was injected subcutaneously into the footpad. The popliteal LN was surgically exposed and MP-IVM was performed as described[40].

Cultured Cell Time-Lapse Imaging and Confocal Images

Cultured FDCs attached to glass cover slides were mounted in a chamber, which is filled with PEIC-bound B cells in FDC media and then installed in a heated stage plate (20/20 Technology) connected to a chamber insert (20/20 Technology) and placed in an environment chamber (Okolab), where temperature, humidity, and CO_2 levels were controlled during imaging. Epifluorescence microscopy was performed with a Mariana system (Intelligent Imaging Innovations) with Axiovert 200M microscope (Carl Zeiss MicroImaging) equipped with 60x oil, 100x oil objectives (Plan Apochromat, 1.4 NA, Carl Zeiss MicroImaging), coolSnap HQ II camera (Photometrics). Xenon lamp (DG-4, Sutter Instruments), with WF HQE and Misc. filter set (485/20, 560/25 and 650/13, Chroma Technology Corp) was used as a light source. Time-lapse movies were acquired in one optical section with between 15 s and 1 min intervals, with multiple positions.
The microscope is connected with a spinning disk confocal head (CSU22, Yokogawa electric) cascade camera (Photometrics) and piezo-driven stage (Application Scientific Instrumentation) for sample axial position control. Three lasers (491 nm, 561 nm, 660 nm) coupled to spinning head through an acoustic-optical tunable filter (AOTF) were used as a light source. Images were analyzed and 3D view rendered by Slidebook 4.2 or 5 (Intelligent Imaging Innovations).

Immunohistochemistry

LNs were dissected out and embedded in optimal cutting temperature compound (TissueTek). Cryosections were prepared at 10 mm thickness and fixed in 1% paraformaldehyde solution. Fixed samples were preincubated with anti-FcR (2.4G2, house produced) and 2% bovine serum albumin for blocking nonspecific and Fc-mediated binding. FDC M1 Alexa 488 (house labeled), donkey anti-rabbit DyLight 405, donkey anti-rabbit DyLight 488, and donkey anti-rabbit DyLight 649 (Biolegend) were used for staining ex vivo cultures of FDCs. Images were acquired with a FluoView FV1000 confocal microscope (Olympus) using a 20x lens (NA: 0.7) and processed with FluoView software (Olympus). Data were analyzed with Volocity software (Perkin-Elmer).

Electron Microscopy

Isolated FDCs were grown on collagen-coated ACLAR coverslips. Cells were preincubated with anti-FcR (2.4G2) and then labeled with anti-Cr2 (clone 8C12, in-house), followed by rabbit anti-rat IgG and protein A gold (Cell Microscopy Center, Department of Cell Biology, University Medical Center Utrecht, the Netherlands). For detection of ICs, cells were pretreated as above and then labeled with protein A gold. Popliteal LNs were harvested and sections were prepared as described[40]. Samples were analyzed on a Tecnai G2 Spirit Bio TWIN electron microscope (FEI Company) at the Harvard Medical School EM facility.

Supplemental Information

Supplemental Information includes three figures and three movies that can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013. 02.023.

Movie S1 | B Cells transport PEICs to FDCs in vivo
By multi-photon intra-vital microscopy, the popliteal LN of an anesthetized mouse was imaged for 60 min. The mouse was pre-injected with anti-Cr2 pacific blue (FDC outlined, blue), passively immunized with rabbit anti-PE antibody, and adoptively transferred with WT fluorescent labeled B cells (green). Imaging was done from 60 min to 120 min after PE injection (red) into the footpad. Green B cells acquire the PE at the sub-capsular sinus (SCS) and then migrate toward the FDC, where they deposit the PE onto the FDC (arrowheads). Time-lapse movie prepared with Volocity showing 12 frames per second. Higher magnification in inset prepared at 1 frame per second. Related to Figure 1.

Movie S2 | PEICs transfer from B Cells to FDCs in vitro
FDCs (green) were incubated with in vitro generated PEICs (red) bound to B cells at 37°C in a culture chamber for 2 hr. With wide-field microscopy, images were captured each minute. Time-lapse movie shows transfer of a cluster of PEICs from B cells to FDCs. PEIC clusters rapidly dissipate and spread on the FDC surface. Time (min:s); scale bar represents 10 μm. Related to Figure 1.

Movie S3 | 3D z-stack view of PEICs internalized by FDCs
Cultured FDCs (green) were incubated with PEIC-bound B cells for 2 hr and then image was taken by spinning disk confocal microscope; each optical plane was approximately 0.4 μm apart. Movie shows from the top to the bottom stacks merged with trans-planes. Related to Figure 1.

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Chapter 3

Follicular Dendritic Cells Retain Infectious HIV in Cycling Endosomes and are Long-Lived Depots in Patients on ART

III

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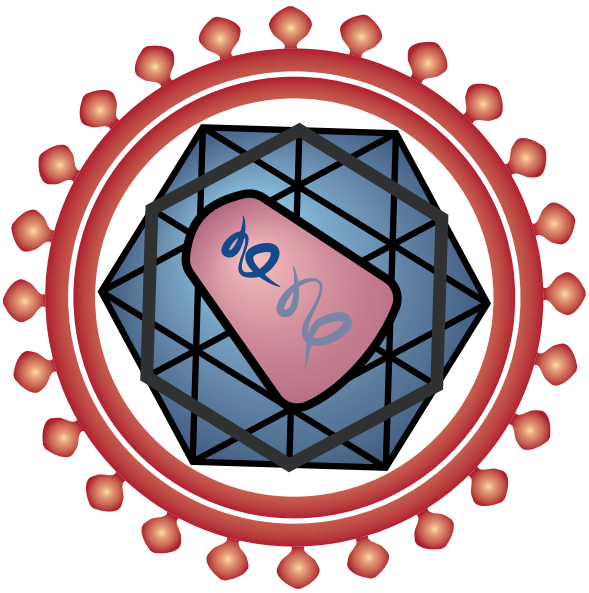
Under review



Abstract

Despite the success of antiretroviral therapy (ART), it does not cure Human Immunodeficiency Virus (HIV) and discontinuation results in viral rebound. Growing evidence implicates lymph nodes (LN) as a major site for continued infection of CD4+ T cells, but the cell sources of a persistent reservoir remain unclear. Follicular dendritic cells (FDC) are in direct contact with CD4+ T follicular helper cells (Tfh), a major subclass of infected T cells. Moreover, they retain intact antigen for prolonged periods. We found that human FDCs isolated from patients on ART retain infectious HIV within a cycling compartment and transmit infectious virus to CD4+ T cells in vitro. Importantly, treatment of the HIV+ FDC with a soluble complement receptor 2 fusion protein (sCD21-Ig) purges the FDC of HIV virions and prevents viral transmission in vitro. Our results suggest that sCD21-Ig could be a potential component of new therapeutic strategies to achieve functional cure or viral eradication in ART-treated HIV-infected humans.

Introduction

Anti-retroviral therapy (ART) is capable of suppressing plasma viral load to undetectable levels and in many cases results in restoration of circulating CD4+ T cell counts to near normal values. Despite the success of ART, when treatment is halted the virus rebounds[1]. Of note, ART is less effective in lymphatic tissues, suggesting that LNs are a major site for residual viral replication in ART-treated individuals[2]. In addition to latently infected memory CD4+ T cells, believed to be the major reservoir of HIV[3-5], other cell types can be latently infected including naive CD4+ T cells, macrophages, astrocytes and thymocytes[6-8]. Latently infected cells harbor replication-competent HIV DNA integrated in the genome, but are not productively infected and thereby escape immune detection. To this day all attempts to activate or alter these reservoirs in order to then deplete them with ART have failed[9-14]. Recently, treatment of HIV-infected humanized mice with broadly neutralizing antibodies (bNab) yielded promising results. The bNab were able to partly prevent seeding of the reservoir and viral rebound after ART was halted and demonstrated the importance of the Fc receptor (FcR) function in protection[15]. However, humanized mice lack certain immune cells, including follicular dendritic cells (FDC), which are known to retain antigen for extensive periods. Classically ,HIV reservoirs have been defined as latently infected cells, however a less restricted definition can include cells that retain replication-competent virus for a long time and are capable of reseeding new pools of susceptible cells, potentially including FDCs.

In secondary lymphoid tissues, FDCs are a source of the chemoattractant (CXCL13) for B cells and T follicular helper cells (Tfh), which is required for maintenance of follicle structure[16,17]. FDCs are stromal derived and long recognized for their ability to retain antigen as an immune complex (IC) for periods of at least one year in mice[18,19]. A mechanism of how FDCs can retain ICs in a non-degraded state for extensive periods was recently identified[20]. Notably, we found that IC opsonized with complement C3d

are internalized via CD21 receptor into a cycling endosomal compartment that co-localizes with the transferrin receptor. Thus, the results provide a mechanism by which IC are retained protected inside the cell but are periodically cycled to the cell surface for access by cognate B cells.

Simian Immunodeficiency Virus (SIV) rapidly seeds the reservoir even before detectable systemic viremia[21]. This observation suggests that cells in lymphoid organs, which are not reflected in systemic measures of viral load, are the first to become infected and constitute the initial reservoir. FDCs, which are located central to the B cell follicle, have been described to contain viral RNA, however their role as a potential reservoir or depot has not been fully explored[22,23]. It has been suggested that FDCs can be a persistent reservoir during ART due to the lower local concentrations of ART in the LN[2,24]. But the mechanism by which FDCs can trap and retain infectious HIV is not fully understood.

HIV is capable of independently fixing complement through complement factor I and, paradoxically, this enhances HIV infectivity in vitro[25-27]. Moreover, the recent finding that HIV coat proteins contain mannose groups suggests the lectin pathway of complement could participate via mannan binding protein in opsonization of viral particles[28]. Alternatively, HIV specific antibodies can, through the classical complement pathway, activate and deposit complement on the viral surface[29,30]. We propose that complement-opsonized HIV might exploit the recently identified mechanism of FDC uptake and cycling of IC. This mechanism could provide a safe-haven for the virus, allowing it to escape both ART and detection by the immune system while remaining infectious. We sought to test this hypothesis by specifically determining the ability of FDCs from LNs of HIV- and HIV+ donors on prolonged ART to carry infectious virions. We show that human FDCs cycle antigen and retain infectious HIV but, unlike CD4+ T cells, do not harbor provirus in their genome. Strikingly, the virus can be displaced by treatment with a soluble form of human CD21 receptor fused to murine IgG1 (sCD21-Ig). In FDCs from HIV+ donors on ART, treatment with sCD21-Ig prevents infection of healthy CD4+ T cells.

Results

Human FDC internalize and cycle immune complexes

To determine if human FDCs take-up and cycle antigen similar to murine FDCs, complement C3 opsonized phycoerythrin immune complexes (PEIC) were used as a model antigen[20]. The fluorescence characteristics and the availability of specific antibodies make PEICs easy to track inside and outside the cell. PE was incubated with serum and anti-PE antibody, leading to activation of complement components in the serum. A single cell suspension of cells was prepared from LNs of healthy donors and FDC were positively selected to approximately 80% purity using anti-CD35 magnetic beads and cultured on collagen-coated coverslips for four days, until the FDCs regained their dendritic morphology. Naive, non-cognate, allogeneic B cells loaded with surface bound PEICs were added to the cultures to mimic in vivo conditions and

select for successfully opsonized PEIC. FDCs acquire the PEIC from donor B cells in a CD21-dependent manner[20]. Following co-culture with B cells, the cultures were fixed with or without an acid wash treatment that strips the surface of PEIC. Allowing a recovery period after acid wash and before fixation allows restoration of surface levels from an intracellular source (Figure 1 a). To quantify PEIC on the cell surface versus total cellular levels, the ratio of the mean fluorescence intensity (MFI) of anti-PE to total PEIC was determined for each FDC (Figure 1 b). The acid wash treatment efficiently stripped the surface of PEIC and after recovery PEIC was detected on the surface of the FDC in a similar ratio as without acid wash. These data indicate that the PEIC was protected inside the cell during acid wash but cycled to the surface during recovery phase. These data show that human FDCs, similar to their murine counterparts, cycle immune complexes.

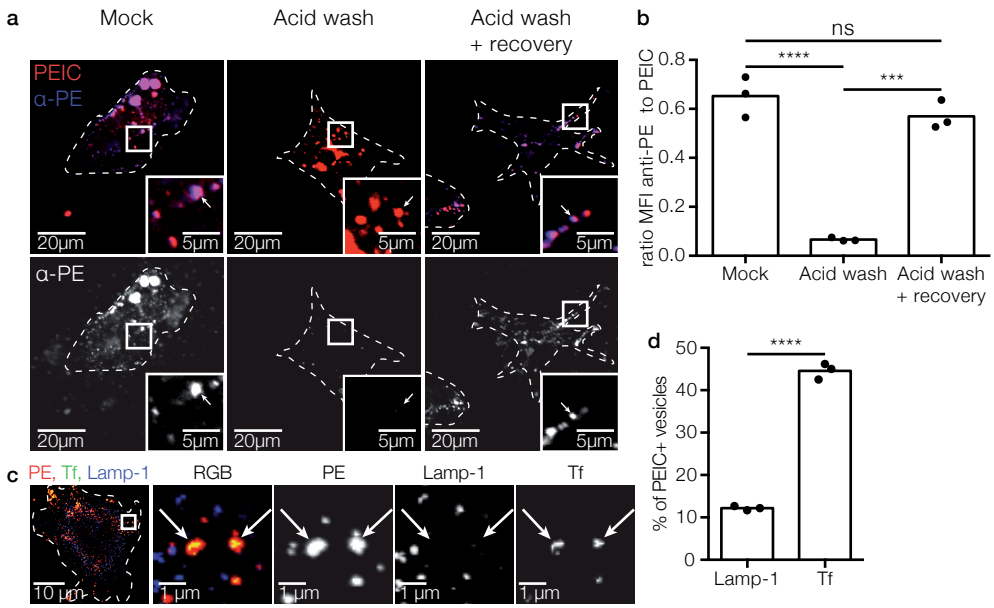


Figure 1 | Human FDCs recycle immune complexes in a transferrin (Tf) positive non-degradative endosomal compartment

a | Human FDCs from lymph node biopsies of HIV-negative volunteers were loaded with complement C3-opsonized phycoerythrin immune complexes (PEICs), then either (1) fixed, (2) acid washed and fixed, or (3) acid washed, incubated in media for 30 min (recovery), and then fixed. Fixed FDCs were stained with an antibody against PE. PEICs detected on the surface of the FDC after acid wash and recovery must have come from the inside of the cell. **b |** Quantification of the results shows efficient stripping of PEICs by acid wash treatment and a robust recovery of PEICs on the surface after 30 min. ***P<0.001, ****P<0.0001 (Two-way ANOVA, multiple comparisons) n=3 (3 subjects, 4 replicates each). **c |** On human FDCs PEIC reside mainly in Tf positive compartments, as is the case in mice. The recycling Tf compartment was visualized by incubation of live cells with fluorescent Tf for 8 minutes. **d |** The percentage of Tf or Lamp-1 positive vesicles within the PEIC positive vesicles was quantified. This shows ~45% of PEICs in Tf positive compartments and only ~12% in Lamp-1 positive compartments. ****P<0.0001 (Two-way ANOVA) n=3 (3 subjects, 6 replicates each).

To examine whether model IC are taken up and transported into the cycling or the lysosomal compartment, transferrin (Tf) and Lamp-1, respectively were used as endosomal markers. Lamp-1 primarily localizes with the degradative compartment, whereas Tf localizes mainly with the recycling endosomal compartment. The majority, about 45%, of PEICs resides in vesicles co-localized with Tf staining whereas only 12% was associated with Lamp-1 vesicles ($p<0.0001$). This indicates that the majority of PEIC cycle through a non-degradative endosomal compartment, while only a fraction of the PEIC is being degraded (Figure 1 c, d). This observation is in line with earlier observations in mice where immune complexes are degraded at early time points, until a steady state is reached[19,20]. In short, upon binding the FDC there are two potential fates for the immune complex: degradation in a lysosomal compartment or retention in a periodically cycling endosome. Over a period of time a stable state is reached in which the antigen retention remains at an apparent constant level. Thus, human FDCs appear to continuously cycle the majority of the antigen to their surface in a non-degradative endosomal compartment, similar to the pattern observed in murine FDCs.

Human FDC internalize HIV in vivo

To assess if human FDCs retain HIV and localize it to a similar compartment as observed with PEIC, inguinal LN tissue was harvested from HIV+ patients classified as chronic progressors (CP) and undergoing therapy with ART (Supplemental Table 1). FDCs were isolated and cultured for 5 days before imaging to regain their dendritic morphology. HIV was detected by confocal microscopy analysis of permeabilized cells after staining with antibody specific for HIV protein p24 (Figure 2 a, b). Not all FDCs were positive for HIV antigens, however when detected, HIV+ vesicles within FDC co-localized in 3 dimensions with transferrin but not with Lamp-1 (Figure 2 a, b; Supplemental Table 2). Thus, despite the prolonged ART (1 to 24 years, median = 8 years) a fraction of the FDC remained positive for HIV antigen in all subjects examined, and the HIV is primarily detected within the cycling endosomal compartment, and not in the lysosomes (23.3 ± 5.9%). No contaminating T cells were detected in the cultures.

In order to determine if human FDCs retain viral RNA in addition to viral protein, cellular RNA was prepared from enriched FDC harvested from HIV+ and HIV- donor LNs and analyzed by digital droplet RT-PCR (ddPCR). The copy number was normalized among similar samples using cell number as a baseline. As expected, no viral RNA was observed in FDCs from HIV- patients. By contrast, FDCs isolated from HIV+ donors retained significant levels of viral RNA (Figure 2 c). These samples are enriched for FDCs but potentially contain contaminating HIV-infected CD4+ T cells. As a control, fibroblast reticular cells (FRC), which are known to be important in the maintenance of T cell homeostasis in LNs, were isolated and cultured from the same LNs as the FDC using a similar protocol. Analysis of RNA extracted from the cultures of FRCs showed no viral RNA suggesting that contaminating HIV+ CD4+ T cells are unlikely to explain the presence of viral RNA in the FDC cultures (Figure 2 c).

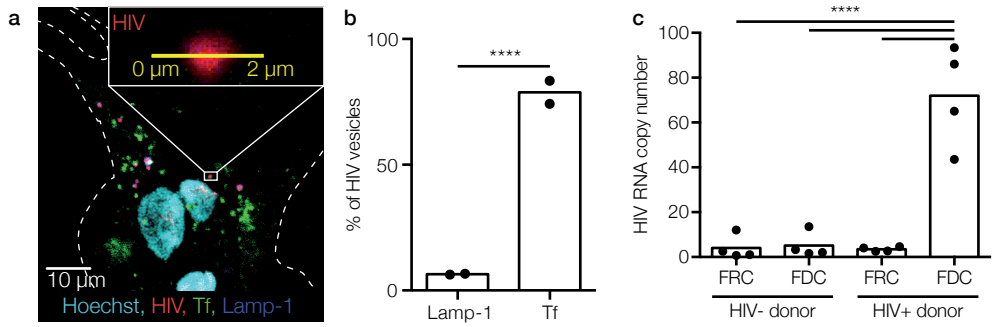


Figure 2 | Human FDCs isolated from HIV+ subjects on ART treatment retain HIV in a Tf+ compartment
a | Cultured human FDCs from HIV+ subjects on ART were stained for Tf (green), HIV (p24, red), Lamp-1 (blue) and Hoechst (cyan). (inset) A single vesicle was enlarged and only the red (HIV) channel was shown. The yellow line was used for line profile analysis, which measures the fluorescent intensity over that line in all channels. Line profiles were also made in the z direction to ensure co-localization of HIV+ vesicle in the X, Y and Z direction with Tf or Lamp-1. **b |** Quantification of line profile measurements of HIV positive vesicles. The vast majority of HIV containing vesicles (~80%) were positive for Tf. ****P<0.0001 (Two-way ANOVA) n=2 (2 subjects, 4 replicates each). **c |** FDCs and FRCs from HIV positive individuals on ART treatment and HIV negative volunteers were cultured and viral RNA levels were quantified. FRC and FDC were isolated from the same persons to control for contaminating CD4+ T cells. Each data point presents an individual subject. ****P<0.0001 (Two-way ANOVA, multiple comparisons) n=4 (4 subjects, 3 replicates).

Infectious HIV is retained by FDC via complement receptor CD21

To determine if HIV retained by FDCs is infectious, FDCs isolated from ART-treated HIV+ patients or healthy HIV- donors were co-cultured with activated CD4+ T cells prepared from PBMC of healthy volunteers. After 5 days of co-culture, CD4+ T cells were selectively removed and RNA was extracted. Analysis of viral RNA from T cells co-cultured with HIV+ FDC identified presence of HIV RNA (Figure 3 a). Moreover, DNA from CD4+ T cells co-cultured with HIV+ FDC from one volunteer were analyzed for viral DNA integration. This analysis confirmed viral integration in the genomic DNA of healthy CD4+ T cells after co-culture with HIV+ FDCs (Figure 3 b). Notably, the HIV+ FDCs did not have viral integration in the genomic DNA (Figure 3 c). Thus, FDC, unlike known reservoirs for HIV, do not retain HIV provirus. As expected, CD4+ T cells co-cultured with FDC isolated from HIV- donors were negative for HIV RNA and DNA. Thus, FDCs isolated from ART- treated patients not only retained viral RNA but were able to transmit virus to healthy CD4+ T cells, functionally rendering them an infectious reservoir.

Complement receptor CD21 is expressed primarily on B cells and FDCs in both human and murine tissues. Earlier studies identified an important role for CD21 in HIV retention in murine lymphoid tissues[31]. Likewise, human B cells have been identified in the blood of HIV+ patients with surface bound C3d-opsonized HIV[32]. Treatment of such HIV+ B cells with a blocking antibody to the C3d-binding site releases the HIV particles, suggesting that CD21 binding to C3d is a major mechanism for viral uptake[32]. Similarly, treatment of LN extract prepared from HIV+ donors with a blocking CD21 antibody reduced infection of T cells, although the cellular source of HIV was not determined[31].

To determine if human FDC retain HIV via CD21, FDCs positive for virus were cultured overnight with either a fusion protein of the C3d binding domain of human CD21 and

murine immunoglobulin (sCD21-Ig) or an isotype control. Strikingly, levels of both virus antigen and RNA were reduced to background following treatment (Figure 4 a-c). These results demonstrate that FDCs retain HIV in ART-treated patients primarily via CD21, and that sCD21-Ig can "purge" FDCs of virus. We hypothesize that sCD21-Ig competes for CD21 binding of C3d on the virion, which is then released in the media. Analysis of the supernatant by ddPCR yielded no presence of HIV virions; however there could be numerous explanations for the inability to detect the virus. Although the exact mode of action has not been shown, treatment with sCD21-Ig empties the cycling compartment of FDCs and removes HIV from the system.

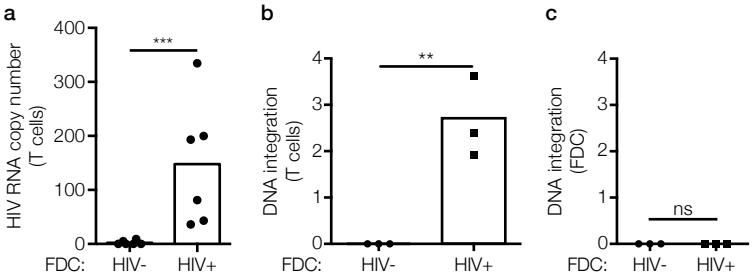


Figure 3 | FDCs are a source of infection for T cells

FDC cultures (10^4 to 10^5 cells) of HIV+ subjects on ART were co-cultured with activated CD4+ T cells (10^6 cells) from PBMC of healthy donors for 5 days. **a |** RNA from CD4+ T cells was collected for analysis. T cells co-cultured with FDCs from HIV+ subjects contained viral RNA, in contrast to the T cells co-cultured with FDCs from healthy HIV- donors. This indicates that HIV on the T cell originated from the FDC. Each data point presents an individual subject. ***P<0.001 (Two-way ANOVA) n=6 (6 subjects, 2 replicates each). **b |** DNA from CD4+ T cells was collected for analysis. T cells co-cultured with FDCs from HIV+ subjects had viral DNA integrated in their genome, in contrast to the T cells co-cultured with FDCs from healthy donors. This indicates active infection of T cell. **P<0.01 (Students *t* test) n=1 (1 subject, 3 replicates). **c |** DNA from FDCs was collected for analysis. FDCs from HIV+ subjects had no viral DNA integrated in their genome, just as FDCs from healthy donors. ns = not significant (Students *t* test) n=1 (1 subject, 3 replicates).

Treatment of FDC with soluble CD21-Ig blocks transmission of infectious virus

To test whether blockade of C3d binding by CD21 could prevent transmission of infectious virus, sCD21-Ig was added to FDC cultures derived from HIV+ LNs that were then co-cultured with uninfected CD4+ T cells as described above (Figure 3). Notably, treatment of FDC cultures with sCD21-Ig reduced viral infection of CD4+ T cells to an undetectable level (Figure 4 d). In vivo this approach could reduce infection of T follicular helper cells (Tfh) by acting on two levels. First, transfer of HIV from B cell to Tfh could be blocked by sCD21-Ig and second, transfer of HIV from FDCs to CD4+ T cells could also be blocked by sCD21-Ig, as shown with these experiments here.

As further evidence that FDCs retain infectious HIV, a cocktail of broadly neutralizing antibody (bNab) was added to HIV+ FDC cultures prior to co-culturing with CD4+ T cells. The bNabs used (VRC01, PG16 and PGT121) all bind to the CD4 binding site of gp120[33]. As expected, the bNab cocktail, which will not block CD21 binding to C3d, failed to purge FDC of viral antigen (Figure 4 b). By contrast, the presence of bNabs reduces transmission of virus to CD4+ T cells to a background level of detection (Figure 4 d). This suggests that the virions are accessible to bNabs during the overnight

treatment and this neutralizes the virions so that no infection then can occur during the co-culture. The FDC storage compartment might be accessible to the fluid phase or it might be exposed during recycling. The purging of the human FDC is likely not FcR mediated since the addition of neither human bNab nor the murine isotype control IgG1 showed blocking activity.

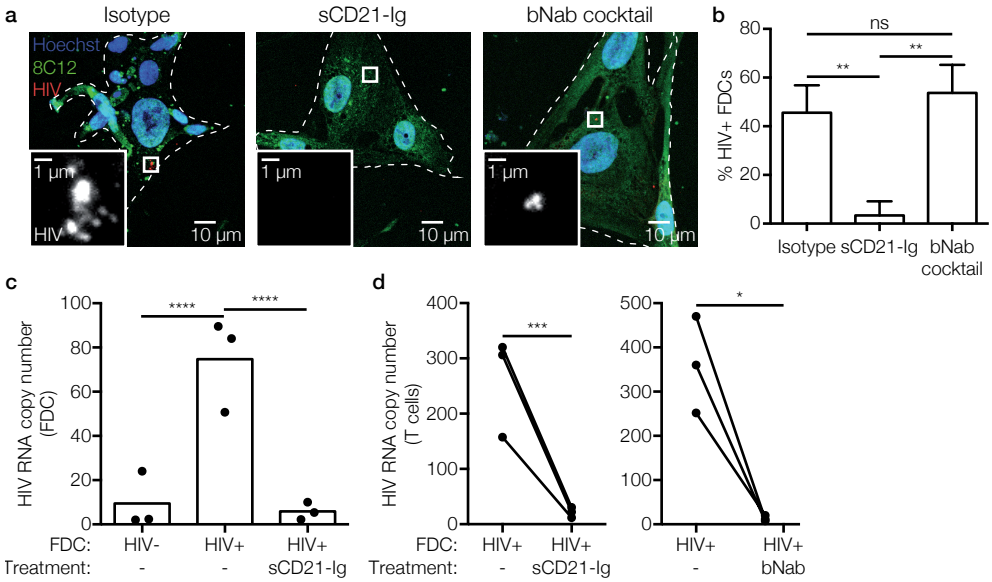


Figure 4 | sCR2-Ig can purge the HIV cycling compartment in FDCs

FDCs were isolated from LNs of HIV subjects on ART and cultured for 4 days. CD4 T cells from HIV negative individuals were activated in parallel. On day 4 FDC cultures were treated overnight with an isotype control antibody, soluble CD21-Ig (sCD21-Ig) or a cocktail of 3 broadly neutralizing antibodies (bNab; VRC01, PG16 and PGT121). Then cultures were washed and co-cultured for 5 additional days with 10^6 activated CD4 T cells. On day 5 samples were split into 3 groups; FDCs for imaging **(a, b)**, FDCs for RNA quantification **(c)** and T cells for RNA quantification **(d)**. **a |** FDCs were fixed, stained for p24 (HIV, red), 8C12 (FDC, green) and Hoechst (blue) and imaged by confocal microscopy. Virions were detected in the isotype and bNab cocktail treated cultures, but not in the sCD21-Ig treated culture. **b |** Percentage of HIV containing FDCs. 10 random field of views were collected and quantified per sample. ns: not significant, **P<0.01 (Two-way ANOVA, multiple comparisons) n=3. **c |** HIV RNA quantification of FDCs after sCD21-Ig treatment. Each data point presents an individual subject. ****P<0.0001 (Two-way ANOVA, multiple comparisons) n=3 (3 subjects, 3 replicates each). **d |** HIV RNA quantification of T cells incubated with FDCs treated with sCD21-Ig (left panel) or bNab cocktail (middle panel). *P<0.05, ***P<0.001 (Two-way ANOVA) n=3 (3 subjects, up to 3 replicates).

Discussion

Despite the success of ART in reducing HIV viral loads and partial restoration of circulating CD4+ T cells, cessation of the drugs results in acute viral rebound, suggesting the importance of a viral reservoir. While it is generally held that latently infected CD4+ T cells are a major reservoir for HIV, there is growing evidence that LNs are partly refractory to ART and include one of the earliest seeded reservoirs.

We find that FDCs retain infectious virus in HIV+ subjects undergoing long-term treatment with ART who have low to undetectable plasma viral loads. Earlier findings of

Schmitz et al., who analyzed FDCs extracted from LNs of HIV-infected donors not on ART, showed HIV RNA on FDC, but not proviral DNA[24]. Although they did not show if the virus on the FDC was infectious, they did show that the FDC itself was not infected. Novel insights in the mechanism by which FDCs retain antigen showed a more dynamic process than retention on the surface. FDCs cycle antigen through a non-degrading endosomal compartment, which allows the antigen to be protected inside the FDC while remaining available on the surface[20]. We show human FDCs utilize a similar mechanism for antigen and that HIV resides in the same compartment in subjects on ART. Furthermore we confirmed that, in donors on ART, FDCs contain viral RNA, but no proviral DNA and thus are not infected. Despite not being productively infected, we showed that FDCs are a source of infection for CD4+ T cells. Dendritic cells (DC), which are unrelated to the stromal derived FDC, are also capable of recycling HIV and form a source of infection for CD4+ T cells. However, unlike FDCs, DCs are not long lived and thus don't retain antigen for extensive periods. FDCs are a unique reservoir because they are not infected by HIV, long-lived and are a source of infection.

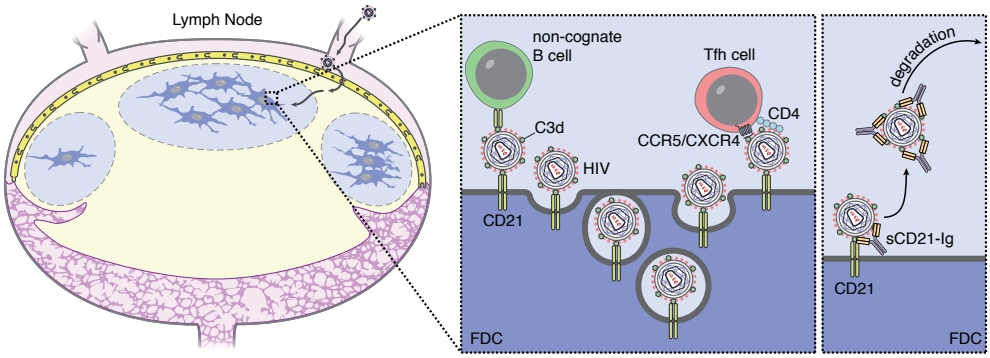


Figure 5 | Model of FDC HIV retention, transfer to T cells and sCD21-Ig purging
Schematic model. HIV is captured and subsequent cycled by complement receptor 2 (CD21) on the follicular dendritic cell (FDC). The HIV virion resides in the protective recycling endosome of the FDC. Upon emerging from the endosome at the cell surface, HIV can infect surrounding T follicular helper (Tfh) cells that have been attracted to the FDC by a CXCL13 gradient. Infection occurs through binding of CD4 and either CXC-chemokine receptor 4 (CXCR4) or CC-chemokine receptor 5 (CCR5) as a co-receptor by gp120. The soluble CD21 receptor fusion protein (sCD21-Ig) competes with the CD21 on the FDC for binding of complement C3d on the virion. This facilitates the release of the virion and makes it available for degradation by other cells. To prevent infection of T cells at this stage the treatment should be combined with broadly neutralizing antibodies (bNab).

Moreover, we found that the sCD21-Ig fusion protein can purge FDC of virus and thereby prevent transmission of virus to CD4+ T cells in vitro. This supports a mechanism for viral retention in which CD21 binds and internalizes C3d-opsonized virus and retains it for extended periods in a non-degradative cycling endosome.

We hypothesize that C3-opsonized HIV particles are transported to the FDC by B cells, as has been shown with immune complexes, and recycled until it encounters a CD4+ Tfh (Figure 5). Tfh have been described as the cell type most infected by HIV and are attracted to the FDC by a CXCL13 gradient.

Although the transfer event has not been studied here, we propose a mechanism in which the integrins LFA and VLA4 on the Tfh can bind to respectively ICAM and VCAM on the FDC[35]. This would provide the proximity needed for the interaction of gp120 with CD4 and CCR5.

Our results suggest that sCD21-Ig could provide an adjunct therapy to ART or could be used in combination with bNab and provide a potential component of new therapeutic strategies to achieve a functional cure or viral eradication in ART-treated HIV-infected humans.

Patient ID	Phenotype	CD4 count	viral load	ART (yrs)
1	Negative	ND	0	0
2	Negative	ND	0	0
3	Negative	ND	0	0
4	CP	522	61	1
5	CP	1258	79	10
6	CP	712	<20	24
7	CP	417	<20	8
8	CP	554	<20	7
9	CP	918	<20	6
10	CP	392	<20	13

Table 1 | Subject data

Phenotype negative is HIV- healthy subjects. CP phenotype is chronic progressor. ART (anti-retroviral therapy). CD4 count is per ml of blood. Viral load was determined one week before surgery with a method that has a detection limit of 20.

Patient ID	FDCs/well (calculated)	# FDCs analyzed	# HIV+ FDC	% HIV+ FDC	# HIV+ vesicles	HIV+ vesicles per HIV+ FDC
1	100k	112	0	0	0	0
2	40k	48	0	0	0	0
3	70k	79	0	0	0	0
4	75k	86	17	19.8	24	1.4
5	50k	56	7	12.5	12	1.7
6	60k	68	11	16.2	17	1.5
7	20k	-	-	-	-	-
8	35k	-	-	-	-	-
9	30k	36	8	22.2	23	2.9
10	165k	187	86	46.0	206	2.4

Table 2 | Table summarizes number of FDCs analysed by confocal per subject sample

The number of FDCs per well was calculated by counting FDCs in multiple field of views (FOV) with a known area and extrapolating this number to the surface area of the well. Microscopy combined with p24 staining determined HIV positive vesicles.

Experimental procedures

Mice

C57BL/B6 background mice were purchased from Jackson Laboratories and maintained in specific- pathogen-free facilities at Boston Children's Hospital Program in Cellular and Molecular Medicine (PCMM), Harvard Medical School. Institutional Animal Care and Use Committees (IACUC) at Harvard Medical School and PCMM approved animal experimental protocols.

Human subjects

Written, informed consent, approved by the Partners Human Research Committee of the Massachusetts General Hospital, was provided and signed by study participants before enrollment in the study. Research conformed to ethical guidelines established by the ethics committee of the Massachusetts General Hospital, University of Montreal Health Center. Inguinal lymph nodes were excised under anesthesia by a surgeon at Massachusetts General Hospital according to normal surgical procedures. Tissue was then transported on ice to the BL2+ facility at the Ragon Institute.

Immune Complex Generation

B-phycoerythrin (PE) (Anaspec) was used as a model Ag. ICs were generated by mixing 5 µg of PE, 5 µg of rabbit anti-PE IgG (Rockland), and 10 ml freshly isolated C57BL/B6 or human serum (as a source of complement) in GVB++ buffer (Complement Tech) for 30 min at 37°C. Splenocytes from a C57BL/B6 mouse were then incubated with the immune complex mix for 30 min at 37°C to generate IC-bound B cells. Alternatively the human B cell line Raji was used.

FDC Isolation and Ex Vivo Culture

FDC isolation and culture procedures were modified from that described to be compatible with human[20]. In short, inguinal LNs were surgically excised from volunteer subjects or mice and digested with 0.26 U Liberase DH and 0.2 mg/ml DNase I (Roche). FDCs were enriched with magnetic bead sorting (Stem cell technologies) according to manufacturer's protocol, with 50 µg biotinylated anti-CD35 antibody (Biolegend). Positively selected FDCs were suspended in FDC media (RPMI supplemented with 10% FBS, 20 mM HEPES buffer, 0.2 mM MEM nonessential amino acids, 2 mM L-glutamine, and 1 mg/ml gentamicin) and plated on collagen-coated (Rat tail derived collagen; Roche) glass coverslips (#1.5, 25 mm diameter, Warner Instruments). 5–7 day cultured FDCs were used for different assays.

ddPCR

HIV-1 RNA was quantified using the QX100™ Droplet Digital™ PCR system (Bio-Rad, Pleasanton, CA). The ddPCR mix consisted of: 10 µl 2x ddPCR™ super mix for probes (Bio-Rad); 500 nM of Forward (5'-CATGTTTTCAGCATTATCAGAAGGA-3') and Reverse (5'-TGCTTGATGTCCCCCCACT-3') primers; 250 nM probe mix (5'FAM-CCACCCCACAAGATTTAAACACCATGCTAA-TAMRA-3') and 3 µl of the cDNA into a final volume of 20 µl. The total mix was placed into the 8-channel cartridge, 70 µl of droplet generating oil was added and droplets were formed in the QX100™ droplet generator (Bio-Rad). Droplet in oil suspensions were transferred to a 96 well plate

and placed into the T100™ Thermal Cycler (Bio-Rad). Cycling conditions were as follows: 95°C for 10 min, followed by 45 cycles of 94°C for 30 sec and 60°C for 60 sec. Subsequently, the droplets were automatically read by the QX100™ droplet reader and the data was analyzed with the QuantaSoft™ analysis software 1.3.2.0 (Bio-Rad).

Immunohistochemistry

FDCs were fixed in 1% paraformaldehyde solution. Fixed samples were pre-incubated with anti-FcR (2.4G2, house produced) and 2% bovine serum albumin for blocking nonspecific and Fc-mediated binding. Anti-PE (Rockland), donkey anti-rabbit DyLight 405, anti-CD35, anti-Lamp-1 (Biolegend), fluorescent transferrin and anti-p24 (KC57-RD1, Beckman-Coulter) were used for staining ex vivo cultures of FDCs. Images were acquired with a FluoView FV1000 confocal microscope (Olympus) with a 20x lens (NA: 0.7) and processed with FluoView software (Olympus). Data were analyzed with Fiji software.

Broadly neutralizing antibodies and soluble CD21-Ig

The broadly neutralizing antibodies used in this study are available through aidsreagent.org and were obtained through our collaboration with the Ragon Institute. VRC01, PG16 and PGT121 all have the human IgG1 isotype. sCD21-Ig consists of a full murine IgG1 antibody (hybridoma B1-8) bearing the two N-terminal CD21 SCR (short consensus repeat) domains that bind human C3d[36]. As isotype control the B1-8 IgG1 hybridoma was used.

Author Contributions:

B.A.H. initiated the study, designed, and performed the experiments, analyzed the data and contributed to the writing of the manuscript. M.L. performed part of the experiments. P.A.V. performed the surgeries. E.P.S. was crucial in the recruitment of patients. M.A. provided crucial resources and gave crucial advice. B.D.W. provided resources and was essential for the completion of the study. D.E.K. provided access to the patient material, gave advice and consulted on HIV related material. M.C.C. supervised the study and contributed to the writing of the manuscript.
M.L., P.A.V., E.P.S., M.A., B.D.W. and D.E.K. all contributed to the editing of the manuscript.

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Chapter 4

Do Follicular Dendritic Cells Regulate Lupus-Specific B Cells?

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IV

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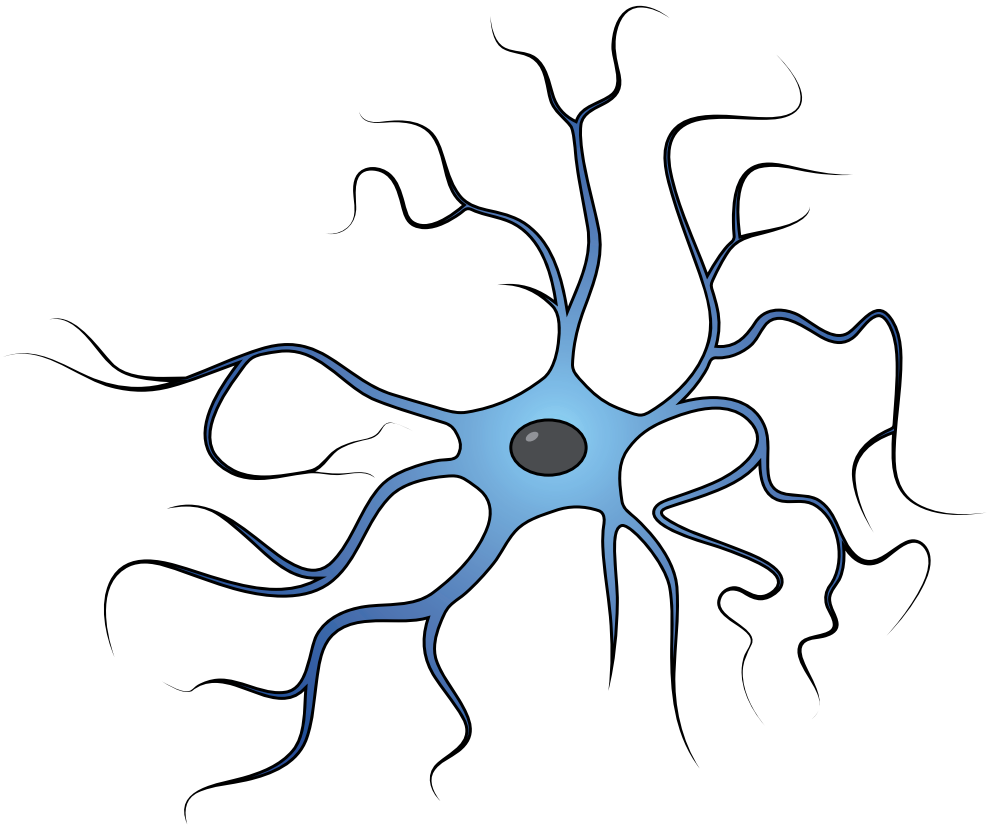

Abstract

The factors that allow self-reactive B cells to escape negative selection and become activated remain poorly defined. In this review we describe recently published results in which a B cell receptor-knock-in mouse strain specific for nucleolar self-antigens was bred with mice deficient in complement C4 and discuss the implications for the lupus field. Absence of C4 leads to a breakdown in the elimination of autoreactive B cell clones at the transitional stage. This is characterized by a relative increase in their response to a range of stimuli, entrance into follicles and a greater propensity to form self-reactive germinal centers. In this review, a model is proposed in which, in the absence of complement C4, inappropriate clearance of apoptotic debris promotes chronic activation of myeloid cells and follicular dendritic cells, resulting in secretion of Type I interferon. This allows for the maturation and activation of self-reactive B cell clones leading to increased spontaneous formation of germinal centers and subsequent generation of auto-antibodies.

Introduction

Systemic lupus erythematosus (SLE) represents a multigenic autoimmune disease for which there is no current cure[1]. It is characterized by auto-antibodies specific for nuclear antigens found in apoptotic blebs such as ribonuclear proteins (RNP), histones and dsDNA that form immune complexes (IC) with self-antigen and deposit in tissues. Given this phenotype, SLE is considered an immune complex disease in which failure to clear apoptotic blebs or IC containing nuclear material from apoptotic cells can lead to alteration in negative selection of autoreactive B cells and production of autoantibody. One of the major pathways for clearance of apoptotic cells and IC is the complement system (C′)[2]. In particular, the classical pathway becomes activated when the first component (C1q) binds to IC containing IgM or IgG. This leads to an activation of C1-associated serine proteases that cleave the fourth component (C4) exposing an internal thioester and results in covalent attachment to the IC[3]. Subsequently, the central component of complement, i.e. C3, is activated and forms a covalent bond with the IC complex[4,5]. In normal individuals, IC and apoptotic debris are cleared efficiently through a combination of scavenger receptors and specific complement receptors that bind activated fragments of complement C3, such as CR1 (CD35) and the CR-Ig receptor[6,7].

Deficiency in C1q or C4 but not C3 is a major risk factor for SLE[1,8,9]. Although relatively rare, genetic or acquired deficiency in C1q leads to SLE in 90% of individuals identified. Total deficiency in complement C4 also results in SLE at a high frequency, i.e. 75%, among affected individuals[1]. Interestingly, in contrast to the normal population where women have a much higher susceptibility to disease than men (9:1), men and women deficient in C4 are equally susceptible to lupus. Mice deficient in C1q or C4 are also predisposed to a lupus-like phenotype[10,11]. Although spontaneous disease is mild,

crossing either C1q or C4 deficient strains with mice bearing another susceptibility locus such as lpr (lymphproliferation and autoimmunity; CD95 or Fas) on the C57Bl/6 background lead to elevated anti-nuclear antibodies (ANA) and glomerulonephritis[10-13]. How activation and binding of C1q and C4 to IC bearing apoptotic debris is protective is not known. Earlier hypotheses suggested that direct opsonization of apoptotic IC with C1q or C4 enhanced binding and direct clearance through phagocytic receptors. One developing model is that opsonization of apoptotic debris with C1q or C4 acts to dampen activation of myeloid cells following phagocytosis of the debris. Thus, C1q or C4 may "mark" IC for clearance without inducing inflammation. Support for this novel role comes from several recent reports. The Elkon group reported that uptake of apoptotic debris by human peripheral blood monocytes (PBMC) or dendritic cells (DC) is relatively non-activating when pretreated with lupus sera of subjects in the presence of C1q[14,15]. In contrast, when the source of lupus sera is C1q deficient, uptake of the apoptotic IC leads to activation of human PBMCs and DCs. In their system, activation was assayed by cell expression of proinflammatory cytokines such as Type I interferon (IFNα). Thus, they proposed that C1q acted to suppress the activation of inflammation and secretion of IFNα. Further support for this hypothesis comes from the finding of Diamond and colleagues that the leukocyte-associated Ig-like receptor 1 (LAIR-1) binds the collagen stalk of C1q and mediates negative signaling through its ITIM (immunoreceptor tyrosine-based inhibitory motif) of plasmacytoid DC (pDC)[16]. In a more recent study, Means and colleagues report that the scavenger receptor SCARF-1 (scavenger receptor expressed by endothelial cell 1) is required for efficient uptake of dying cells; and mice deficient in the receptor develop an autoimmune phenotype similar to that of C1q deficient strains[17]. In their study, SCARF-1 interacts on the cell surface of DC with C1q bound to apoptotic cells via exposure of phosphatidylserine. C1q cannot only bind Ig-coated IC or apoptotic cells through the Fc region of Ig but also via its affinity for phosphatidylserine. Thus, similar to calreticulin-CD91[18], MFG-E8[19,20], TIM 3-TIM-4[21], C1q recognizes dying cells through exposure of phosphatidylserine and promotes phagocytosis without triggering of inflammation.

Whether C4 interacts directly with scavenger receptors similar to C1q is not clear. One possible interaction is with the TAMs (Tyro3, Axel, and c-Mer), which are a family of tyrosine kinases that act as negative regulators of myeloid cell activation following phagocytosis of apoptotic debris. A combined deficiency of all 3 family members results in severe lupus-like disease[22]. Alternatively, deficiency in c-Mer alone leads to a dysregulation of B cell tolerance and a mild lupus phenotype[23]. The primary ligands for TAMs are Gas 6 and Protein S, which recognize apoptotic cells through phosphatidylserine[24]. In the latter example, Protein S is known to interact with C4 binding protein (C4bp) in human sera. Interestingly, while Protein S promotes clearance of apoptotic cells, the complex of Protein S and C4bp is inhibitory[25]. One explanation for a protective role for C4 is that it may compete with Protein S to bind C4bp. Thus, C4 may displace Protein S-bound apoptotic debris from C4bp and promote clearance via scavenger receptors and activation of TAMs.

Loss of B cell tolerance in absence of C4

Mice deficient in C1q or C4 not only have impaired clearance of IC but develop elevated ANA suggesting a loss of B cell tolerance to lupus antigens[10,11,26]. A current paradigm to explain dysregulation of lupusspecific B cells is based on the observation that in general lupus antigens are ligands for TLRs such as TLR-7 and 9. Thus, uptake and internalization of nuclear debris containing ribonucleolar proteins, such as Ro and La, can activate cytoplasmic TLRs and result in activation of NFκβ and induction of downstream pathways such as IFNα and IL-6[27-29]. TLR signaling can overcome intrinsic anergy leading to expansion and differentiation of the autoreactive B cells to plasma cells and possibly memory B cells. Whether this pathway allows for escape of tolerance at the immature stage has not been reported.

To test if deficiency in C4 results in loss of tolerance to a known lupus antigen, $C4^{-/-}$ mice were crossed with the B cell receptor (BCR) knock-in line 564 Igi. These mice bear an insertion of the rearranged Ig heavy (VH DH JH) and light (Vk Jk) chain genes. The line was developed originally from a hybridoma (mAb) isolated from an autoimmune strain of mice that spontaneously develop a lupuslike phenotype[30]. Initial characterization of the knock-in mice identified normal negative selection of the autoreactive B cells at checkpoint I and II (clonal deletion and clonal anergy in the periphery respectively). However, a fraction of the autoreactive B cells escape tolerance and expand in a TLR-7-dependent pathway and secrete ANA. The 564 Igi strain is one of the first in vivo models that confirm the paradigm of TLR-7 signaling leading to loss of anergy and secretion of pathogenic autoantibody.

Characterization of the 564 mAb identified a nucleolar staining pattern as expected. Likewise, sera isolated from the $C4^{+/+}$ and $C4^{-/-}$ 564 Igi mice gave a similar staining pattern as the mAb (Figure 1). The nucleolus is a major site for RNA splicing and accumulation of ribonucleoproteins (RNP). Biochemical analysis of immune precipitates prepared from 564 Ig serum, identified multiple RNP including the known lupus antigen SSB/LA. 564 Ig binding is dependent on both the RNA and protein components suggesting that the antibody recognizes an RNA binding domain[31].

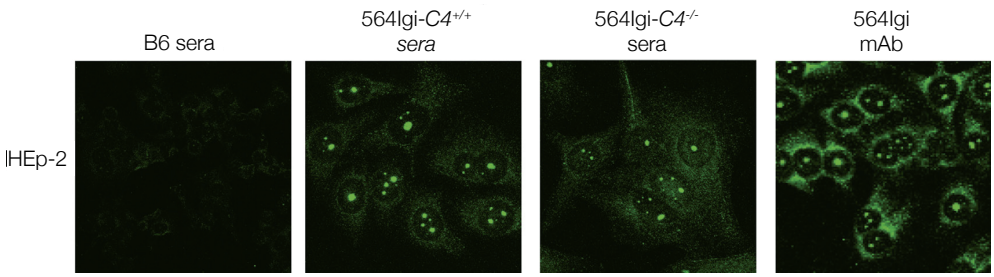

Figure 1 | 564 serum and mAb shows a nuclear staining patern
Sera from B6, $C4^{+/+}$564 Igi and $C4^{-/-}$564 Igi-mice (1/100) were incubated on Hep2 slides. 564 Igi mAb was used as control. Images were acquired at 20× magnification

To characterize B cell tolerance, splenic B cells isolated from $C4^{+/+}$ and $C4^{-/-}$ 564 Igi mice heterozygous for Ig H and L chains were analyzed by flow cytometry. Autoreactive B cells were identified using an antibody that was specific for the 564 Igi BCR (idiotype or Id). Comparison of Id+ B cells isolated from the $C4^{+/+}$ and $C4^{-/-}$ 564 Igi mice identified a relatively low frequency of autoreactive B cells in the former strain as expected[30,31]. By contrast, a significantly higher frequency of mature Id+ B cells was observed in the spleen in the $C4^{-/-}$ 564 Igi mice suggesting that in the absence of C4 negative selection was less efficient (Figure 2). Further analysis of Id+ splenic B cells using cell surface markers such as AA4.1 to distinguish transitional from mature identified the defect at the checkpoint II stage. Notably, a similar frequency (approximately 80%) of Id+ B cells was observed in the immature compartment of peripheral B cells in the $C4^{+/+}$ and $C4^{-/-}$ 564 Igi mice. However, analysis of the Id+ B cells at the transitional and mature stage indicated a dramatic reduction in the Id+ cells in the $C4^{+/+}$ mice as expected from the earlier studies of Berland and Chatterjee[30,31]. By contrast, there was some reduction in frequency and number of mature B cells isolated from the $C4^{-/-}$ 564 Igi mice but it was less dramatic than observed in the $C4^{+/+}$ 564 Igi mice[31]. Thus, in the absence of C4, negative selection against the lupus-antigen specific B cells was less efficient relative to WT background.

To determine if the autoreactive B cells that escape tolerance and mature are anergic, multiple assays were performed including stimulation with TLR ligands specific for TLR-7 and 9, cross-linking of BCR and analysis for expression of activation markers. The results indicate that Id+ B cells in the $C4^{+/+}$ 564 Igi mice bear an anergic phenotype while those isolated from the $C4^{-/-}$ 564 Igi mice appeared responsive to stimulation. An in vivo assay for B cell anergy is exclusion from splenic follicles or "follicular exclusion"[32]. Immunohistochemistry (IHC) analysis using confocal microscopy showed that Id+ B cells were excluded from the follicles in the $C4^{+/+}$ but not the $C4^{-/-}$ 564 Igi mice (Figure 3). Thus, consistent with the in vitro analyses, autoreactive B cells in the $C4^{+/+}$ mice developed an anergic phenotype. By contrast, in the absence of C4, Id+ B cells remained responsive and were included within the splenic follicles.

A hallmark of immune response to foreign antigen is development of germinal centers (GC) within B cell follicles. Here, activated B cells undergo class switch recombination and somatic hypermutation and differentiate into effector and memory B cells[33]. Recent studies have identified GC in mice with a lupus-like phenotype suggesting that autoreactive B cells may be regulated and differentiate similar to foreign antigen stimulated B cells[34,35]. Analysis of splenic and lymph node tissues in the 564 Igi mice by both flow cytometry and IHC identified GC in both strains. However, a greater frequency of Id+ GC B cells was identified in the $C4^{-/-}$ 564 Igi mice[31]. Moreover, flow cytometry analysis identified an increase in the frequency of CD4+ T follicular helper cells further supporting a more robust immune response to self-antigen in the absence of C4[31].

The complement system not only participates in clearance of IC and apoptotic cells but activation of the classical pathway leads to enhanced humoral immunity. Binding of C3d (a split product of C3) to the IC acts to lower the threshold of B cell activation and promotion of B cell survival in germinal centers (GC) through co-stimulation of the B cell

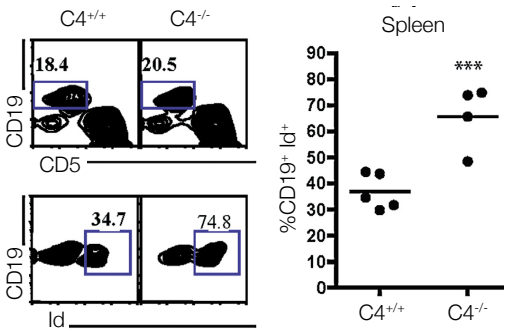

Figure 2 | C4 deficiency leads to greater accumulation of mature self-reactive B cells in the spleen

Cells from spleen of C4$^{+/+}$and C4$^{-/-}$564 Igi mice were stained with anti-AA4.1, B220, CD19 and Id antibodies and analyzed by flow cytometry. The meanfrequency of mature Id cells is indicated in the panel on the right. Asterisk indicatessignificance p value <0.001.

co-receptor and retention of antigen on follicular dendritic cells (FDC)[36,37]. Interestingly, coupling of C3d to a self-antigen (ArsCCG/C3d) leads to break in tolerance of anergic B cells in the Ars/A1 Ig transgenic model[38]. Mice deficient in C4 or C3 have an impaired secondary immune response characterized by reduced GC and differentiation into B memory and effector cells[39]. C3-deficient mice immunized with model protein antigens develop GC within secondary lymphoid compartments but they fail to support differentiation of memory B cells and the B cells die at the GC stage. For example, immunization of mice bearing a mutation in CD21 or CD19 in which the co-receptor signaling is impaired have a defect at the class switch recombination (CSR) stage[40,41]. Thus, B cells in mice bearing impaired co-receptor signaling become activated and express activation-induced cytidine deaminase (AID) but fail to undergo isotype switch. Therefore, it was unexpected to find an increase in GC in the C4$^{-/-}$ 564 Igi mice relative to C4$^{+/+}$ 564 Igi[31]. Analysis of GC B cells in the C4$^{-/-}$ 564 Igi mice revealed a relative high turnover and cell death suggesting a high frequency of activation and initiation of GC phenotype but a block in their differentiation to memory or effector cells[31]. Whether an alternative pathway activates C3 is not clear; but one explanation for the presence of GC in the C4$^{-/-}$ 564 Igi mice is that excess of the RNP self-antigen stimulates TLR-7 to drive self-reactive B cells to form GC in the absence of C4 activation.

FDC internalize and cycle foreign antigen

Examination of splenic follicles of the C4$^{+/+}$ and C4$^{-/-}$ 564 Igi mice by IHC identified PNA+ Id+ 564 Igi B cells in the GC as discussed above (Figure 3). A hallmark of GC formed in response to immunization with foreign antigen is retention of IC by FDCs where contact with antigen and C3d ligand is required for survival of cognate B cells[39,42]. GC are common in autoimmune mice as well but whether retention of self-antigen is also required for maintenance of self-reactive GC has not been reported. To analyze for the presence of 564 Ig-IC, FDC were isolated from the spleen and LNs of C4$^{+/+}$ 564 Igi mice and grown in culture on collagen-coated cover slips. Confocal analysis of fixed sections identified co-localization of Id+ IC and FDC-M1 (FDC marker) on FDC suggesting retention of the self-antigen-IC similar to that observed with foreign antigen (Figure 4).

How foreign antigen is retained by FDC for extensive periods has been a long-standing enigma until recently. By combining live cell imaging and analysis of FDC ex vivo, Heesters et al. found that C3d-coated IC were taken-up via CD21 receptor and rapidly internalized into a cycling endosomal compartment that co-localizes with the transferrin receptor[43]. They found that IC remained intact within the cycling compartment but periodically returned to the FDC surface where antigen was available for recognition and binding by cognate B cells. Thus, an internalization and cycling mechanism could explain how antigen is retained for extensive periods by FDC but readily available for sampling by circulating B cells.

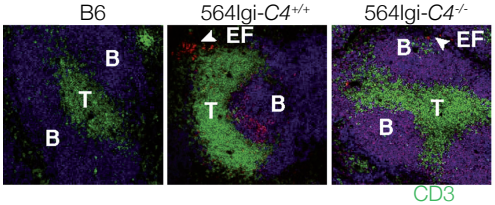

Figure 3 | Follicular exclusion in C4$^{+/+}$, but not in C4$^{-/-}$ 564-Igi mice
Spleen sections from B6, C4$^{+/+}$ and C4$^{-/-}$ 564 Igi mice were stained with anti-CD3 (T-cell area (T)), anti-B220 (B cell follicles (B)) and anti-564 antibody (self-reactiveB cells (Id)). 'EF' indicates extrafollicular foci of Id-producing cells. Data shown are representative of 4 independent experiments performed with 5 mice total.

IV

In a preliminary set of experiments, FDC isolated from 564 Igi secondary lymph nodes were characterized for retention of Id+ IC. To determine if 564 Igi IC are internalized and degraded or cycled as found with foreign antigens, ex vivo cultures of FDC were analyzed using labeled antibodies specific for the transferrin receptor or LAMP-1 compartments and anti-Id. The transferrin receptor cycles from the cell surface to the cytoplasm via the cycling endosomal compartment and then returns to the cell surface undegraded. By contrast, IC within vesicles that are internalized and delivered to the LAMP-1+ lysosomal compartment are degraded. Our preliminary results identify a significant association of the autoimmune IC with the transferrin receptor relative to the degradative LAMP-1 compartment in the C4$^{+/+}$ 564 Igi mice (Figure 5). This suggests that 564 Igi IC are indeed partially retained in this cycling non-degradative endosomal compartment. In future experiments, it will be important to determine if RNP-IC taken-up by FDC in the C4$^{-/-}$ 564 Igi mice are also retained in a similar cycling compartment or directed to an alternative compartment. This possibility is discussed further below.

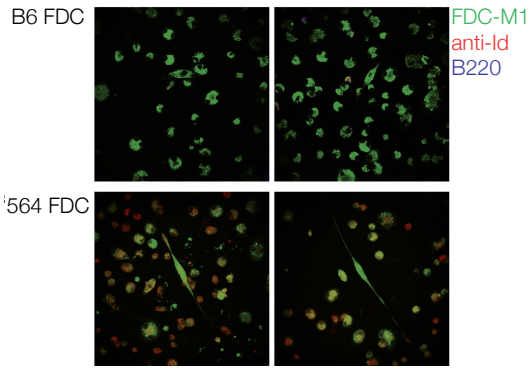

Figure 4 | FDC retain 564 antigen
Follicular dendritic cells (FDC) isolated from 564 Igi mice bear RNP-immune complexes. FDC were isolated from C4$^{+/+}$and C4$^{-/-}$564 Igi mice and grown on collagen-coated slides for 5–10 days. Slides were fixed and stained with antibody specific for FDC-M1 (green), anti-Id (red) or B220 (blue) and analyzed by confocal fluorescentmicroscopy. Upper panels: Representative images of FDC isolated from WT mice. Lower panels: representative images of FDC isolated from C4$^{+/+}$564 Igi mice.

Besides retention of self-antigens, FDC participate in regulating autoimmunity by other pathways such as secretion of MFGE8 which enhances clearance of apoptotic cells as noted above[20]. Alternatively, expression of self-antigen by FDC can lead to negative selection of auto-reactive B cells at the immature stage. Rickert et al. developed an elegant model in which duck egg lysozyme (mDEL) was expressed conditionally on FDC, in the presence of Ig transgenic B cells bearing the anti-HEL B cell receptor. In their model, induction of expression of mDEL by FDC led to anergy and elimination of the self-reactive B cells at the transitional stage[44].

Model for FDC as a source of Type I interferon

A current paradigm proposes that internalization of lupus antigens by B cells via the BCR triggers the TLR pathway resulting in induction of IFNα and activation and differentiation of anergic B cells to secrete auto-antibody[28,45]. Alternatively, or in parallel with B cell stimulation, clearance of RNP-IC by dendritic cells in the absence of C1q or C4 or in models of excess IC can result in activation and secretion of IFNα especially by pDC[15]. Moreover, IFNα taken-up via the IFNα receptor (IFNAR) on autoreactive B cells can stimulate increased expression and sensitivity of TLR-7 to ligand internalized via BCR resulting in expansion and secretion of auto-antibody[28,46,47]. Therefore, understanding the source and regulation of IFNα release is an important question.

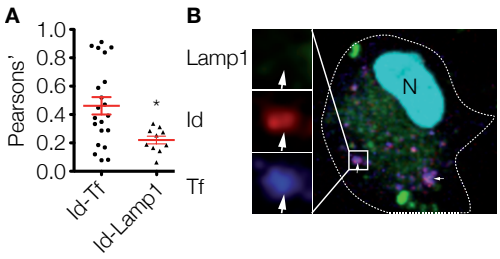

Figure 5 | RNP-IC localize to cycling compartment
RNP-immune complexes are internalized by FDC into the cycling endosomal compartment. FDC were isolated from C4$^{+/+}$564 Igi mice and cultured on collagencoated slides for 5–10 days. Cells were stained with antibody specific for Id (red), transferrin receptor (Tf) (blue) or Lamp-1 (green) fixed and imaged by confocal fluorescentmicroscopy. Each individual symbol represents co-localization of Id+IC with either Tf or LAMP-1 staining; horizontal bar indicates mean ± standard error. * = $p < 0.05$.

We speculate that FDC may be a significant source of IFNα in the C4$^{-/-}$ 564 Igi autoimmune mice. It is proposed that inappropriate uptake and internalization of RNP-IC by FDC in the absence of C4 could lead to intersection with the TLR-7 compartment and result in expression of IFNα and downstream interferon stimulated genes (ISG). Preliminary results identify elevated expression of TLR-7 and ISG by FDC isolated from C4$^{-/-}$ 564 Igi mice (A. Das and M Carroll, unpublished results). Therefore, FDC could be a major source of IFNα signal driving escape of self-reactive B cells from negative selection and maturation into auto-antibody secreting cells (Figure 6).

Given the importance of the TLR-7 pathway and induction of type I interferon in viral infections such as influenza A it is possible that FDC may participate in viral immunity. In future studies, it will be important to test this hypothesis and determine if FDC are triggered to release IFNα in WT mice or only in mutant mice in which an excess of self-antigen or viral RNA IC develop.

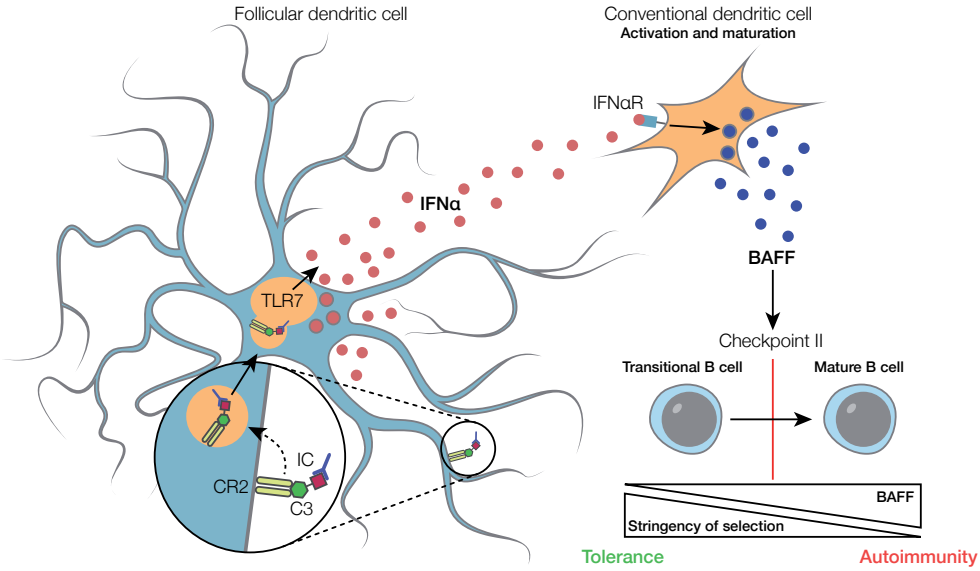

Figure 6 | Suggested model for IFNα release
FDC model as source of IFN stim DC to produce BAFF and together they induce survival in 564 Igi B cells.

Summary

A major breakthrough in the lupus field came over the past decade with the finding that internalization of lupus antigens via the BCR of cognate, self-reactive B cells could lead to escape of anergy and differentiation into autoantibody producing cells[29]. In this review, we attempt to explain a long-standing enigma referred to as the "lupus paradox", i.e. that humans (and mice) deficient in complement C4 are highly predisposed to development of lupus despite the absence of a key component of innate immunity[48]. We propose that in addition to pDC, FDC are an important source of IFNα that drives escape of tolerance by self-reactive B cells in the $C4^{-/-}$ 564 Igi mice. It will be important in future studies to test this hypothesis in vivo using $C4^{-/-}$ 564 Igi mice in which the TLR-7 and/or IFNα pathway is blocked.

Acknowledgments

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Chapter 5

Trafficking of *Streptococcus pneumoniae* in the Lymph Node

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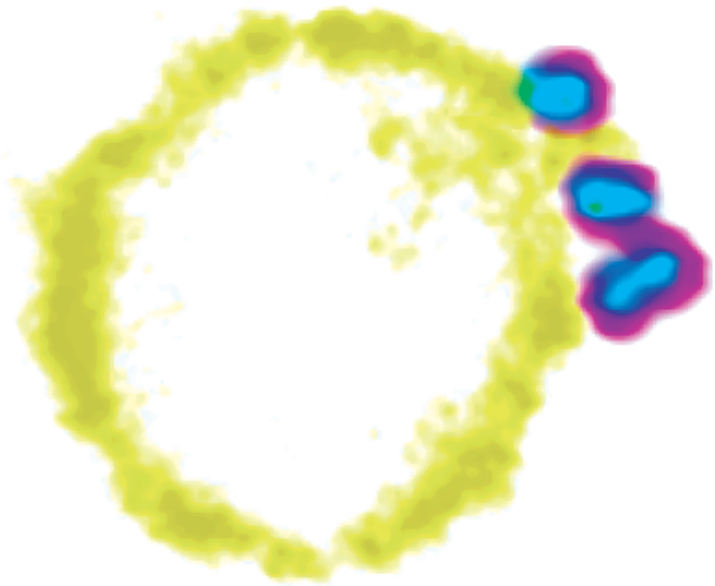

High magnification image of a B cell binding complement C3d-coated *Streptococcus pneumoniae* (SP) on its surface. The B cell surface is marked with B220 (yellow), SP (blue) and C3d (purple).

Abstract

As a pathogen *Streptococcus pneumoniae* is well equipped to evade the immune system and can quickly establish a systemic infection. The primary host defense mechanism is the complement system, which is effective in clearing the pathogen. SIGN-R1, a receptor on macrophages in the spleen, plays an important role in the initiation of complement opsonization of SP. Marginal zone macrophages in the spleen and their counterparts in the medullary region of lymph nodes (LNs) express SIGN-R1. Here we show that S. pneumonia is captured by both medullary macrophages and subset of LN resident dendritic cells (DC) that expresses SIGN-R1. We propose a mechanism in which DCs and B cells are required for the transport of C3-opsonized SP to the follicular dendritic cell (FDC). A better understanding of this process can contribute to improvement of future vaccine design.

Introduction

The Gram-positive pathogen *Streptococcus pneumoniae* (SP), commonly known as the pneumococcus, is the predominant cause of community acquired pneumonia and causes many cases of Otis media, sinusitis, meningitis and septicemia[1-3]. Invasive pneumococcal disease leads to high mortality and morbidity rates, especially in young, elderly, debilitated or immunosuppressed individuals[4]. Worldwide more than 1 million people die from pneumococcal infections each year, mostly in the developing world[5,6]. However, the rate in which resistance of SP to antibiotics is increasing in the United States and the rest of the developed world is alarming[7,8].

SP resides in the nasopharynx as a commensal in up to 20% of healthy adults and up to 50% of healthy children. Besides being commensal SP is also opportunistic and occurs often after another respiratory tract infection. During influenza pandemics the leading cause of death is often a secondary infection with SP[9]. Clearance of SP is mediated through opsonization by Igs and by Ig-independent components now known as complement[10,11]. In 1969 it was shown by Johnston et al. that complement is also important in pneumococcal disease by increasing phagocytosis[12]. Since SP binds C3d, and C3d binds to B cells, it is no surprise that SP polysaccharides immune complexes (IC) bind to CD21[13].

The classical complement pathway is the major contributor to SP opsonization[14]. Although the pathway used is the CP, the initiation is not standard. Instead of Ig activation of C1q it is SIGN-R1, a lectin with a high binding specificity for SP, that interacts with C1q[15]. Opsonization of SP with C3d and deposition on the FDC is required for humoral immunity to SP[15].

Despite these elegant findings on the mechanism of antigen binding and complement activation, it is unknown how the cognate B cell eventually acquires the antigen. To

track delivery of SP to the follicular dendritic cell (FDC) and its subsequent acquisition by cognate B cells we used a model in which mice were immunized s.q. with a fluorescent labeled inactivated SP. Unexpectedly we found that sinus lining macrophages in the lymph node (LN) are not required for humoral immunity to SP but instead C3d-opsonized bacteria are captured by LN-resident DCs and either handed-off to naive B cells or transported directly to the FDC.

We propose a mechanism in which B cells require DC mediated opsonization of SP and in which DCs are capable (but inefficient) in transport of SP to the FDC independent of macrophages.

Results

Medullary macrophages capture lymph-borne SP

In order to identify the mechanism behind the capture of lymph-borne SP in the LN, we injected mice in the footpad with fluorescent-labeled, heat-inactivated SP strain D39. This will result in drainage of the bacteria to the popliteal lymph node (pLN)[16]. Surprisingly, cryosections of pLNs show localization of SP in the medullary macrophage region and

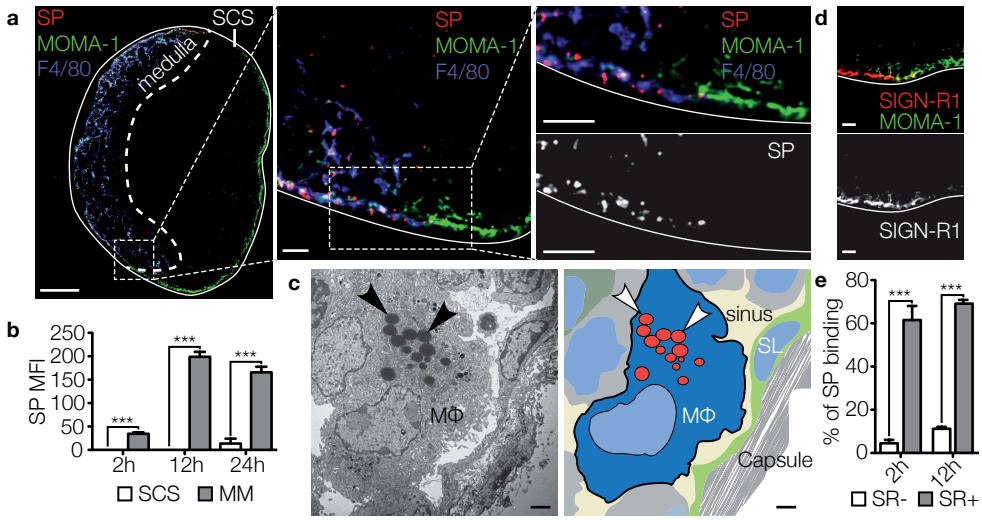

Figure 1 | Streptococcus pneumoniae is captured in the medulla and not in the sub-capsular sinus

a | Sub-capsular macrophages are stained with MOMA-1 (green), medullary macrophages with F4/80 (blue) and SP with CMTPX (red). Left panel shows whole lymph node after 2 hours. Scale bar indicates 100 µm. Middle panel shows a magnification of the transition from medulla to sub-capsular sinus. Scale bar indicates 10 µm. Right panel shows a further magnification, which enables detection of single bacteria. Gray scale image of bacteria shows exclusion in the sub-capsular sinus. Scale bar indicates 10 µm. **b |** Using confocal microscopy, the binding of SP to the medulla (MM) and the sub-capsular sinus (SCS) was quantified using the mean fluorescence intensity (MFI). The arrival of the bacteria is gradual over the course of 12 hours and bacteria are accumulating in the MM. After two hours bacteria can be detected in the MM. The SCS shows no binding at all, even at 12 hours. **c |** EM of a medullary macrophage that has engulfed multiple bacteria near the medullary sinus. The scale bar indicates 2 µm. **d |** SIGN-R1 (SR1) expression is limited to the medulla. **e |** SIGN-R1 positive areas bind significantly more bacteria.

not in the sub-capsular macrophage region, which are considered the gatekeepers of the LN (Figure 1 a). Capture is apparent at two hours after injection and quantification of the data shows an increase in binding in the medullary region over time (Figure 1 b). In contrast to our published study of Influenza virus, SP does not bind the sub-capsular sinus macrophages[17]. Electron micrographs show internalization of the bacteria by macrophages within the medullar, but not the sub-capsular region (Figure 1 c). In addition, at later time points SP was observed in the follicular region, co-localizing with the FDC marker 8C12 (Figure 3 d, Supplemental figure 1). FDCs are thought to be important in the presentation of antigens to cognate B cells.

Lymph node DCs and macrophages bind SP

To determine which cell types are responsible for the binding and possibly for the transport of SP to the FDC, a flow cytometric analysis of single cell suspensions of pLN was performed. Heat-inactivated fluorescent-labeled SP was injected in the footpad at different time points before harvesting the popliteal lymph node.

Binding by DCs and macrophages was shown at 2 hours and 12 hours post injection (Figure 2 a). Binding increases over time, indicating the gradual arrival of SP in the pLN (Figure 2 b). A closer look at the DC population reveals a major and initial role for SIGN-R1 positive CD4+ DCs. At later time points also the CD8α+ DCs pick up the bacteria (Figure 2 c). However, CD8α+ DC binding seems to be less SIGN-R1 dependent while CD4+ DC binding is mainly SIGN-R1 mediated (Figure 2 d and Supplemental figure

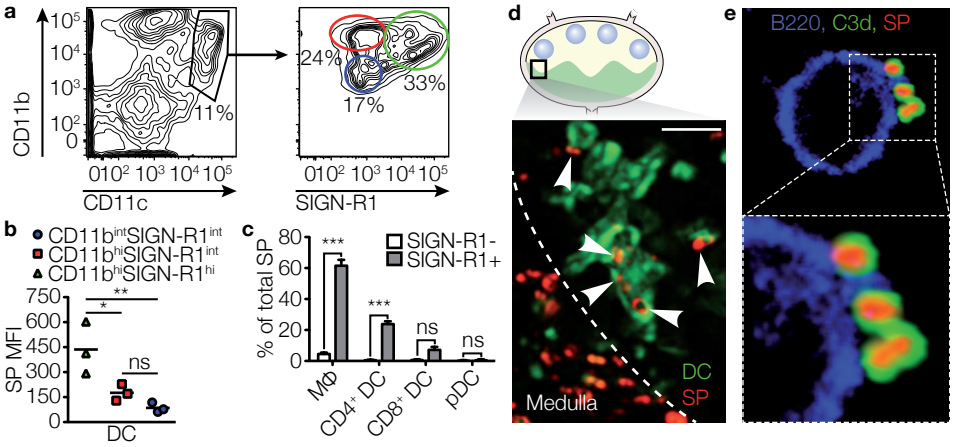

Figure 2 | Dendritic cells and macrophages bind SP

a | Flow cytometry was used to distinguish DC subsets based on SIGN-R1 expression. **b |** In the 3 groups defined there is a clear correlation between SIGN-R1 expression and SP (D39) binding. **c |** Within the DC population distinction can be made between CD4 positive DCs, CD8 positive DCs and plasmacytoid DCs (pDC). It is clear that SIGN-R1 positive macrophages and SIGN-R1 positive CD4+ DCs account for the majority of the bacterial binding. **d |** In vivo multi-photon micrograph of a DC loaded with SP (D39, white arrows) near the medullary border. Scale bar indicates 10 µm. **e |** High magnification image of a B cell binding complement C3d-coated SP on its surface. The B cell surface is marked with B220 (blue), SP D39 (red) and C3d (green).

2). CD4+ DCs bind SP via SIGN-R1. As CD8α+ DCs are known for their phagocytic properties, this binding is probably less specific and due to phagocytosis of debris.

To determine capture of SP by LN resident DCs, mice expressing eYFP under the CD11c promotor were immunized with labeled, inactivated SP and uptake was visualized in real-time by multi-photon intra-vital microscopy (MP-IVM) of the pLN (Figure 2 e). The DC is in close proximity of the medulla and is loaded with several bacteria. DCs appear to capture bacteria by direct sampling of the medullary lymphatics via SIGN-R1 as receptor.

Lymph node DCs are required for immune response against SP

To assess whether DCs or macrophages or both are required for an immune response against SP, we constructed chimeric mice in which CD11c-Dtr bone marrow (BM) was transferred into lethally irradiated Wt mice. In these mice, expression of the diphtheria toxin receptor is under control of the CD11c promotor, making dendritic cells susceptible to diphtheria toxin (Dtx). CD11c-Dtr-BM mice were first treated with 5 µg of Dtx to eliminate CD11c+ cells and subsequently immunized with heat inactivated SP in the footpad. IgM titers were assessed by ELISA at day 10 post-immunization (Figure 3 a). As expected, CD11c-BM chimeric mice not treated with Dtx responded similar to Wt mice. By contrast, the IgM response was severely diminished in CD11c-

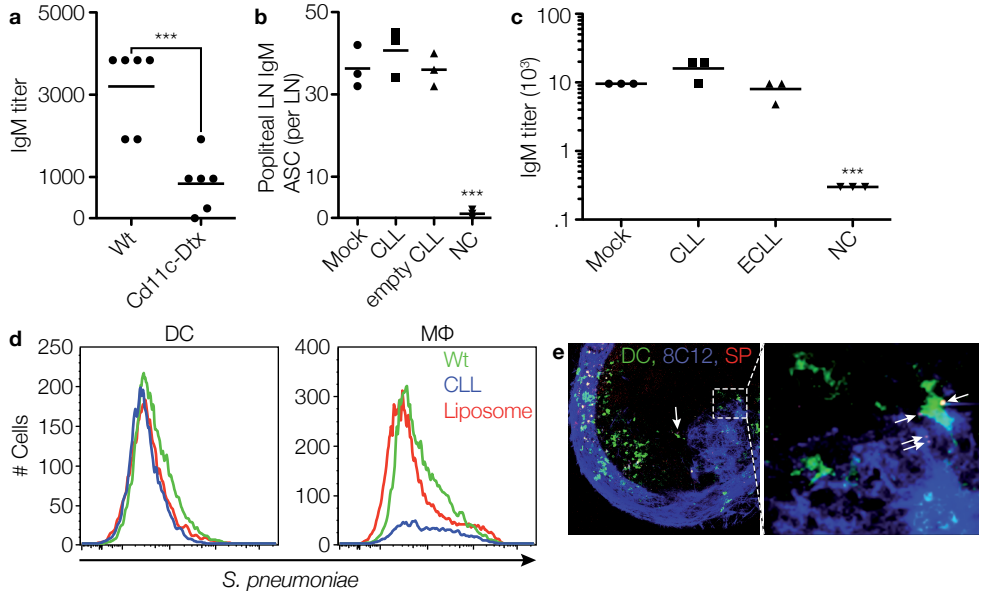

Figure 3 | DCs and not macrophages are important for humoral immune response.
a | Cd11c-Dtr BM in Wt chimeric mice were injected locally with diphtheria toxin (Dtx) prior to immunization with D39. DCs were effectively depleted after 2 days. IgM titers were determined at day 10 post immunization. **b |** Mice were treated locally with CLL in order to deplete macrophages. IgM antigen secreting cells (ASC) were counted at day 10. **c |** In the same experiments as in b IgM titers were determined. **d |** The efficiency of CLL depletion was measured using flow cytometry. There was a minimal effect on DC, while almost all macrophages were depleted. **e |** Still from a MP-IVM movie in which DCs hand-off D39 to an FDC.

BM chimeras administrated Dtx. These results support the requirement of DCs in the transport of SP to the follicle and for the initiation of a humoral immune response.

Medullary macrophages where depleted in mice prior to immunization to assess their contribution in the immune response against SP. Clodronate liposomes (CLL) were used to deplete the macrophages. Importantly, under the conditions used in this study CLL does not deplete DCs (Figure 3 d). When immunized with SP there was no difference in the immune response among Wt mice, CLL treated mice or mice treated with empty liposomes (ECLL) (Figure 3 b, c). Taken together, DCs, but not macrophages, are required for an immune response against SP. Previous studies show that deposition of C3-opsonized SP on FDCs is correlated with B cell memory response against SP[15].

To determine if CD11c+ cells transport SP directly to the FDC , MP-IVM was used in combination with the CD11c-eYFP reporter mice. Using MP-IVM we observed transfer of SP from the DC to the FDC. However, it is most probable this pathway cannot account for all of the accumulation of SP on the FDC network (movie stills; Figure 3 e).

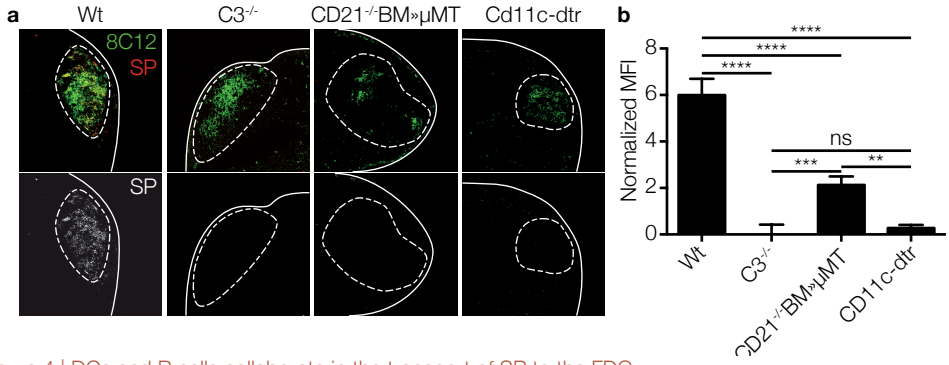

Figure 4 | DCs and B cells collaborate in the transport of SP to the FDC.
Mice were injected with fluorescent labeled D39 in the footpad as described earlier. We used 4 different mouse strains; wild type, complement C3 knockout, complement receptor 2 knockout bone marrow in µMT B cell deficient recipients and Cd11c-dtr mice injected with Dtx 48h prior to immunization. The FDC network was stained using 8C12. **a |** Lymph nodes were harvested 12h after immunization, fixed and frozen in OCT. Cryosections were stained and imaged using confocal microscopy. **b |** Confocal imaging from a was quantified using Cell Profiler. The D39 MFI was normalized to the 8C12 MFI and only co-localized signal was measured. These data show a complete dependence of transport on complement and DC. There is, however, diminished transport in the absence of CR2 bearing B cells.

DCs and B cells collaborate to transport SP to FDCs

Although MP-IVM identified direct transport of labeled SP to FDCs, it seems more likely that B cells do the heavy lifting. Previous studies demonstrated that naive B cells efficiently pick-up C3d-bound immune complex (IC) via the CD21 receptor and transport ICs to the FDC. To examine whether transport of C3d-opsonized SP is dependent on CD21 expression on B cells we constructed BM chimeras in which CD21$^{-/-}$ BM was reconstituted into lethally irradiated µMT mice, which lack mature B cells, to guarantee that none of the residual B cells have CD21. CD21$^{-/-}$ BM-µMT chimeras were used to retain CD21 expression on FDCs, which are radio-resistant.

In contrast to CD21+ BM chimeric mice, we observed a severe reduction in SP deposition on the FDCs. Interestingly, the phenotype was not as severe as in $C3^{-/-}$ mice (Figure 3 a, b). Therefore, it is probable that CD21-dependent transport does not explain all transport to the FDC.

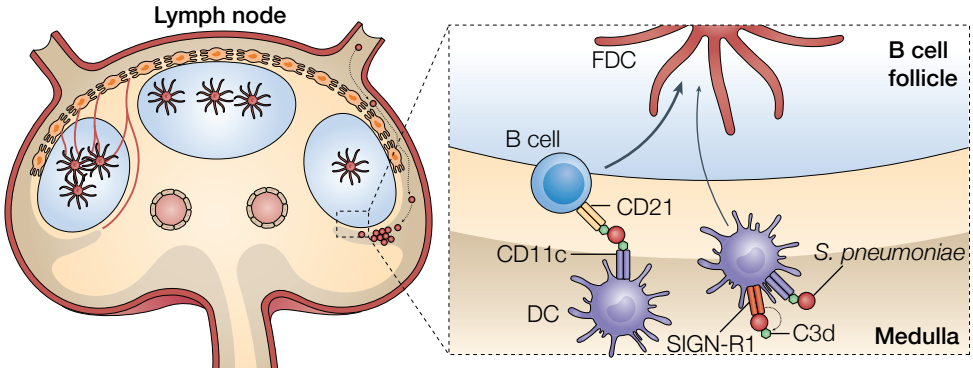

Figure 5 | Proposed model of antigen transport to the FDC.
SP enters the lymph node through the afferent lymphatics coming from the draining site. Due to its thick sugar capsule SP has not been opsonized by complement yet and thus does not bind CR3 on sub-capsular sinus macrophages. Cells in the medulla are capable of binding SP through the SIGN-R1 receptor on macrophages and dendritic cells (DC). Macrophages can endocytose and digest the bacterium, while DCs can opsonize SP via the classical pathway mediated by SIGN-R1 which can bind C1q. C3d opsonized SP can then bind CR3 (or CR4 perhaps) on the DC. The DC can do two things, it can crawl towards the FDC and hand-off the bacterium, or it can transfer the cargo to the CR2 receptor of a B cell. The B cell in turn can transport and hand-off SP to the FDC. The FDC then cycles the antigen in order to display it to cognate B cells in the GC reaction.

From earlier results we know that DCs are one of the early cells to bind SP, therefore we decided to utilize the Cd11c-dtr system to deplete DCs as described above. Notably, the phenotype was similar as in the $C3^{-/-}$ mice and there was negligible deposition on the (Figure 4 a, b). Based on combined results we propose a model in which both DCs and B cells are necessary for the efficient transport of SP to the FDC.

Discussion

Acquisition of SP antigen by FDCs is crucial to humoral immunity and retention is dependent on complement opsonization, supporting a critical role for C3. Binding of antigen by B cells is also dependent on complement opsonization. There are different routes a pathogen can follow from the moment it enters the lymph node. This work suggests, in combination with recent papers, that the routes within the lymph node can vary for different pathogens[18]. For instance the difference observed between Influenza and SP might be subscribed to the ability of Mannan Binding Lectin (MBL) to bind Influenza and not SP (data not shown).

However, even for a single pathogen different routes can be available. Here we hypothesize that at least two routes exist for SP transport to the FDC (Figure 5).

The CD21$^{-/-}$BM in μMT recipient experiment suggests a transport mechanism independent of B cells, even though it is less robust than the B cell dependent transport. We propose that this residual transport is mediated by DCs since we captured this event by MP-IVM. However, without DCs deposition on FDCs is negligible, as shown by our CD11c-dtr experiments. Therefore we propose a mechanism in which DCs are involved in two independent pathways. DCs can transport SP to the FDC directly and are required for the facilitation of SP transport by B cells by mediating opsonization of SP.

We show transport of SP to FDCs to be a collaborative effort between DCs and B cells, which depends on C3 opsonization most likely mediated by SIGN-R1 on DCs [19]. Complement receptor 3 (CD11c), which is also present on DCs, could be involved by stabilizing the binding of SP after opsonization by SIGN-R1. We hypothesize that SP is bound by SIGN-R1 and then opsonized with C3d after which CD11c mediates stabilization. At this point the DC can transport and hand-off SP to the FDC; or hand-off SP to a B cell which then transports and hands-off SP to the FDC (Figure 5). In conclusion we show that DCs, besides B cells, are capable of transferring antigen to the FDC network.

Materials and Methods

Mice

Mice were bred in house or were from Jackson Laboratories or Charles River Laboratory. The following strains were used: wild type (C57BL/6 and BALB/c), CD11c-eYFP, C3$^{-/-}$, C1q$^{-/-}$, CD21$^{-/-}$ and CD11c-DTR[20,21]. All mice were on the C56BL/6 background. Mice were maintained in specific pathogen–free facilities at Immune Disease Institute, Dana Farber Cancer Institute or Harvard Medical School. All animal experiments were in accordance with protocols approved by the Subcommittee on Research Animals Care at Harvard Medical School and The Immune Disease Institute, and were in accordance with guidelines set by the National Institutes of Health.

Bacterial strains and growth conditions

Streptococcus pneumoniae strain D39 was grown in Todd Hewitt broth supplemented with yeast extracts and horse blood until log phase (OD650=1.5)[22]. SP was then labeled by incorporation of CMTPX dye and heat-inactivated at 65°C for 30 minutes

Antibodies

The following antibodies were purchased from BioLegend: anti-IgMa (DS-1), anti-B220 (RA3-6B2), anti-CD11c (N418), anti-CD11b (M1/70). CD169 (36.112 and MOMA-1)

was acquired from AbD Serotec. Anti-CD35 (8C12), anti-SIGN-R1 (22D1 and ERTR-9), anti-CD11b (M1/70) and anti-F4/80 (HB-198; American Type Culture Collection) were produced in-house and were purified by affinity chromatography. Secondary antibodies, including streptavidin–Alexa Fluor 488 (S11223), streptavidin–Alexa Fluor 568 (S11226), streptavidin–Alexa Fluor 633 (S21375) and Alexa Fluor 633–conjugated anti-rat (A21094; all from Invitrogen). Purified antibodies were labeled with Alexa Fluor 488 (A10235), Alexa Fluor 568 (A10238), Alexa Fluor 633 (A20170) or Pacific blue (P30012) according to the manufacturer's instructions (Invitrogen).

Mouse pretreatment.

For labeling of FDCs, mice received 5 µg anti-CD35 intraperitoneally 24 h before MP-IVM. For labeling of SSMs in vivo, 1 µg fluorescence-labeled CD169 was injected into the footpad 3–5 h before MP-IVM.

Enzyme-linked immunosorbent and immunospot assays.

Mice were immunized with 1×10^5 SP bacteria subcutaneously. At day 10, blood was collected and serum was obtained. Enzyme-linked immunosorbent assays were done as described[23]. For enzyme-linked immunospot assays, popliteal lymph nodes and spleens were removed aseptically and disrupted by passage through 70-µm mesh, and antibody-secreting cells were quantified as described[24].

Flow cytometry, data analysis and statistics.

A FACSCanto II, FACSCalibur or FACSAria (BD Biosciences) was used for flow cytometry. Dead cells were excluded by sytox blue or 7-amino-actinomycin D (Invitrogen). Data were analyzed with FlowJo software (Tristar). Prism 5 (Graphpad Software) was used for statistical analyses.

Bacteria injection.

Anesthetized mice were injected with 1×10^6 heat-inactivated CMTPX labeled bacteria in the hock (in a volume of 10 µl). At various time points, draining and non-draining lymph nodes were isolated for analysis of bacterial trafficking and SP-specific immune responses.

Histology and microscopy.

Cryosections of lymph nodes embedded in optimal cutting temperature compound (TissueTek) were prepared, then sections were washed with Hank's balanced salt solution and incubated with anti-FcR (2.4G2) before incubation with antibody as described[16]. Transmission electron microscopy of lymph nodes injected with SP was performed as described[16]. MP-IVM was performed as described[17]. For all mouse pretreatments, the hocks were injected with a volume of no more than 10 µl.

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Chapter 6

Summarizing Discussion

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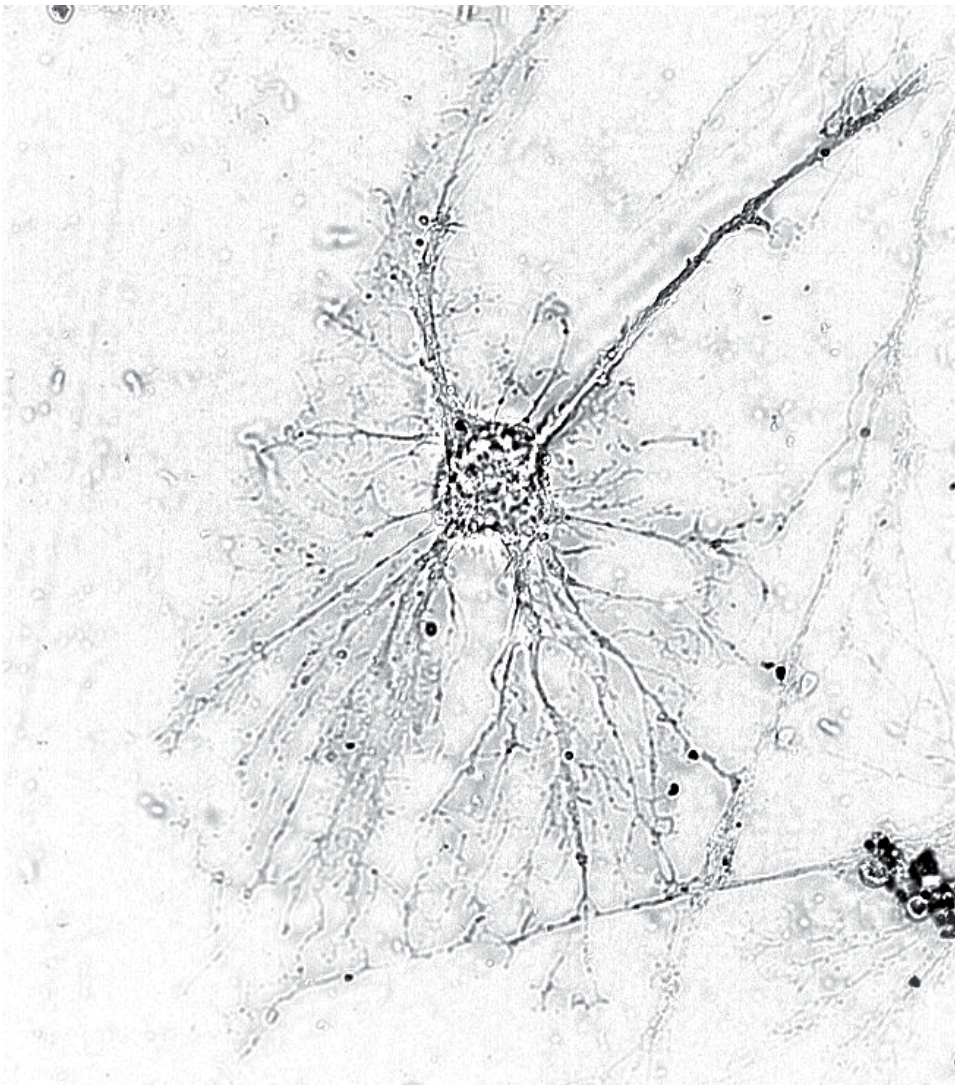

Single FDC in 2D culture on collagen coated glass. This extensive morphology is very rare, eventhough this cell is reminiscent of a neuron it is not and stains for multiple FDC markers.

Antigen retention in B cell follicles was first described 1950, however the cell type responsible was only observed in 1963 and named 'antigen-retaining reticular cell' in 1965. The name follicular dendritic cell (FDC) has been the consensus since 1978. A connection with the complement system and antigen retention was quickly made. Surprisingly the cell biology of this retention has been an enigma ever since and many other functions of FDCs are poorly studied.

The hallmarks of adaptive immunity are the ability to learn and remember such that when a pathogen is encountered for a second time the response will be quicker, stronger and more specific. These abilities of the immune system are what vaccination exploits by mimicking a primary infection. B cells are key players in the humoral adaptive immune response, which effector mechanism is based on the production of antibodies. In most vaccination strategies the serum levels of specific antibodies are used as readout to determine whether it was successful. Antibodies are secreted by B cells and are a soluble form of the B cell receptor (BCR).

The BCR is a trans-membrane receptor protein located on the surface of B cells. The receptor is composed of a membrane-bound antibody that, like all antibodies, has a unique and randomly determined antigen-binding site. During the immune response, upon encounter of its specific antigen, the receptor acquires a higher affinity for the antigen while in the germinal center (GC). In the GC B cells vary their BCR specificity by somatic hypermutation (SHM) a process that causes mutation in the DNA that encodes BCR binding site. As these mutations are semi-random, they can lead to higher or lower affinity of the BCR to the antigen. Generally, for an immune response to be effective, the mutations that lead to higher affinity should be selected. The BCR with the highest affinity for the antigen requires stimulation of the native antigen, preferably within the B cells follicle, to get selected. This affinity maturation within the GC reaction is an iterative process that is driven by the interaction between antigen on the FDC and the BCR on naive B cells. In turn, FDCs provide B cells with survival- and proliferation signals, inducing B cell clonal expansion.

FDCs form a unique cell population, located central to the B cells follicles of secondary lymphoid organs. Contrary to what their name might suggest these cells are not related to the myeloid dendritic cell population and are in fact stromal in origin. FDCs are an integral part of the extensive stromal network found in lymphoid organs, which is crucial for LN structure and homeostasis. FDCs retain antigen in a complement dependent manner and present it to B cells in GCs. Antigen that is opsonized by the complement protein C3 is captured from the lymph by macrophages or dendritic cells and transported to the FDCs by complement receptor 2 (CR2) on B cells.

A higher affinity of the BCR for an antigen is represented by an increase in their dissociation constant (Kd). On average, the interaction time between B cells and FDCs increases with an increased BCR affinity, allowing for more survival signals from FDCs to B cells. This will result in the cell death of B cells clones with unfavorable mutations and expansion of the clones with higher affinity. As this is an iterative process, B cells can undergo several rounds of SHM. Additionally, in a feed forward mechanism, affinity

maturated B cells will occupy all available antigen within the GC, competing out any clones with lower affinity.

The retention of antigen by FDCs is essential for an efficient GC reaction, especially in conditions where antigen is limited. Our current understanding of the GC reaction and the role of FDCs in this process would make us predict that FDCs retain antigen for the duration of the GC. However, FDCs can retain antigen for much longer periods (up to years). The immunologic function of this observation is poorly understood and hypotheses range from maintenance of memory to the retention being an artifact of the GC reaction. In order to begin to understand the immunological significance of this long-term retention phenomenon, we first need to understand the mechanistic underpinnings of FDC antigen retention, and the way they protect their cargo from degradation and damage. Maintaining the integrity of the antigen is a key feature for B cell function, as the antigen should mimic the native antigen in order to achieve the proper antibody specificity.

The body of work represented in this manuscript reports on our insights in the mechanism by which FDCs retain antigen for extended periods. We also describe potentially clinically relevant tools that we have developed to functionally manipulate this process. Manipulation of the system was tested in vivo and in vitro in mouse studies and confirmed in vitro in human studies. Future studies will focus on deeper understanding of the mechanism and application of our tools in primate studies.

All mammals have FDCs central to the B cell follicles within their secondary lymphoid organs. This system is very conserved in mammals; therefore it is justified to study the function and biology of FDCs in a murine model system. Interestingly, the Bursa of Fabricius, a primary lymphoid organ in birds, also contains antigen retaining FDC-like cells. The integral character of GC reactions did not allow us to make our observations solely in vitro, we therefore were obliged to utilize in vivo murine systems. However, to study how FDCs retain antigen is a cell biological question, for which an in vitro system is most suitable considering the technical limitations of in vivo research. Since there is no FDC cell line available, we choose to use an ex vivo system where FDCs were isolated from mice and cultured on collagen-coated glass coverslips. The experiments described in this thesis use both in vivo and ex vivo systems to study FDC biology.

A comprehensive overview of FDC biology, which introduces the topics discussed in this dissertation, can be found in chapter 1. Even though the focus is on FDCs, it is important to realize there are 4 other major stromal populations that can be distinguished on the basis of their morphology and function; fibroblastic reticular cells (FRCs), marginal reticular cells (MRCs), lymphatic endothelial cells (LECs) and blood endothelial cells (BECs). Together with these cells FDCs are part of an interconnected stromal network. Here we discuss the implications of dynamic antigen cycling on various processes and propose a model in which FDCs are the catalyst of the GC reaction[1].

That antigen is retained on FDCs has been known for a long time, however how the antigen gets there and how it is retained was not clear. In chapter 2 we show by

multi-photon intra-vital microscopy that non-cognate B cells directly transfer immune complexes (IC) to FDCs[2]. Earlier studies show that macrophages capture ICs from the lymph using complement receptor 3 (CR3) and transfer them to B cells, which capture the antigen through complement receptor 2 (CR2)[3,4]. Co-crystal structures of the CR3-C3d-CR2 complex shed light on the molecular mechanism behind this transfer[1,5]. Here we show that after the initial hand-off, the B cell travels to the FDC and hands the IC off to CR2 on the FDC. Then, contrary to the dogma, the FDC internalizes the IC into a non-degradative cycling endosome and retains it there for extended periods. This finding changed our thinking about FDCs from being a passive antigen depot to a dynamic antigen library with a lot of opportunities for regulation and control.

The previous finding suggested a general mechanism, however the study was only performed in mice. We show in chapter 3 that cycling is not restricted to mouse FDCs, but that human FDCs are also capable of cycling antigen. When we obtained LNs from people with HIV on anti-retroviral therapy (ART) we observed HIV within the same type of vesicle that cycle antigen. Co-cultures of FDCs from subjects with uninfected CD4 T cells showed that HIV retained by the FDC remains infective. When treating FDCs with a sCR2 decoy receptor we were able to displace HIV from the FDCs, preventing infection of CD4 T cells. However, displacement alone is not enough and to truly come a potential successful therapy sCR2 treatment should be combined with broadly neutralizing antibody treatment. If displacement of HIV from the FDC works in vivo we are one step closer to curing HIV.

For the FDC all antigen seems equal as long as it is opsonized by complement and self-antigen is nothing special for the FDC[6]. We review the possible implications of FDC cycling of self-antigen in autoimmune diseases such as lupus in chapter 4. We show that FDCs retain nuclear auto-antigens in the 564Ig transgenic lupus model. We propose a model in which inappropriate clearance of apoptotic debris promotes chronic activation of myeloid cells and FDCs. The inability to properly clear apoptotic debris arises in the absence of complement C4 and will eventually resulting in secretion of Type I interferon. This allows for the maturation and activation of self-reactive B cell clones leading to increased spontaneous formation of germinal centers and subsequent generation of auto-antibodies. These auto-antibodies then fuel the formation of more ICs, completing the vicious circle.

Once on the FDC there seems to be no difference between antigens as long as they are opsonized. However, there might be major differences in the transport towards the FDC. In chapter 5 we will discuss the trafficking of larger antigens (bacterial) in the LN, to complement earlier chapters that discussed small antigens (protein and viral). Interestingly Streptococcus pneumoniae localizes exclusively to the medulla of the LN where it is captured by dendritic cells (DCs) and macrophages. Eventually bacterial antigen is transported to the FDC where it is retained. Here we show that B cells are largely responsible for the transport of S. pneumoniae to the FDC area. The residual capture of S. pneumoniae by FDCs is mediated by DCs, which are capable of direct transport and hand-off to the FDC. Surprisingly, when DCs were ablated there was no more transport of S. pneumoniae to the FDC, not even by B cells. Therefore we

propose a model in which DCs are capable of transport, but crucial for the opsonization of S. pneumoniae, which is required for transport by B cells. In short, DCs and B cells collaborate in order to transport antigen to the FDC.

It will be interesting to see which receptors besides CR2 are involved in the cycling of antigen on FDCs and what signaling cascades regulate internalization, cycling and retention. There might be differences depending on the cargo or the type of antibody used to form the IC. Receptors capable of sensing these differences in ICs are Fc-receptors, which are up-regulated in a GC response, and Toll-like receptors (TLRs). However, if and how they are involved remains undetermined.

Understanding the precise mechanism of cycling will potentially create opportunities to manipulate FDCs and thus the GC reaction. How antigen behaves on the FDC and in the GC could steer the antibody response in different directions. FDCs could potentially regulate the amount of antigen available to B cells or express different survival factors or cytokines. Currently it is unclear what determines the quantity of antigen retained on FDCs or the rate at which it cycles. Altering any of these variables might have an effect on the type and length of the GC. The ability to understand how these parameters work, how to change them and what the consequences of such changes are, could be the platform for effective vaccination therapies. One thing is clear, removing unwanted antigens from the FDC has great potential in treatment of autoimmune disease and HIV infection. For both these diseases removal of antigen from FDCs cannot be a stand-alone therapy and they will need to be supplemented with additional therapies, such as ART or bNab in HIV.

In conclusion, FDCs are still an enigmatic cell type and additional research is needed to fully understand how this cell operates. The availability of reliable RNA sequencing data as well as a specific reporter mouse would greatly benefit the field. We still do not know exactly how the GC reaction is maintained, regulated and what determines the outcome. Nevertheless the concept of cycling antigen in FDCs does teach us that the process of antigen retention is much more dynamic than previously thought.

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Hoofdstuk 6

Samenvattende Discussie

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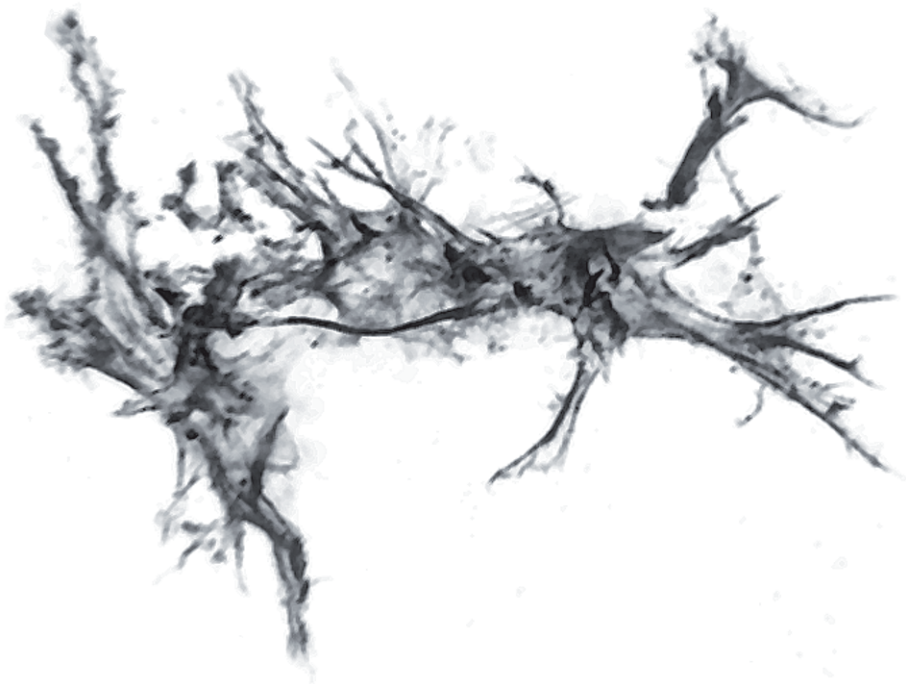

Single FDC in 3D culture in a collagen-based matrix gel. False coloring and image manipulation using Fiji.

Samenvatting

Wanneer het lichaam een pathogeen voor de tweede keer tegenkomt is de respons beter dan de eerste keer en wordt het niet meer ziek. Dit komt omdat het immuunsysteem een geheugen heeft en in staat is om te leren van voorgaande ervaringen. B-cellen spelen een sleutelrol in de humorale adaptieve immuunrespons. Dit effector-mechanisme is gebaseerd op de productie van antilichamen. Antilichamen zijn B-cel receptoren (BCR) die worden uitgescheiden in het extra cellulaire milieu. Tijdens een immuun respons vermenigvuldigen de B cellen die specifiek voor het antigeen zijn. Tijdens deze deling muteert de bindingsregio van de BCR en is dus onderhevig aan verandering. Sommige mutaties zullen leiden tot een zwakkere en sommige tot een sterkere affiniteit voor het antigeen. De BCR met de sterkere affiniteiten worden geselecteerd door middel van competitie voor beschikbaar antigeen. Alleen de sterke binders ontvangen overlevingssignalen.Folliculaire dendritische cellen (FDC) maken deel uit van het stromale netwerk in de lymfeknoop en houden antigeen vast voor lange periodes. FDCs geven overlevingssignalen aan de B-cellen. Paradoxaal genoeg houden deze cellen antigeen vast op een manier dat het beschikbaar is voor B cellen, maar ook onbeschadigd blijft. Dit laatste is erg belangrijk aangezien het lichaam alleen een antilichaam respons moet ontwikkelen tegen het originele pathogeen. Wij laten zien dat FDCs antigeen beschermen in een compartiment dat cyclisch naar het membraanoppervlak komt. Dit biedt de mogelijkheid voor de FDC om de immuunreactie te reguleren. Verder laten wij zien hoe HIV de FDC gebruikt als reservoir in patiënten die aan de antiretrovirale therapie zitten en hoe dit reservoir te legen is.

Retentie van antigeen in B cel follikels werd voor het eerst beschreven in 1950, echter het celtype verantwoordelijk voor deze retentie werd pas in 1963 waargenomen en in 1965 de naam “antigeen behoudende reticulaire cel” gegeven. De naam folliculaire dendritische cel (FDC) is sinds 1978 de consensus. Een verbinding tussen het complement systeem en de retentie van antigeen was snel gemaakt. Verrassend is dat de celbiologie van deze retentie een raadsel is en dat vele andere functies van FDCs slecht onderzocht zijn.

De voornaamste kenmerken van adaptieve immuniteit zijn leervermogen en geheugen, zodat wanneer een pathogeen voor een tweede maal wordt gezien, de respons sneller, sterker en meer specifiek is. Vaccinaties maken gebruik van deze aspecten van het immuunsysteem door een primaire infectie na te bootsen. B cellen spelen een sleutelrol in de humorale adaptieve immuunrespons, hun effectormechanisme is gebaseerd op de productie van antilichamen. In de meeste vaccinatiestrategieën worden de serumniveaus van specifieke antilichamen gebruikt als validatie. Antilichamen worden uitgescheiden door B- cellen en zijn een vorm van de B cel receptor (BCR) die niet meer verbonden is aan de B cel. De BCR is een transmembraan receptoreiwit op het oppervlak van B cellen. De receptor bestaat uit een membraangebonden antilichaam dat, zoals alle antilichamen, een unieke en willekeurig bepaalde antigeen bindingsplaats heeft. In de immuunrespons, bij de ontmoeting van een specifiek antigeen, krijgt de

receptor een hogere affiniteit voor het antigeen gedurende de kiemcentrum (KC) reactie.

In het KC variëren B cellen specificiteit van hun BCR door somatische hypermutatie (SHM), een proces dat mutatie veroorzaakt in het DNA dat de BCR bindingsplaats codeert. Aangezien deze mutaties semi-willekeurig zijn, kunnen ze leiden tot hogere of lagere affiniteit van de BCR voor het antigeen. In het algemeen, voor een effectieve immuunrespons, worden mutaties die leiden tot hogere affiniteit geselecteerd. De BCR heeft stimulering van het natieve antigeen nodig, bij voorkeur in de B cel follikel, om geselecteerd te worden. Door de gelimiteerde beschikbaarheid van het antigeen wordt er geselecteerd voor de meest specifieke BCR. Deze affiniteitsrijping in de KC-reactie is een iteratief proces dat wordt gestuurd door de interactie tussen enerzijds het antigeen op de FDC en anderzijds de BCR op naïeve B cellen. Dus, FDCs verschaffen B cellen met overlevings- en proliferatiesignalen die B cel klonale expansie mogelijk maakt.

FDCs vormen een unieke cel populatie, gelegen centraal in de B cel follikels van secundaire lymfoïde organen. In tegenstelling tot wat hun naam suggereert hebben deze cellen geen verband met de myeloïde dendritische cel populatie en zijn in feite van stromale oorsprong. FDCs zijn een integraal onderdeel van het uitgebreide stromale netwerk in lymfoïde organen en zijn cruciaal voor de structuur en homeostase van de lymfeknoop (LK). FDCs behouden antigeen in een complement-afhankelijke wijze en presenteren die aan B cellen in het KC. Antigeen dat geopsoniseerd is door complement eiwit C3 wordt gevangen uit de lymfe door macrofagen, dendritische cellen en B cellen. Op B cellen bindt complement receptor 2 (CR2) het antigeen en deze transporteren het naar de FDCs.

Een hogere affiniteit van de BCR voor een antigeen wordt weergegeven door een toename van de dissociatieconstante (Kd). Gemiddeld is de interactie tussen FDC en B cel met een grotere BCR-affiniteit langer, waardoor meer overlevings signalen van FDC aan deze B cellen wordt gegeven. Dit leidt tot apoptose van B cel klonen met ongunstige mutaties en vermenigvuldiging van de klonen met hogere affiniteit. Aangezien dit een iteratief proces is, kunnen B cellen verscheidene rondes van SHM ondergaan. Bovendien, zullen in een feed forward mechanisme, affiniteit gerijpte B cellen al het beschikbare antigeen binnen het KC bezetten en daardoor klonen met lagere affiniteit wegconcurreren. Het behoud van antigeen door FDC is essentieel voor een efficiënte KC- reactie, vooral in omstandigheden waar antigeen beperkt is. Ons huidige begrip van de KC-reactie en de rol van FDC hierin zou voorspellen dat FDCs antigeen behouden voor de duur van de KC. Echter, FDCs behouden antigeen voor veel langere periodes. De immunologische functie van deze waarneming is slecht begrepen en hypotheses variëren van het onderhoud van B cel geheugen tot een artefact van de KC reactie.

Om vat te krijgen op de immunologische betekenis van dit lange termijn retentie-fenomeen, moeten we eerst de mechanistische onderbouwing van FDC antigeen-retentie begrijpen, en de manier waarop ze hun lading beschermen tegen afbraak en schade. De integriteit van het antigeen op de FDC is belangrijk voor het functioneren van B cellen, zonder intact antigeen zal de antilichaamspecificiteit waarvoor geselecteerd

wordt niet tegen het daadwerkelijke pathogeen zijn.

Het werk vertegenwoordigd in dit proefschrift rapporteert onze inzichten in het mechanisme waarmee FDCs antigeen behouden voor langere tijd. We beschrijven ook potentieel klinisch relevante middelen die we hebben ontwikkeld om dit proces functioneel te manipuleren. Manipulatie van het systeem is getest in in vivo en in vitro studies in muizen, en bevestigd in vitro in humane studies. Toekomstig onderzoek zal zich richten op beter begrip van het mechanisme en toepassing van onze gereedschappen in onderzoek bij primaten.

Alle zoogdieren hebben FDCs centraal in de B cel follikels binnen hun secundaire lymfoïde organen. Dit systeem is zeer geconserveerd in zoogdieren. Daarom is het gerechtvaardigd om de functie en de biologie van FDCs te bestuderen in een muizenmodel. Interessant is dat ook de Bursa van Fabricius, een primair lymfoïde orgaan in vogels, antigeen behoudende FDC-achtige cellen bevat. De integrale eigenschappen van de KC-reactie laten ons niet toe om alle waarnemingen alleen in vitro te maken, daarom hebben we gebruik moeten maken van een in vivo muismodel. Echter, om te bestuderen hoe FDCs antigeen behouden is een celbiologische vraag. Door de technische beperkingen van een in vivo systeem, is een in vitro systeem hiervoor het meest geschikt. Aangezien er geen FDC cellijn beschikbaar is hebben we gekozen voor een ex vivo systeem waarbij FDCs geïsoleerd zijn uit muizen en gekweekt op een met collageen bedekt dekglas. De in dit proefschrift beschreven experimenten gebruiken zowel in vivo als ex vivo systemen om FDC biologie te studeren.

Een uitgebreid overzicht van FDC-biologie, waarin de onderwerpen van dit proefschrift geïntroduceerd worden is te vinden in hoofdstuk 1. Hoewel de focus ligt op FDCs, is het belangrijk om te realiseren dat er vier andere grote stromale populaties zijn in de LK. Deze populaties kunnen onderscheiden worden op basis van hun morfologie en functie; fibroblastische reticulaire cellen (FRCs), marginale reticulaire cellen (MRCs), lymfatische endotheelcellen (LECs) en bloed endotheelcellen (BECs). Deze cellen maken, samen met de FDCs, deel uit van een onderling verbonden stromaal netwerk. Hier bespreken we de gevolgen van de dynamische antigeen-recycling op verschillende processen en stellen we een model waarin de FDC een katalysator van de GC reactie is voor[1].

Dat antigeen lang op FDCs behouden wordt is al langer bekend, maar hoe het antigeen daar komt en hoe het wordt vastgehouden is niet bekend. In hoofdstuk 2 laten we door multi-photon intra-vital microscopy zien dat niet-specifieke B cellen direct immuuncomplexen (IC) over dragen aan FDCs[2]. Eerdere studies tonen aan dat macrofagen ICs uit de lymfe vangen met behulp van complement receptor 3 (CR3) en overgeven aan B cellen, die het antigeen door middel van complement receptor 2 (CR2) vangen[3,4]. Co-kristalstructuren van het CR3-C3d-CR2 complex werpen licht op het moleculaire mechanisme achter deze overdracht[1,5]. Hier laten we zien dat na de eerste overdracht, de B cel naar de FDC afreist en het IC afgeeft aan CR2 op de FDC. Vervolgens, in tegenstelling tot het dogma, internaliseert de FDC het IC in een niet-degraderend recycling endosoom en blijft daar gedurende zeer langer periodes. Deze bevinding veranderde ons denken over FDCs van een passief antigeen magazijn

naar een dynamische antigeen bibliotheek met veel mogelijkheden voor de regulering en controle.

De eerdere bevinding suggereert een algemeen mechanisme, maar de studie werd alleen uitgevoerd in muizen. We tonen in hoofdstuk 3 aan dat recycling niet beperkt is tot de muis FDC, maar dat de menselijke FDC ook antigeen kan recyclen. Toen we LKs van mensen met HIV op anti-retrovirale therapie (ART) verkregen observeerden we HIV binnen dezelfde soort compartimenten als diegenen die IC's recyclen. Co-culturen van FDCs van HIV patiënten met gezonde CD4 T cellen toonden aan dat HIV bewaard door de FDC besmettelijk blijft. Bij de behandeling van FDCs met een sCR2 decoy receptor konden we HIV van de FDC af krijgen, waardoor infectie van CD4 T cellen voorkomen werd. Echter, verwijdering alleen is niet genoeg en om tot een potentieel succesvolle therapie te komen zal sCR2 behandeling moeten worden gecombineerd met een breed neutraliserende antilichaam therapie. Indien verwijdering van HIV van de FDC werkt in vivo zijn we een stap dichterbij het genezen van HIV.

Voor de FDC lijkt antigeen gelijk zolang het geopsoniseerd is door complement en zelf-antigeen. Dat is niets bijzonders voor de FDCs[6]. We bekijken de mogelijke implicaties van FDC-recycling van zelf-antigeen in auto-immuunziekten zoals lupus in hoofdstuk 4. We laten zien dat FDCs nucleaire auto-antigenen vasthouden in het 564Ig transgene lupus muismodel. Wij stellen een model voor waarin ongepaste klaring van apoptotische vuil chronische activatie van myeloïde cellen en FDCs bevordert. Het onvermogen om goed apoptotische afval op te ruimen ontstaat in de afwezigheid van complement C4 en resulteert uiteindelijk in de secretie van type I interferon. Dit leidt tot de rijping en activering van auto-reactieve B cel klonen en tot spontane vorming van KC. Dit bevordert de generatie van auto-antilichamen. Deze auto-antilichamen zijn dan de brandstof voor de vorming van meer IC, dit voltooit de vicieuze cirkel.

Eenmaal op de FDC lijkt er geen verschil tussen antigenen zolang ze geopsoniseerd zijn. Echter, er zouden grote verschillen kunnen zijn in het vervoer naar de FDC. In hoofdstuk 5 zullen we het transport van grotere (bacteriële) antigenen in de LK, bespreken om eerdere hoofdstukken aan te vullen die het transport van kleine antigenen (eiwitten en viraal) besproken hebben. Interessant genoeg lokaliseert *Streptococcus pneumoniae* (SP) uitsluitend in de medulla van de LK waar het wordt opgevangen door dendritische cellen (DCs) en macrofagen. Uiteindelijk wordt bacterieel antigeen getransporteerd naar de FDC waar het wordt vastgehouden. Hier laten we zien dat B cellen grotendeels verantwoordelijk zijn voor het transport van SP naar de FDC. Het resterende transport van SP naar de FDC wordt gemedieerd door DCs, die in staat zijn om antigeen direct naar de FDC te transporteren. Verrassend genoeg, toen we DCs verwijderden was er geen transport van SP naar de FDC meer, zelfs niet door B cellen. Daarom stellen we een model voor waarin DCs SP kunnen vervoeren en cruciaal zijn voor de opsonisatie van SP, wat vereist is voor transport door B cellen. Kortom, DC's en B cellen werken samen om antigeen naar de FDC te vervoeren.

Het zal interessant zijn om te zien welke receptoren naast CR2 betrokken zijn bij de recycling van antigeen op FDCs en welke signaleringscascades internalisatie, recycling

en retentie reguleren. Afhankelijk van de lading, of het type antilichaam gebruikt om de IC te vormen kunnen er verschillen zijn. Receptoren die in staat zijn om deze verschillen in IC's waar te nemen zijn Fc-receptoren en Toll-like receptoren (TLRs). Echter, of en hoe zij betrokken zijn blijft onbekend.

Inzicht in de precieze mechanismen van het recyclen kan mogelijkheden om FDCs en dus ook de KC-reactie te manipuleren creëren. Hoe antigeen zich gedraagt op de FDC en in de KC kan de antilichaam-respons in verschillende richtingen sturen. FDCs zouden de hoeveelheid antigeen dat ter beschikking is voor B cellen of de expressie van verschillende overlevingsfactoren of cytokines kunnen regelen. Momenteel is het onduidelijk wat de hoeveelheid antigeen op FDCs of de snelheid waarmee het recycled bepaalt. Verandering van elk van deze variabelen kan een effect op de aard en lengte van de KC hebben. Door te streven naar een goed begrip van deze parameters en hoe deze parameters werken en wat de gevolgen van de veranderingen zijn, kan een platform voor effectieve vaccinatie therapieën worden gevormd. Echter, een ding is duidelijk, het verwijderen van ongewenste antigenen van de FDC heeft een grote potentie in het begrijpen van auto-immuunziektes en HIV-infecties. Voor beide ziekten kan verwijdering van het antigeen van de FDC een therapie zijn, ook al moeten ze waarschijnlijk worden aangevuld met additionele therapieën, zoals ART of bNab in HIV.

In conclusie, FDCs zijn nog steeds een slecht begrepen celtype en aanvullend onderzoek is nodig. De beschikbaarheid van betrouwbare RNA sequencing data, evenals een specifieke reporter muis zou veel baat hebben voor het veld. We weten nog steeds niet precies hoe de KC-reactie wordt gehandhaafd, gereguleerd en wat de uitkomst bepaalt. Echter, het begrip van antigeen-recycling in FDC heeft ons geleerd dat het proces van antigeen-retentie veel dynamischer is dan voorheen werd gedacht.

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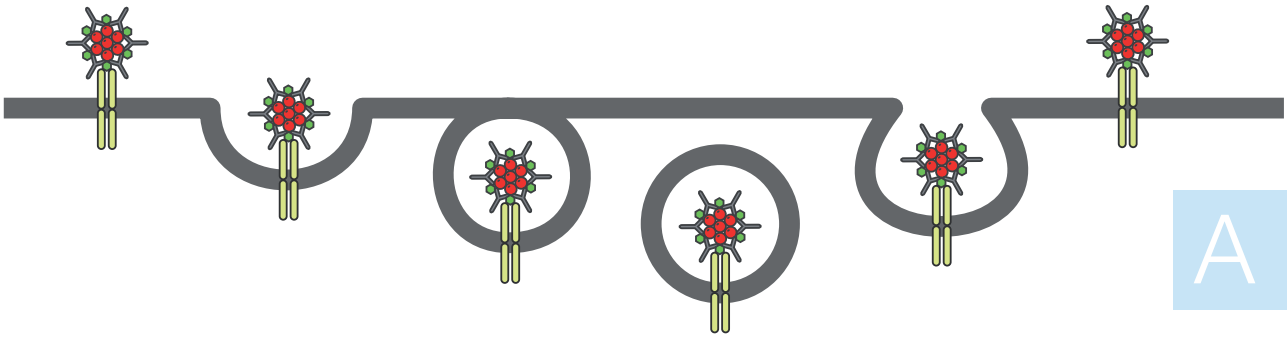
Addendum

Acknowledgments

Curriculum vitae

List of Publications



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Acknowledgments

Dear Mike, somewhere in the spring of 2009 I met you in Boston as part of a foreign excursion. I was fascinated by the multi-photon microscopy featured in your talk and afterwards I asked if I could rotate in your lab for six months. You told me: 'You can always email', so that's what I did and you agreed to let me rotate in your lab for six months. Near the end of this fruitful internship you offered me to join your lab as a PhD student, I had some reservations, so we agreed on a joint supervision between Jos and you. In retrospect I can safely say that joining your lab was one of the best decisions I have made. You taught me so many things; I particularly enjoyed our talks about the non-scientific aspects of running a lab, our scientific ski-trips and all the opportunities you gave me to meet so many people. Thank you for making me feel at home in your lab and for all the freedom you gave me to collaborate and explore. I couldn't have asked for more.

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Dear Elisabeth, it was great to joke with you in the lab. I especially enjoyed all the "at the Ragon" jokes whenever I arrived at lab a little later than everyone else. Thank you for keeping the lab in order and helping me on my many treasure hunts for old and long-forgotten reagents. It was a pleasure having you as a lab manager and I will miss your delicious baked goods.

Dear Matt, as my bench-buddy for the past 4 years you're by far the person I've seen the most. I enjoyed our friendship tremendously in and outside of the lab. I especially enjoyed our elaborate conversations about the 3-dimensional structure of the lymph node and I'm sure we'll keep in touch to discuss our science in the future.

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Another great collaboration was with Isaac, my future boss and a Carroll lab alumnus. Isaac, I think we form a great team that is very complimentary and I'm looking forward to work with you again. I also want to thank Clifford and Mike for supporting us during our bacteria-pain adventure.
The work on immune complex cycling would have taken a lot longer without the very helpful advice from Patrick and Tom. Patrick, thank you for always being there to listen to me and for you advice, both scientifically and career trajectory wise.
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Gabriel, I always enjoy the joint lab meetings with your lab, the beers afterwards and especially our talks about how the scientific world works on our way back home. Luka, thank you for your patience during our sorts and for all the sequencing, I hope we've figured it out by the time you'll read this.

Dear present and past members of the Carroll laboratory, you all have made coming to 'work' everyday a pleasure. Our numerous coffee breaks, lunches and discussions together were always a lot of fun. We goofed around a lot, but when you needed to be serious, you were there with solid advice. Thank you for answering my silly questions and telling me time and time again where I could find that one reagent I needed.

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My housemates and old-housemates, there isn't a more pleasant house that throws such incredible parties. You are all wonderful people and all your different backgrounds have taught me so much about life, thank you. Nancy, special thanks go out to you, without you I wouldn't have had a place to live, thank you for finding me a place while I was still in the Netherlands and for being an awesome friend!

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Shaida, I'm not going to iterate all the things I like about you because I don't think this is the place for that, but I do want to acknowledge your help with my thesis. You truly are the chief editor of this thesis, thank you for reading it and giving me honest feedback.

Mijn ouders, Ans en Balt (maar voor mij altijd gewoon Mam en Pap), al mijn vrienden zeggen het al jaren, jullie zijn echt twee van de vriendelijkste en fijnste mensen die ik ken. Het is zo fijn om jullie steun te hebben, ik kan me geen betere ouders voorstellen, bedankt voor alles.

Curriculum vitae

On February 14, 1987 I was born in Amsterdam, the Netherlands and named Balthasar Adrianus Heesters. In 2005 I obtained my high school diploma at the S.G.G. Nehalennia in Middelburg, after which I began my studies in Biomedical Sciences at Utrecht University. In 2008 I successfully completed this undergraduate degree. The same year I enrolled in the master program in Immunity and Infection at Utrecht University. As part of the master program I first did a nine-month research internship at the Virology department in Utrecht. In the research group of Prof. Dr. Raoul de Groot my focus was on O-acetylated sialic acids under the supervision of Dr. Martijn Langereis. I then wrote a literature thesis in the group of Prof. Dr. Jos van Strijp on Complement evasion of pathogens. In January 2010 I started a six-month internship in the group of Prof. Dr. Michael Carroll at Harvard University, Boston, USA, where I worked on the transport of S. pneumoniae in the lymph node. I concluded the master program in December of 2010. In January 2011 I returned to the research group of Prof. Dr. Michael Carroll at Harvard University to start as a PhD student. The results of this study are described in this thesis. In the spring of 2015 I will continue my research career at Harvard Medical School in Boston in the group of Dr. Isaac Chiu. There I will work on neuro-immune interactions, with a focus on pain neurons.

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