Published in final edited form as:

Sci Immunol. 2016 December ; 1(6): . doi:10.1126/sciimmunol.aah4447.

The human thymus perivascular space is a functional niche for viral-specific plasma cells

Sarah Nuñez^{1,6}, Carolina Moore², Baoshan Gao², Kortney Rogers¹, Yessia Hidalgo⁶, Pedro J. del Nido³, Susan Restaino⁴, Yoshifumi Naka⁵, Govind Bhagat⁷, Joren C. Madsen², María Rosa Bono⁶, and Emmanuel Zorn^{1,*†}

¹Columbia Center for Translational Immunology, Department of Medicine, Columbia University Medical Center, New York, NY, USA

²MGH Transplant Center and Division of Cardiac Surgery, Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

³Department of Surgery, Boston Children Hospital, Boston, MA, USA

⁴Department of Medicine, Columbia University Medical Center, New York, NY, USA

⁵Department of Surgery, Columbia University Medical Center, New York, NY, USA

⁶Department of Biology, University of Chile, Santiago, Chile

⁷Department of Pathology and Cell Biology, Columbia University Medical Center, New York, NY, USA

Abstract

The human thymus is susceptible to viral infections that can severely alter thymopoiesis and compromise the mechanisms of acquired tolerance to self-antigens. In humans, plasma cells residing primarily in the bone marrow confer long-lasting protection to common viruses by secreting antigen-specific antibodies. Since the thymus also houses B cells, we examined the phenotypic complexity of these thymic resident cells and their possible protective role against viral infections. Using tissue specimens collected from subjects ranging in age from 5 days to 71 years, we found that starting during the first year of life, CD138⁺ plasma cells (PC) begin accumulating in the thymic perivascular space (PVS) where they constitutively produce IgG without the need for additional stimulation. These, thymic PC secrete almost exclusively IgG1 and IgG3, the two main complement-fixing effector IgG subclasses. Moreover, using antigen-specific ELISpot assays, we demonstrated that thymic PC include a high frequency of cells reactive to common viral proteins. Our study reveals an unrecognized role of the PVS as a functional niche for viral-specific PCs.

*Corresponding author: ez2184@cumc.columbia.edu.

†Address: Columbia Center for Translational Immunology, Columbia University Medical Center, 650 West 168th Street, Black Building, room BB1701F, New York, NY 10032. Tel (212) 342-3453

Authors contribution: S.N. processed donor tissue, carried out all experiments, analyzed data and wrote the paper. C.M. processed donor tissue and contributed to immunochemistry and flow cytometry experiments. B.G. processed donor tissue. K.R., S.R. and J.C.M. coordinated tissue acquisition. P.N. and Y.N. performed surgical acquisition of donor tissue. Y.H. performed ELISpot data acquisition and analysis. G.B. assisted with histological analysis. M.R.B. contributed to the study design. E.Z. coordinated tissue acquisition, designed experiments, analyzed data and wrote the paper.

Competing interests: The authors declare no competing interests.

The PVS is located between the thymic epithelial areas and the circulation. PCs located in this compartment may therefore provide internal protection against pathogen infections and preserve the integrity and function of the organ.

Introduction

The thymus is a common target organ for infectious pathogens. Viral, bacterial and fungal infection of the thymus often results in severe atrophy, which can have dramatic consequences for the integrity and function of this crucial lymphoid organ (1). In mice, influenza infection triggers extensive thymocyte apoptosis causing atrophy of large part of the gland (2). In humans, the measles virus can also be potentially harmful to the thymus, infecting cortical thymic epithelial cells and affecting their function in T cell development (3). As described in several animal studies, viral infections of the thymus can interfere with central tolerance through the modulation of both positive and negative thymocyte selection (3–6). The recruitment of antimicrobial immunity directly to the thymus can help resolve local infection. For instance, it was reported that effector T cells specific to influenza (2), lymphocytic choriomeningitis virus (7), and *Mycobacterium tuberculosis* (8) homed to the thymus following infection and efficiently controlled the viral burden in the organ.

B cells are essential elements in the establishment of protective immunity to pathogens. The thymus contains a significant subset of resident CD20⁺ B cells (9). Although initially described as being mostly IgM⁺ naïve B cells in mice (10, 11), the normal human and mouse thymus also contains class-switched membrane-IgG⁺ cells (12–14). The thymus is a highly dynamic organ that undergoes profound structural and functional changes throughout life. The size of the thymus progressively decreases with age together with its output of naïve T cells through a process known as thymic involution (15). In addition to the cortex and medulla, the thymus also contains a third compartment called perivascular space (PVS), which surrounds blood vessels within the capsule but is separate from the thymic epithelial space. This third compartment is often overlooked as it only represents a minimal area of the thymus during infancy and does not appear to be a site of thymopoiesis. As the thymus ages, however, the PVS enlarges and, progressively replaces the epithelial area (16). Although most studies have concluded that B cells are restricted to the medulla where they can participate in negative selection, Flores et al. have shown that lymphoid cells, including B cells are also present in the PVS. The function of PVS B cells, however, has not been examined (17). We investigated whether B cells located in the thymic PVS include pathogen-specific clones. We present here a detailed analysis of the distribution, phenotype and key functional aspects of human thymic B cells from 35 donors throughout seven decades of life. Our findings reveal an unrecognized role of the thymic PVS as a niche for viral antigen-reactive plasma cells. Because of its location at the interface between the circulation and thymic epithelium, antigen-experienced B cells within this niche may confer protection to the thymus gland from a host of viruses.

Results

The human thymus contains B cells in two distinct compartments: medulla and perivascular space

Previous studies have described abundant B cells in the medulla of the human thymus. An analysis of the distribution of B cells among thymic compartments using specimens from donors aged 5 days to 71 years showed that B cells were distributed throughout the thymic medulla, occupying 10–60% of the area in that compartment (Fig. 1A and Fig. S1, S2). In children older than 1 year, abundant clusters of B cells could also be detected in areas adjacent to but distinct from the medulla (Fig. 1A and Fig. S1). In adults, where thymic morphology was showing evident signs of atrophy, B cells were abundantly distributed throughout the tissue where cortex and medulla could not be clearly defined (Fig. 1A and Fig. S1). To determine the localization of these clustered B cells with greater accuracy, we performed immunofluorescence staining of the cytokeratin network. This staining allowed visualization of the cytokeratin⁺ medullary thymic epithelial network and exposed the non-epithelial perivascular space (PVS) (Fig. S3). In all ages we observed B cells co-localizing with cytokeratin⁺ medullary thymic epithelial cells (mTECs). However, children older than 1 year and adults also contained prominent B cell clusters located in the non-epithelial areas of the thymus (Fig. 1B, Fig. S4), which appeared in early childhood and replaced most of the thymic epithelial areas in adults (Fig. S3). These well-defined non-epithelial areas correspond to the PVS as evidenced by positive staining for the endothelial marker CD31 (PECAM-1) (Fig. 1B, Fig. S5). The presence of CD20⁺ B cells in virtually all areas devoid of cytokeratin (Figure 1 and S4, children and adults) strongly suggested that these were located in the PVS. Based on the differences in the distribution of B cells and thymic morphology observed among donors (Fig. S1), we empirically defined three age groups for the remainder of the study: infants 0–1 year (N=15; actual range 5 days–11 months; median age 3 months), children 1–20 years (N=13; actual range 1.1 years–15 years; median age 3 years), and adults 20–60 years (N=7; actual range 27 years–71 years; median age 40 years).

Memory B cells accumulate in the aging thymus

We next examined the phenotype of thymic B cells in relation to the age of the donors. The CD19⁺ B cell frequency among total thymocytes increased significantly from <5% in infants and children to 5–15% in adult thymuses (Fig. 2A). In infants, the vast majority of thymic B cells were naïve cells with only a minimal fraction (2–15%) expressing the memory marker CD27. In children and adults, the frequency of CD27⁺ memory B cells significantly increased in an age-dependent manner and this subset became predominant in adult thymus (Fig. 2B). We also examined membrane immunoglobulin expression to assess the presence of IgD⁺IgM⁺ cells, IgD⁺IgM^{low} B cells, IgG⁺ and IgA⁺ class-switched B cells, as well as Ig[−] B cells. In line with the accumulation of memory B cells, B cells in infants were largely IgD⁺IgM⁺ naïve B cells (40–70%) whereas this population decreased with age and was replaced by class-switched IgG⁺ cells (Fig. 2C). Class-switched IgA⁺ B cells were also present at a minor but steady frequency (9.9±5.1 %) in all age groups. Although most CD27⁺ memory B cells express IgG or IgA immunoglobulins, a minor fraction corresponded to unswitched IgM⁺ memory B cells (Fig. S6B). The size of all thymic B cell subsets was different from that of the same subsets observed in the blood of healthy donors

of the corresponding age (18). We compared the frequency of memory B cells in thymus and peripheral blood in adult donors from which matched blood samples were available. The proportion of class-switched IgG⁺ and CD27⁺ memory B cells was significantly higher in the thymus compared to blood (Fig. 2D), indicating a tissue-specific enrichment of memory B cells rather than a reflection of circulating B cell subsets. Together, these data provide evidence of a previously overlooked group of B cells that accumulate in the thymic PVS, consistent with the accumulation of memory B cells in this organ.

Additionally, age-associated changes in the thymic B cell phenotype were not limited to the composition of naïve and memory B cells. Since previous studies in mice have shown that thymic B cells can present antigens to developing T cells in the medulla and contribute to negative selection, we also investigated the expression of molecules involved in this function, including MHC-II, co-stimulation molecules CD80, CD86 and CD83, and CD40. All molecules were highly expressed by thymic B cells from infant thymuses but their expression decreased on B cells from older thymuses (Fig. S7A). Moreover, we observed that CD19⁺ cells present in infant thymuses expressed significantly higher levels of MHC-II than CD11c dendritic cells isolated from the same specimens (Fig. S7B, C).

The aging thymus harbors antibody-producing plasma cells

In addition to memory B cells, we investigated whether the thymus might also harbor fully differentiated antibody-secreting plasma cells. This was examined by immunofluorescence staining of the adhesion proteoglycan CD138. While plasma cells were scarcely detected in infant thymus, sections from children and adults specimens showed CD138⁺ cells that were clustered in cytokeratin-negative non-epithelial areas corresponding to the PVS (Fig. 3A, Fig. S8). Plasma cells within the PVS were also detected by the staining of the secretory form of IgG in child and adult thymuses (Fig. 3B, Fig. S9). Simultaneous staining of CD20, CD138 and secretory IgG, revealed that most CD138⁺ cells, which contained cytosolic IgG, had lost expression of CD20, consistent with the phenotype of terminally differentiated plasma cells (Fig. 3C).

The persistence of plasma cells in the bone marrow is controlled by cell-intrinsic mechanisms as well as the existence of nurturing cells such as eosinophils within the niche that provide key external signals (19). We asked whether any of these mechanisms were involved in the maintenance of thymic plasma cells. Following hematoxylin and eosin staining, we observed the presence of plasma cells in proximity to eosinophils in all sections we analyzed from 7 donors ranging from 3 to 39 years of age (Fig. 4A, Fig. S10). Moreover, we found that thymic plasma cells expressed CD28, known to promote the survival of bone marrow plasma cells (19), at significantly higher levels compared to circulating plasma cells (Fig. 4B).

We further confirmed the presence of functional plasma cells in the human thymus by examining their ability to secrete antibodies. IgM, IgG and IgA-producing plasma cells were enumerated using ELISpot. Thymocytes from infants, children and adults contained cells that spontaneously secreted IgM, IgG and IgA albeit at different ratios (Fig. 5A), while few Ig-secreting cells were detected in unstimulated PBMCs (Fig. S11). Infant thymuses contained predominantly IgM-secreting cells along with few IgG- and IgA-secreting cells. In

contrast, the frequency of IgM-secreting cells decreased among thymocytes from children and adults, which contained mostly IgG-secreting cells and to a lesser extent IgA-secreting cells (Fig. 5A). To gain further insight into the functional properties of these antibodies, we analyzed the subclass profile of IgG secreted by thymic plasma cells. Most IgG-secreting cells produced IgG3 and to a lesser extent IgG1 (Fig. 5B). These two IgG subclasses are the most abundant type of IgG produced in the course of viral infections and are most efficient at complement fixation and antibody-dependent cellular cytotoxicity (20, 21). No IgG2- or IgG4-secreting cells could be detected. In addition to fully differentiated plasma cells, we also found that the thymus contained plasmablasts identified as CD19⁺ cells expressing IRF4, a key transcription factor driving the plasma cell fate. As previously described, this factor is upregulated at the plasmablast stage (22). In most donors, plasmablasts represented less than 5% of total thymic B cells. However in some cases, particularly among children close to 1 year of age, this subset was present at a significantly higher frequency (Fig. 5C). CD19⁺IRF4⁺ cells also displayed high expression of CD27, consistent with a plasmablast phenotype (Fig. S12).

The thymus contains viral-specific plasma cells and memory B cells

Humoral immunity is maintained by pathogen-specific long-lived plasma cells residing primarily in the bone marrow. We reasoned that the newly identified pool of plasma cells maintained in the thymic PVS may also include pathogen-specific clones resulting from infectious experience or vaccination. To test this possibility, we assessed the frequency of IgG-producing plasma cells specific to prevalent viral pathogens and vaccine antigens among thymic B cells from infants younger than 12 months (N=3; median age 2 months), thus prior to trivalent measles-mumps-rubella vaccination, and children aged 13 months to 15 years (N=10; median age 3 years). Antigen-specific ELISpots were carried out by coating plates with either viral immunodominant epitopes or whole virus preparations of influenza, cytomegalovirus (CMV), Epstein-Barr virus (EBV), rhinovirus B, adenovirus C, measles and rubella. Viral specific cells were detected in multiple donors (Fig. 6A,B). The frequency of plasma cells specific to influenza and measles was significantly higher in children compared to infants ($p<0.01$ and $p<0.05$ respectively). Two of the three subjects displaying a high response to measles (>5% of all ASC) also displayed high response to rubella, albeit, because prior health information was not available for these subjects, we could not relate the data to their vaccination history. Reactivity to EBV, adenovirus C and rhinovirus B was also detected in few donors and did not show statistical difference between infants and children.

Since the thymus progressively accumulates memory B cells, we examined if these cells could also provide an antiviral response upon reactivation. We found that following brief polyclonal stimulation, thymic memory B cells in children and adults acquired the phenotype of activated plasmablasts, upregulating CD27, CD19 and the IRF4 transcription factor (Fig. 7A). We tested the reactivity of IgG-producing activated memory B cells against the panel of antigens detailed previously and found that cells with antiviral specificity were also contained in this subset (Fig. 7B,C).

We next investigated whether a particular chemokine axis could have directed the homing of activated effector B cells to the thymus PVS. We analyzed the expression of chemokine

receptors CXCR3, CXCR4 and CXCR5 receptors, which are required for the recirculation of B cells through sites of inflammation, bone marrow, and lymphoid tissues (23). While CXCR4 and CXCR5 are expressed by virtually all thymic B cells, regardless of age, (Fig. 8B,C) the expression of CXCR3 showed a different pattern. Most thymic B cells in infants did not express CXCR3. However, a distinct CXCR3⁺ B cell subset appeared in the thymus of children and gradually increased with age (Fig. 8A). These CXCR3⁺ cells located within the PVS as revealed by immunofluorescence (Fig. 8D).

Discussion

Long considered an immune privileged site, the thymus is now recognized as a target of viral, bacterial and parasitic infections. As shown in multiple animal studies, these infections can lead to thymic atrophy and reduce the overall thymopoiesis (3). Local viral-induced inflammatory reactions can also compromise the fine mechanisms of T cell selection, resulting in the export of improperly matured T cells in the periphery or the tolerance of new thymocytes towards the infecting agents (3). While less studied, consequences of thymic infections in humans are also detrimental. An example is measles infection. This viral infection is initiated in the respiratory tract and spreads systemically to multiple target organs, including the thymus. There, the viral tropism for thymic epithelial cells results in severe thymocyte loss and immunosuppression (24–26). For its central role in the development of T cell immunity, the thymus warrants efficient protective mechanisms against infections. The common view is that such protection is provided by serological immunity accrued through immunizations. Using a collection of thymus specimens collected from neonates to elderly donors we reveal here an unknown characteristic of the human thymus and more specifically the perivascular space, to house memory B cells and plasma cells with anti-viral specificities. Because of its location, the PVS is a natural buffer zone between the circulation and the thymic cortex and medulla. Local production of viral-specific antibodies within this space therefore represents an immune barrier to free viral particles. More generally, this unrecognized intrathymic B cell immunity is a likely natural defense against blood borne pathogens.

Evidence of B cells as a normal component of the thymus was reported more than four decades ago, by the identification of an unusual subset of thymocytes that expressed surface immunoglobulin (27). Subsequent characterization of human and mouse thymus confirmed the existence of thymic resident B cells confined to the medulla. In humans, thymic B cells have been investigated mainly in fetal and pediatric donors (9, 12, 28). Likewise, studies in mice have been conducted using juvenile or young adults. As a result, the evolution of the thymic B cell population with age has not been thoroughly investigated. Our studies used a collection of human thymus specimens collected from donors of different ages to address this question. Our findings reveal the existence of two distinct subsets of B cells in the human thymus located in the medullary and the perivascular space respectively. Medullary B cells co-localize with mTEC and immature thymocytes. We could detect this subset as early as five days after birth, however previous studies indicates that B cells colonize the thymus even earlier, during fetal development (12). They have a characteristic CD27⁺IgD⁺IgM⁺ unswitched naïve phenotype with remarkably high expression of MHC class II and co-stimulatory molecules, supporting a role as antigen-presenting cells (Fig. S7). This

observation supports recent studies that have used transgenic mouse models with targeted presentation of foreign and self-antigen to demonstrate the contribution of thymic B cells to negative selection (13, 14, 29) while other studies have also pointed to a possible role in regulatory T cell differentiation (30, 31).

As revealed in our studies, the second subset of B cells starts to appear in the thymic PVS after the first year of life. It is noteworthy that the PVS enlarges with age, allowing an increasing number of B cells to accumulate in this area. PVS B cells include switched IgG⁺CD27⁺ memory cells together with terminally differentiated plasma cells secreting primarily IgG. We confirmed the function of the plasma cells by demonstrating their ability to spontaneously secrete antibodies ex vivo without the need for additional stimulation. This ability is characteristic of bone marrow-resident plasma cells (32, 33). In contrast, thymic-derived memory B cells required in vitro stimulation during a 3-day culture in order to initiate antibody secretion. All classes of immunoglobulins, except IgE, are secreted by PVS B cells, however, IgG secreting cells gradually become a predominant subset. Antibody production in the thymus has been documented in early studies in mice and pigs (34, 35). However our studies are the first to comprehensively characterize these cells in human thymic specimens in relation to reactivity and age.

The time of appearance of this second contingent of effector B cells in the thymic PVS appears to coincide with the ability to develop strong and persistent IgG antibody responses. Such humoral immunity is relatively weak during the first 12 months of life but progressively strengthens throughout childhood (36, 37). The detection of high frequency viral antigen-specific cells suggests that thymic memory B cells and PC result from anti-viral B cell responses elicited in early life. The predominance of IgG1 and IgG3 among IgG secreted by thymic plasma cells is consistent with such protective T cell-dependent humoral responses to pathogens and vaccine antigens (21, 38–42). The increasing frequency of CXCR3⁺ thymic B cells, which localize in the PVS, also supports this view. Expression of this chemokine receptor is upregulated on activated memory B cells and controls the migration of B cells to the bone marrow and sites of ongoing inflammation (23, 43, 44). A comparable recruitment of pathogen-specific effector T cells expressing CXCR3 is observed in the mouse thymus during mycobacterial infection (8). Collectively, these observations support the hypothesis that memory B cells and plasma cells accumulate in the PVS following peripheral immune responses and therefore reflect past immunization history.

Altogether, several lines of evidence support the existence of a PC niche in the aging human thymus: 1) the density of IgG-secreting, CD138⁺ PC observed in the PVS is consistent with a specialized niche, 2) the ability of thymic PC to spontaneously secrete immunoglobulins without the need for additional stimulation in vitro is characteristic of fully differentiated long-lived PC, 3) the frequency of viral-specific PC is comparable to that seen in bone marrow PC populations, 4) the persistence of measles and rubella-specific PC in 3, 6 and 15 year old donors (supplementary Table 6), i.e. years after the presumed time of MMR vaccination, suggests the presence of long-lived PC, 5) Eosinophils are detected in the thymic PVS in proximity to PC. These polymorphonuclear cells are essential for the maintenance of long-lived PC in the bone marrow (19), 6) Thymic PC express high levels of CD28, a necessary receptor required for the long-term maintenance of PC the bone marrow

niche (45). While the thymic and bone marrow niche and PC share a number of characteristics, it is also evident that they differ by others. For instance, the two niches have different anatomical locations, which may influence the function of their resident PC.

Our study has a number of limitations in part due to the difficulty of obtaining other tissue specimens from the same donors from whom we collected the thymus fragments. As a result, we could not compare thymic PC with bone marrow or spleen PC from the same individuals. Additionally, while the phenotypic and functional features of thymic PC suggest their involvement in long-term protective immunity, we could not formally demonstrate this capacity. Nevertheless, our observations raise several questions. First, is there a quantitative contribution of thymic PC to the amount of serum immunoglobulins, particularly IgG? A comparison between the number of long-lived thymic PC to that of the bone marrow could help evaluate this contribution. While the volume of the bone marrow far outweighs that of the thymic tissue in adults, it is conceivable that this ratio be inverted during the first few years of life. Consequently, the infant thymus may represent a significant, even primary reservoir for plasma cells during this early life period. The second question is whether thymic PC have a different repertoire of antigen specificities and therefore produce different immunoglobulins than bone marrow PC. In that scenario, the contribution of thymic PC would be more qualitative than quantitative.

In conclusion, we provide here evidence that pathogen-specific antibody-producing plasma cells accumulate in the PVS of the human thymus. These cells are ideally poised to act as sentry against blood-borne infectious agents.

Materials and Methods

Study design

The objective of the research study was to characterize the localization, phenotype and functional properties of B cells located in the human thymus in relation with age. The working hypothesis was that thymic B cell subsets evolve with age in a manner consistent with their role. The study used thymus specimens collected randomly from pediatric and adult patients undergoing corrective heart surgery. A total of thirty-five specimens were collected from two separate institutions that were included in three age groups: infants (N=15), children (N=13) and adults (N=7). The sample size was evaluated as to provide sufficient statistical power to analyze all biological variables examined between the three age groups. None of the subject had a condition known to affect the immune system. Functional assays were carried out on a restricted number of samples because of cell availability. No randomization or blinding was used in this observational study. All data were reported, including outliers. All Elispot assays were carried out in duplicates.

Collection of human thymus

Thymus tissue was obtained from 35 subjects (age 5 days – 71 years) detailed in Table S1. Pediatric donors (age 5 days – 20 years) were patients undergoing corrective cardiac surgery at Boston Children's Hospital. Adult donors (age 20 – 71 years) were patients undergoing cardiac surgery (with the exception of donor #29 (27 years old) who was a deceased organ

donor) at Massachusetts General Hospital and Columbia Presbyterian Hospital. Collection of bona fide thymic tissue was verified by assessing characteristic CD4/CD8 single positive and CD4+CD8+ double positive thymic subsets in all donors (Fig. S14). Since collected thymus was discarded tissue from partial thymectomy performed during cardiac surgery, this study followed a non-human subject protocol approved by the MGH and Columbia University IRB, respectively. Blood was also obtained from adult donors after informed consent.

Sample processing

Thymic tissue was maintained in ice-cold PBS and processed immediately after removal. Following extensive washing with PBS to remove blood, part of the tissue was fixed in a 10% paraformaldehyde solution and paraffin-embedded for histological analysis. The remaining tissue was disrupted and homogenized using a gentleMACS tissue dissociator (Miltenyi Biotec) and filtered through a 40µm Cell Strainer (BD Biosciences). PBMCs from adult donors were isolated by Ficoll (GE Healthcare Lifesciences) density gradient. Thymocyte and PBMC cell suspensions were frozen in heat inactivated fetal bovine serum (Atlanta Biologicals) supplemented with 10% DMSO and kept in liquid nitrogen.

Immunohistochemistry and tissue immunofluorescence

Immunohistochemical and immunofluorescence stainings were performed on 4µm-thick FFPE tissue sections. Sections were treated with xylene and graded alcohols and antigen retrieval was performed in citrate buffer (Biocare Medical) using a Decloaking Chamber (Biocare Medical). Regular immunohistochemical staining was performed using Cell and Tissue Mouse HRP kit (R&D) and counterstained with Mayer's hematoxylin (Sigma-Aldrich). For immunofluorescence, background staining was blocked with Background Sniper solution (Biocare Medical) after antigen retrieval. The following antibodies were used for immunohistochemistry and immunofluorescence: anti-human CD20 (L26, Dako), CD20 (BV11, Abcam), Cytokeratin (AE1/AE3, Abcam), CD31 (EPR3094, Abcam), CD34 (EP373Y, Abcam), CD138 (SP152, Abcam), Secretory IgG (EPR4421, Abcam). Primary antibodies were diluted in TBS with 1% BSA and incubated overnight at 4°C. For immunofluorescence, control stainings with fluorochrome-conjugated secondary antibodies only are presented in Fig. S15.

Flow cytometry

The following fluorochrome-conjugated antibodies were used for flow cytometry: anti-human CD4 (Phycoerythrin/Cy7, SK3, BD Biosciences), CD8 (Phycoerythrin, SK1, Tonbo Biosciences), CD11c (Brilliant violet 510, B-ly6, BD Biosciences), CD19 (Brilliant violet 711, SJ25C1, BD Biosciences), CD19 (Allophycocyanin, SJ25C1, Tonbo Biosciences), CD20 (Brilliant ultraviolet 737, 2H7, BD Biosciences), CD27 (Allophycocyanin, O323, Tonbo Biosciences), CD40 (Allophycocyanin/H7, 5C3, BD Biosciences), CD80 (Brilliant violet 605, L307.4, BD Biosciences), CD83 (Phycoerythrin, HB15e, BD Biosciences), CD86 (Brilliant violet 650, 2331, BD Biosciences), IgM (Brilliant violet 650, MHM-88, Biolegend), IgG (Phycoerythrin/Cy7, G18-145, BD Biosciences), IgA (FITC, IS11-8E10, Miltenyi), MHC-II (HLA-DR; FITC, G46-6, BD Biosciences), IRF4 (Phycoerythrin, IRF4.3E4, Biolegend), CXCR3 (Phycoerythrin, G025H7, Biolegend), CXCR4

(Phycoerythrin, 12G5, Biolegend), CXCR5 (Brilliant violet 421, J252D4, Biolegend). DAPI solution (3uM, Invitrogen) was used as a viability exclusion dye. For fixed and permeabilized cells, Fixable Viability stain 450 (BD Biosciences) was used to exclude dead cells. Cells ($1-2 \times 10^6$) were stained in FACS buffer (PBS supplemented with 2% FBS) for 30 minutes at 4°C protected from light. Cells were washed twice and resuspended with FACS buffer for acquisition. For intracellular staining, after surface staining, cells were fixed and permeabilized with Nuclear Factor fixation and permeabilization Buffer Set (Biolegend). Cells were acquired and analyzed on a FACSCanto II cytometer (BD Biosciences) or LSR II cytometer (BD Biosciences). Data was analyzed using FlowJo software (Treestar). Detailed flow cytometry data is presented in Tables S1, S2, S3 and S4.

ELISpot assays

ELISpot plates (MSIPN4510, Millipore) were coated with anti-human IgM (5ug/ml, Mabtech), anti-human IgG (5ug/ml, Mabtech), and anti-human IgA (5ug/ml, Mabtech) for the quantification of total antibody-secreting cells (ASC). For detection of antigen-specific ASC, plates were coated with H1N1 Influenza (3ug/ml, Prospec), CMV immunodominant antigens Pp150, Pp52 and Pp28 (5ug/ml, Prospec), EBV immunodominant antigens EA and EBNA (5ug/ml, Prospec), Rhinovirus B immunodominant peptide (QTDALTEGLSDELEEIVIVEKTKQTLASVSSGPKHTQSVPALANETGATLPTRPSD (46) (20ug/ml, NeoScientific), Adenovirus C immunodominant peptide (MTQGRRGNVYWVRDSVSGLRVPVTRPPRN (46) (20ug/ml, NeoScientific), Measles antigen (50ug/ml, Microbix), Rubella antigen (40ug/ml, Microbix). 50,000 and 200,000 total thymocytes were plated for the detection of total and antigen-specific ASC, respectively, and incubated for 8–12 hours without stimulation. To test the reactivity of memory B cells, thymocytes were stimulated with TLR7/8 agonist R848 (1ug/ml, Mabtech) and rhIL-2 (10ng/ml, Mabtech) for 72 hours prior to plating on coated plates. Bound antibodies were detected using biotinylated anti-human IgM (1ug/ml, Mabtech), anti-human IgG (1ug/ml, Mabtech), and anti-human IgA (1ug/ml, Mabtech). Spots were developed using ELISpot Blue Module and counted using the Elispot Bioreader 6000 (BioSys). Detailed Elispot counts and calculation are detailed in Table S5 and S6.

Fluorospot

Enumeration of IgG1, IgG2, IgG3, and IgG4 secreting cells was done using Human IgG1/IgG2/IgG3/IgG4 Four color Fluorospot (Cellular Technology). Low fluorescence PVDF ELISpot plates were coated with anti-human IgG. 100,000 total thymocytes were incubated overnight without stimulation. Bound antibodies were detected with 4-color fluorochrome-conjugated anti-human IgG1, IgG2, IgG3 and IgG4 (Cellular Technology). Spots were analyzed on a CTL Analyzer (Cellular Technology). Detailed Fluorospot counts and analysis are presented in Table S5.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism (GraphPad Software). Age-dependent changes were analyzed with Spearman's rank correlation coefficient. Statistical significance of the differences between the three age groups in the study (0–1 year, 1–20 years, 20–60 years) was calculated using one-way ANOVA with Bonferroni multiple

comparisons adjustments. Difference in the frequency of B cell subsets between patient-matched blood and thymus were tested using Student's paired t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are indebted to Drs. Megan Sykes, Christian Leguern, Gilles Benichou, Georges Tocco and Roberto Bellucci for their thorough review of the manuscript and to Dr. Bruce Levin for reviewing our statistical analyses.

Funding: This work was supported by NIH/NIAID grant AI110854 awarded to E.Z. S.N. was supported by the Chilean CONICYT Grant for Doctoral Studies and CONICYT Grant for Doctoral fellowship. Research reported in this publication was performed in the CCTI Flow Cytometry Core, supported in part by the Office of the Director, National Institutes of Health under awards S10RR027050. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

1. Savino W. The thymus is a common target organ in infectious diseases. *PLoS Pathog.* 2006; 2:e62. [PubMed: 16846255]
2. Vogel AB, Haasbach E, Reiling SJ, Droebner K, Klingel K, Planz O. Highly pathogenic influenza virus infection of the thymus interferes with T lymphocyte development. *J Immunol.* 2010; 185:4824–4834. [PubMed: 20861351]
3. Nunes-Alves C, Nobrega C, Behar SM, Correia-Neves M. Tolerance has its limits: how the thymus copes with infection. *Trends Immunol.* 2013; 34:502–510. [PubMed: 23871487]
4. Jamieson BD, Ahmed R. T-cell tolerance: exposure to virus in utero does not cause a permanent deletion of specific T cells. *Proc Natl Acad Sci U S A.* 1988; 85:2265–2268. [PubMed: 3258424]
5. Korostoff JM, Nakada MT, Faas SJ, Blank KJ, Gaulton GN. Neonatal exposure to thymotropic gross murine leukemia virus induces virus-specific immunologic nonresponsiveness. *J Exp Med.* 1990; 172:1765–1775. [PubMed: 2147951]
6. Milich DR, Jones JE, Hughes JL, Price J, Raney AK, McLachlan A. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? *Proc Natl Acad Sci U S A.* 1990; 87:6599–6603. [PubMed: 2395863]
7. Gossman J, Lohler J, Lehmann-Grube F. Entry of antivirally active T lymphocytes into the thymus of virus-infected mice. *J Immunol.* 1991; 146:293–297. [PubMed: 1898603]
8. Nobrega C, Nunes-Alves C, Cerqueira-Rodrigues B, Roque S, Barreira-Silva P, Behar SM, Correia-Neves M. T cells home to the thymus and control infection. *J Immunol.* 2013; 190:1646–1658. [PubMed: 23315077]
9. Spencer J, Choy M, Hussell T, Papadaki L, Kington JP, Isaacson PG. Properties of human thymic B cells. *Immunology.* 1992; 75:596–600. [PubMed: 1592434]
10. Miyama-Inaba M, Kuma S, Inaba K, Ogata H, Iwai H, Yasumizu R, Muramatsu S, Steinman RM, Ikehara S. Unusual phenotype of B cells in the thymus of normal mice. *J Exp Med.* 1988; 168:811–816. [PubMed: 3261779]
11. Ferrero I, Anjuere F, Martin P, Martinez del Hoyo G, Fraga ML, Wright N, Varona R, Marquez G, Ardavin C. Functional and phenotypic analysis of thymic B cells: role in the induction of T cell negative selection. *Eur J Immunol.* 1999; 29:1598–1609. [PubMed: 10359114]
12. Isaacson PG, Norton AJ, Addis BJ. The human thymus contains a novel population of B lymphocytes. *Lancet.* 1987; 2:1488–1491. [PubMed: 2447458]
13. Perera J, Meng L, Meng F, Huang H. Autoreactive thymic B cells are efficient antigen-presenting cells of cognate self-antigens for T cell negative selection. *Proc Natl Acad Sci U S A.* 2013
14. Yamano T, Nedjic J, Hinterberger M, Steinert M, Koser S, Pinto S, Gerdes N, Lutgens E, Ishimaru N, Busslinger M, Brors B, Kyewski B, Klein L. Thymic B Cells Are Licensed to Present Self

Antigens for Central T Cell Tolerance Induction. *Immunity*. 2015; 42:1048–1061. [PubMed: 26070482]

15. Dooley J, Liston A. Molecular control over thymic involution: from cytokines and microRNA to aging and adipose tissue. *Eur J Immunol*. 2012; 42:1073–1079. [PubMed: 22539280]
16. Flores KG, Li J, Sempowski GD, Haynes BF, Hale LP. Analysis of the human thymic perivascular space during aging. *J Clin Invest*. 1999; 104:1031–1039. [PubMed: 10525041]
17. Flores KG, Li J, Hale LP. B cells in epithelial and perivascular compartments of human adult thymus. *Hum Pathol*. 2001; 32:926–934. [PubMed: 11567221]
18. Morbach H, Eichhorn EM, Liese JG, Girschick HJ. Reference values for B cell subpopulations from infancy to adulthood. *Clin Exp Immunol*. 2010; 162:271–279. [PubMed: 20854328]
19. Chu VT, Berek C. The establishment of the plasma cell survival niche in the bone marrow. *Immunol Rev*. 2013; 251:177–188. [PubMed: 23278749]
20. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol*. 2014; 5:520. [PubMed: 25368619]
21. Frasca D, Diaz A, Romero M, Mendez NV, Landin AM, Blomberg BB. Effects of age on H1N1-specific serum IgG1 and IgG3 levels evaluated during the 2011–2012 influenza vaccine season. *Immun Ageing*. 2013; 10:14. [PubMed: 23607926]
22. Nutt SL, Taubenheim N, Hasbold J, Corcoran LM, Hodgkin PD. The genetic network controlling plasma cell differentiation. *Semin Immunol*. 2011; 23:341–349. [PubMed: 21924923]
23. Radbruch A, Muehlinghaus G, Luger EO, Inamine A, Smith KG, Dorner T, Hiepe F. Competence and competition: the challenge of becoming a long-lived plasma cell. *Nat Rev Immunol*. 2006; 6:741–750. [PubMed: 16977339]
24. White RG, Boyd JF. The effect of measles on the thymus and other lymphoid tissues. *Clin Exp Immunol*. 1973; 13:343–357. [PubMed: 4701743]
25. Auwaerter PG, Kaneshima H, McCune JM, Wiegand G, Griffin DE. Measles virus infection of thymic epithelium in the SCID-hu mouse leads to thymocyte apoptosis. *J Virol*. 1996; 70:3734–3740. [PubMed: 8648708]
26. Vidalain PO, Laine D, Zaffran Y, Azocar O, Servet-Delprat C, Wild TF, Roubourdin-Combe C, Valentin H. Interferons mediate terminal differentiation of human cortical thymic epithelial cells. *J Virol*. 2002; 76:6415–6424. [PubMed: 12050353]
27. Vitetta ES, Uhr JW, Boyse EA. Immunoglobulin synthesis and secretion by cells in the mouse thymus that do not bear theta antigen. *Proc Natl Acad Sci U S A*. 1973; 70:834–838. [PubMed: 4514995]
28. Punnonen J, de Vries JE. Characterization of a novel CD2+ human thymic B cell subset. *J Immunol*. 1993; 151:100–110. [PubMed: 7686927]
29. Frommer F, Waisman A. B cells participate in thymic negative selection of murine auto-reactive CD4+ T cells. *PLoS One*. 2010; 5:e15372. [PubMed: 20976010]
30. Walters SN, Webster KE, Daley S, Grey ST. A role for intrathymic B cells in the generation of natural regulatory T cells. *J Immunol*. 2014; 193:170–176. [PubMed: 24872190]
31. Lu FT, Yang W, Wang YH, Ma HD, Tang W, Yang JB, Li L, Ansari AA, Lian ZX. Thymic B cells promote thymus-derived regulatory T cell development and proliferation. *J Autoimmun*. 2015; 61:62–72. [PubMed: 26071985]
32. McMillan R, Longmire RL, Yelenosky R, Lang JE, Heath V, Craddock CG. Immunoglobulin synthesis by human lymphoid tissues: normal bone marrow as a major site of IgG production. *J Immunol*. 1972; 109:1386–1394. [PubMed: 4673617]
33. Halliley JL, Tipton CM, Liesveld J, Rosenberg AF, Darce J, Gregoretti IV, Popova L, Kaminiski D, Fucile CF, Albizua I, Kyu S, Chiang KY, Bradley KT, Burack R, Slifka M, Hammarlund E, Wu H, Zhao L, Walsh EE, Falsey AR, Randall TD, Cheung WC, Sanz I, Lee FE. Long-Lived Plasma Cells Are Contained within the CD19(-)CD38(hi)CD138(+) Subset in Human Bone Marrow. *Immunity*. 2015; 43:132–145. [PubMed: 26187412]
34. Andreu-Sanchez JL, Faro J, Alonso JM, Paige CJ, Martinez C, Marcos MA. Ontogenic characterization of thymic B lymphocytes. Analysis in different mouse strains. *Eur J Immunol*. 1990; 20:1767–1773. [PubMed: 2209688]

35. Cukrowska B, Sinkora J, Mandel L, Splichal I, Bianchi AT, Kovaru F, Tlaskalova-Hogenova H. Thymic B cells of pig fetuses and germ-free pigs spontaneously produce IgM, IgG and IgA: detection by ELISPOT method. *Immunology*. 1996; 87:487–492. [PubMed: 8778038]
36. Siegrist CA. Neonatal and early life vaccinology. *Vaccine*. 2001; 19:3331–3346. [PubMed: 11348697]
37. Siegrist CA, Aspinall R. B-cell responses to vaccination at the extremes of age. *Nat Rev Immunol*. 2009; 9:185–194. [PubMed: 19240757]
38. Falconer AE, Carson R, Johnstone R, Bird P, Kehoe M, Calvert JE. Distinct IgG1 and IgG3 subclass responses to two streptococcal protein antigens in man: analysis of antibodies to streptolysin O and M protein using standardized subclass-specific enzyme-linked immunosorbent assays. *Immunology*. 1993; 79:89–94. [PubMed: 8509146]
39. Gregorek H, Madalinski K, Woynarowski M, Mikolajewicz J, Syczewska M, Socha J. The IgG subclass profile of anti-HBs response in vaccinated children and children seroconverted after natural infection. *Vaccine*. 2000; 18:1210–1217. [PubMed: 10649622]
40. El Mubarak HS, Ibrahim SA, Vos HW, Mukhtar MM, Mustafa OA, Wild TF, Osterhaus AD, de Swart RL. Measles virus protein-specific IgM, IgA, and IgG subclass responses during the acute and convalescent phase of infection. *J Med Virol*. 2004; 72:290–298. [PubMed: 14695672]
41. Toptygina AP, Pukhalsky AL, Alioshkin VA. Immunoglobulin G subclass profile of antimeasles response in vaccinated children and in adults with measles history. *Clin Diagn Lab Immunol*. 2005; 12:845–847. [PubMed: 16002633]
42. Hjelholt A, Christiansen G, Sorensen US, Birkelund S. IgG subclass profiles in normal human sera of antibodies specific to five kinds of microbial antigens. *Pathog Dis*. 2013; 67:206–213. [PubMed: 23620184]
43. Hauser AE, Debes GF, Arce S, Cassese G, Hamann A, Radbruch A, Manz RA. Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response. *J Immunol*. 2002; 169:1277–1282. [PubMed: 12133949]
44. Muehlinghaus G, Cigliano L, Huehn S, Peddinghaus A, Leyendeckers H, Hauser AE, Hiepe F, Radbruch A, Arce S, Manz RA. Regulation of CXCR3 and CXCR4 expression during terminal differentiation of memory B cells into plasma cells. *Blood*. 2005; 105:3965–3971. [PubMed: 15687242]
45. Rozanski CH, Arens R, Carlson LM, Nair J, Boise LH, Chanan-Khan AA, Schoenberger SP, Lee KP. Sustained antibody responses depend on CD28 function in bone marrow-resident plasma cells. *J Exp Med*. 2011; 208:1435–1446. [PubMed: 21690252]
46. Xu GJ, Kula T, Xu Q, Li MZ, Vernon SD, Ndung'u T, Ruxrungtham K, Sanchez J, Brander C, Chung RT, O'Connor KC, Walker B, Larman HB, Elledge SJ. Viral immunology. Comprehensive serological profiling of human populations using a synthetic human virome. *Science*. 2015; 348:aaa0698. [PubMed: 26045439]

Figure 1. Histological analysis of B cells in the aging thymus

(A) Representative immunoperoxidase staining of CD20⁺ B cells (brown) in thymus from donors of the indicated ages. Scale bar, 200µm. (B) Representative immunofluorescence staining of B cells in the thymic perivascular space. Thymus sections were stained with anti-cytokeratin (red) and anti-CD20 (green) (top) or with anti-cytokeratin (red) and anti-CD31 (cyan) (bottom). HC, Hassall corpuscles; PVS, perivascular space. Scale bar, 100µm.

Figure 2. Age-associated phenotypic changes in thymic B cells

(A) Frequency of B cells in total live thymocytes in relation to age (N= 34). (B) Representative FACS plots of infant (2 months), child (3 years) and adult (29 years) thymus showing the subset of CD27⁺ memory B cells (left) and frequency of CD27⁺ cells within CD19⁺ thymic B cells in relation to age (N= 34) (right). (C) Representative FACS plots of infant (2 months), child (3 years) and adult (29 years) thymus showing naïve IgD⁺IgM⁺ (top) and class-switched IgG⁺ and IgA⁺ (bottom) B cells (left) and frequency of IgD⁺IgM⁺, IgG⁺, IgA⁺ cells within CD19⁺ thymic B cells in relation to age (N= 34) (right). Average ratios of IgD⁺IgM⁺, IgD⁺IgM^{low}, IgG⁺, IgA⁺, Ig⁻ subsets for the three age groups: infants (0–1 year; N=15), children (1–20 years; N=13) and adults (20–60 years; N=6) (lower right). Correlation coefficients and statistical significance between age and frequency of CD19⁺, CD27⁺, IgD⁺IgM⁺, IgG⁺, and IgA⁺ subsets were obtained with Spearman's rank correlation analysis. (D) Frequency of CD27⁺, IgD⁺IgM⁺, IgG⁺, IgA⁺ subsets within total CD19⁺ B cells in donor-matched thymus and peripheral blood from 5 adult donors. Student paired t-test p values for statistical differences between blood versus thymus are as follows: CD27⁺ p=0.003; IgD⁺IgM⁺ p=0.004; IgG⁺ p=0.002; IgA⁺ p=0.57 (not significant). Flow cytometric gating strategy is detailed in Fig. S6A.

Figure 3. Presence of plasma cells in thymic perivascular space

Representative immunofluorescence staining of plasma cells in the thymic perivascular space. Thymus sections from donors of the indicated ages were stained for (A) cytokeratin (red) CD138 (green). Scale bar, 100µm. (B) Cytokeratin (red) and secretory IgG (green). Scale bar, 100µm. (C) CD20 (green), CD138 (red) and secretory IgG (blue). Scale bar, 75µm. HC, Hassall corpuscles; PVS, perivascular space.

Figure 4. The thymus as a survival niche for plasma cells

(A) Hematoxylin and eosin staining of thymus sections of the indicated ages showing eosinophils (yellow arrow) and plasma cells (white arrows). Scale bar, 25 μm. (B) Representative histograms and compiled data showing CD28 expression in B cells and CD138⁺ plasma cells from peripheral blood (N=5, median age 28 years) and thymus (N=5, median age 3 years). Differences in CD28 expression were determined using one-way ANOVA (p=0.0001). Gating strategy of CD138⁺ plasma cells is shown in Fig. S13.

Figure 5. The thymus contains functional antibody-secreting plasma cells

(A) Representative ELISpots for the assessment of IgM, IgG and IgA-secreting cells. 1×10^5 unstimulated thymocytes isolated from donors of the indicated ages were assessed in each well (left). Frequency and relative ratio of IgM, IgG and IgA antibody-secreting cells (ASC) determined among total thymocytes for the three age groups: infants (0–1 years, N=15), children (1–20 years, N=13) and adults (20–60 years, N=5) (center, right). (B) Frequency of IgG1, IgG2, IgG3, IgG4 secreting cells within total IgG-secreting cells for the three age groups: infants (0–1 years, N=12), children (1–20 years, N=10) and adults (20–60 years, N=5). (C) Intracellular staining for IRF4 in thymic CD19⁺ B cells from donors of the indicated ages (left), compiled frequency of IRF4⁺ thymic B cells for the three age groups (0–1 year N=14; 1–20 years N=13; 20–60 years N=6) (middle), and frequency of IRF4⁺ thymic B cells in relation to age (right). Statistical differences were determined using one-way ANOVA; p values are as follows: 0–1y vs 1–20y p=0.363, 0–1y vs 20–60y p=0.018, 1–20y vs 20–60y p=0.0003.

Figure 6. Viral reactivity among thymic IgG-secreting plasma cells

(A) Representative ELISpot result for total IgG-secreting cells (left) and cells secreting IgG reactive to influenza, CMV, EBV, rhinovirus B, adenovirus C, measles, rubella antigens or uncoated wells for a child donor (2 years). (B) Compiled frequency of antigen-specific IgG-secreting cells in unstimulated thymocytes from infants (0–1 year; N=3) and children (1–20 years; N=10). Statistical differences in the frequency of viral specific ASC were between both groups were determined using a Welch-corrected t-test; p values are as follows: influenza p=0.009, rubella p=0.64, CMV p=0.49, EBV p=0.20, measles p=0.035, rhinovirus B p=0.91, adenovirus C p=0.50.

Figure 7. Phenotype and reactivity of re-activated thymic memory B cells

(A) Representative flow cytometric histograms showing the expression of CD27, CD38, CD19 and IRF4 in thymic B cells of a 2-year old donor after 3-day polyclonal stimulation with R848 and IL-2 (top). Compiled data showing the expression of CD27, CD38, CD19 and IRF4 in unstimulated and stimulated thymic B cells (N=4, median age 1.5 years). Statistical differences between unstimulated and stimulated conditions were determined using a paired t-test; CD27 p=0.01, CD38 p=0.12, CD19 p=0.001, IRF4 p=0.033. (B) Representative ELISpot result for activated memory B cells secreting IgG reactive to influenza, CMV, EBV, rhinovirus B, adenovirus C, measles, rubella antigens or uncoated wells for a child donor (2 years). (C) Frequency of Ag-specific IgG ASCs determined by ELISpot after 3-day stimulation of thymocytes from children (N=4, median age 1.5 years).

Figure 8. Chemokine receptor expression by thymic B cells

Histograms showing surface expression of chemokine receptors (A) CXCR3, (B) CXCR4, (C) CXCR5 on thymic B cells from representative donors of the three age groups: infants (black), children (blue) and adults (red). Background staining with isotype control antibodies is shown as grey histogram (left). Compiled percentages of CXCR3⁺, CXCR4⁺ and CXCR5⁺ cells within CD19⁺ thymic B cells for infants (0–1 years, N=14), children (1–20 years, N=13) and adults (20–60 years, N=6). Statistical differences between age groups were determined using one-way ANOVA; p values are as follows: 0–1y vs 1–20y $p=0.0007$, 0–1y vs 20–60y $p=0.0001$, 1–20y vs 20–60y $p=0.0001$ (middle). Frequency of CXCR3⁺, CXCR4⁺ and CXCR5⁺ cells within CD19⁺ thymic B cells in relation to age. Correlation coefficient and statistical significance between age and frequency of CXCR3⁺ subset was obtained with Spearman's rank correlation analysis (right). (D) Immunofluorescence staining of CXCR3 (red), cytokeratin (green), CD20 (blue) of thymus sections of the indicated ages. Scale bar, 75μm.