Characterization and functional properties of a novel monoclonal antibody which identifies a B cell subpopulation in bursa of Fabricius

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ABSTRACT The bursa of Fabricius (**BF**) plays a central role in the development of B lymphocytes in birds. During embryonic development the BF primordium is colonized by myeloid and lymphoid prebursal stem cells to form the follicle buds, which ultimately develop into lymphoid follicles with a central medullary and an outer cortical region. Lympho-myeloid differentiation within the medulla is fundamental to normal B cell development. In contrast, the complexity of the cellular composition of the follicular cortex and its role in B cell differentiation has only recently begun to be studied. As an effort to characterize the different bursal cells we have produced a large panel of monoclonal antibodies (**mAbs**) by immunizing mice with a BF cell suspension of guinea fowl (*Numida meleagris*). One of these antibodies (clone: 7H3) was found to recognize a 80 kDa cell surface antigen expressed first in the yolk sac blood island of 2-day-old guinea fowl and chicken embryos, and later detected in the embryonic circulation and primary lymphoid organs. Double immunofluorescence revealed that chB6+ (Bu-1+) B cells of embryonic BF co-express the 7H3 antigen. 7H3 immunoreactivity of the bursal follicles gradually diminished after hatching and only a subpopulation of cortical B cells expressed the 7H3 antigen. In addition, in post-hatched birds 7H3 mAb recognizes all T lymphocytes of the thymus, peripheral lymphoid organs and blood. Embryonic BF injected with the 7H3 mAb showed a near complete block of lymphoid follicle formation

In conclusion, 7H3 mAb labels a new differentiation antigen specific for avian hematopoietic cells, which migrate through the embryonic mesenchyme, colonize the developing BF lymphoid follicles, and differentiate into a subpopulation of cortical B cells. The staining pattern of the 7H3 mAb and the correlation of expression with cell migration suggest that the antigen will serve as valuable immunological marker for studying the ontogeny of avian B cells.

Key words: bursa of Fabricius, B cell, monoclonal antibody, embryo

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INTRODUCTION

The bursa of Fabricius (**BF**) is an avian specific primary lymphoid organ that provides an essential microenvironment for proliferation and clonal differentiation for B cells (Glick, 1991) that develop in 2 separate compartments of the bursal follicles: the ectodermal derived medulla and the outer cortex of mesodermal origin. Embryo manipulation and cell transplantation studies have shown that during organogenesis chicken BF primordia are seeded by CD45+ hematopoietic cells between embryonic days (**E**) 8 and E15 colonizing the mesenchyme, accumulate under the surface epithelium, migrate through the basement membrane and initiate the epithelial bud formation (le Douarin et al., 1975; Nagy et al., 2004a). By immigration of the hematopoietic cells, the epithelial buds are transformed to dendroepithelial tissue consisting of CSF1R+/NIC2+/74.3+ dendritic precursor cells and reticular epithelial cells of ectodermal origin (Nagy and Oláh, 2010; Nagy et al., 2016). Each epithelial bud is colonized by a limited number of chB6+/CXCR4+ B cell precursors that undergo productive immunoglobulin gene rearrangement before homing to the epithelial follicle buds (Salant et al., 1989). Epithelial buds are the forerunner of the medullary region of the follicles where B cells expressing a functional immunoglobulin gene are induced to

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proliferate (Masteller and Thompson, 1994). At hatching, medullary B cell precursors intensively proliferate, migrate, and give rise around each follicle bud a cortical B cell population, which is capable of sending B cells through cortical capillaries to the peripheral lymphoid organs.

Monoclonal antibodies are appropriate tools to identify and characterize the function of different B cell subpopulations. Although there is functional evidence for the existence of different avian B cell subpopulations, there are only a few antibodies (Olson and Ewert, 1990; Houssaint et al., 1991; Paramithiotis and Ratcliffe, 1994; Lee et al., 2011) that can selectively recognize the bursal B lymphocyte subsets. Using in situ hybridization, flow cytometry and embryo manipulation methods, recently we have identified 2 distinct chB6+ (Bu-1+) B cell populations in the post-hatched BF based on differential expression of CXCR4 chemokine receptor. Studies at RNA and protein level revealed that CXCR4 expression was specific for chB6+ cortical B cells of the chicken bursal follicles (Nagy et al., 2020).

The majority of our knowledge of avian immunology derives from the domestic chicken, however, ultrastructure comparison of the lympho-myeloid organs of chickens and game birds, such as guinea fowl and quail, has revealed multiple differences in quality of cellular structure of primary lymphoid organs (Nagy et al., 2001, 2004b; Gumati et al., 2003). We report here the generation and characterization of another novel mAb (clone name: 7H3) generated against BF cell suspension of guinea fowl (*Numida meleagris*), which recognize hematopoietic cells in early embryo and B cell precursors in developing guinea fowl and chicken bursal follicles. In post-hatched BF, the 7H3 immunoreactivity is restricted to a subpopulation of the cortical B cells. Embryo manipulation experiments demonstrate that injection of 7H3 antibody blocks B cell colonization of developing BF.

MATERIALS AND METHODS

Animals

Fertilized guinea fowl (*Numida meleagris*) eggs were obtained from National Centre for Biodiversity and Gene Conservation, Institute for Farm Animal Gene Conservation, Gödöllő, Hungary, and hatched guinea fowl chicks were purchased from commercial breeders. Fertilized White Leghorn chicken eggs were obtained from Biovo Ltd, Hungary. Avian eggs were incubated at 38°C in a humidified incubator (60% relative humidity). The hatching time of guinea fowl differs from that of chicken (guinea fowls normally hatch after 26 d of incubation), therefore the early embryonic age was determined according to the Hamburger and Hamilton (**HH**) stage (Hamburger and Hamilton, 1992) and older embryos were staged by the number of incubation days (**E**). Embryonic tissue samples were taken every other day starting from E2 until the day before hatching (E25 for guinea fowl and E20 for chicken). After hatching the birds were sacrificed at weekly intervals up to 10 wk of age by cervical dislocation. Tissue samples from the bursa of Fabricius, thymus, spleen, cecal tonsil, and blood were collected from at least 3 animals per age group. For hybridoma production 4 mo old Balb/c mice were purchased from the National Institute of Oncology, Budapest, Hungary. Avian and mouse experiments were approved by the Institutional Animal Care and Use Committee of Semmelweis University, Budapest, Hungary.

7H3 Antibody Production, Isotyping, and Purification

7H3 is one of the mAbs which were produced against guinea fowl lymphoid cells following the protocol used by Nagy et al. (2001). Briefly, Balb/c mice were immunized with 8-wk-old guinea fowl BF cell suspension. Four injections were given intraperitoneally at 3 to 5 wk intervals. The spleen cells of immunized mice were collected after 3 d of the last boost injection and fused with Sp2/0-Ag14 mouse myeloma cells. The hybridoma supernatants were screened by immunohistochemistry using cryostat sections of 3 to 5-wk-old guinea fowl lymphoid organs. The selected hybridomas were recloned at least twice before using the supernatant for immunohistochemistry and Western blotting. The isotype of the mAb was determined by ELISA using an immunotyping kit (MilliporeSigma, Budapest, Hungary). 7H3 mAb has mouse IgG2b heavy chain isotype. 7H3 monoclonal antibody was purified from hybridoma culture supernatants on Protein G columns (Nab Protein G Spin Kit, Thermo Scientific) according to the manufacturer's recommendation. The concentration of the purified antibody was defined by spectrophotometry (Hitachi-2001, serial nr: 9826-010, Tokyo, Japan).

SDS-PAGE and Western Blot

Bursa and thymus of 6-wk-old guinea fowls were removed and cut into small pieces in ice-cold PBS. Lymphoid cell suspension was prepared by passing through double-layered Whatman lens cleaning paper (MilliporeSigma) in PBS. The harvested lymphocytes were centrifuged at 520 *g* at 4°C for 25 min and dissolved in lysis buffer containing 150 mM NaCl; 50 mM Tris pH 7.5; 1% Triton X-100, 0.1% SDS and a protease inhibitor mixture (Roche Complete Mini, Roche, Sigma-11836153001), kept for 1 h on ice. Lysates were centrifuged at 21,000 *g* at 4°C for 10 min then supernatants were collected. The samples were mixed with the same amount of reducing TRIS-SDS (0.5M TRIS pH:6.8, 10% glycerol, 2% SDS, 0.5% mercaptoethanol and 0.00125% bromophenol blue) buffer and boiled for 4 min. SDS-PAGE was performed on 10% gels and separated proteins were electroblotted to nitrocellulose membranes (Amersham Hybond ECL, Germany). Protein migration was visualized by indirect immunostaining methods. Five percent milk powder in PBS-Tween was used to wash membranes before and between every step. The

incubation started with 7H3 mAb followed by goat anti-mouse IgG-horseradish peroxidase (Vector Laboratories, Inc., Burlingame, CA) as a secondary antibody. The binding sites of the antibodies were visualized by using enhanced chemiluminescence (**ECL**, Luminate Crescendo Western HRP substrate, Merck-Milipore) according to the manufacturer's instructions.

Histological Procedures

For cryostat section the tissue samples were fixed in 4% buffered paraformaldehyde (**PFA**) in phosphate-buffered saline (**PBS**) for 1 h at room temperature. After fixation the samples were washed in PBS and transferred into 15% sucrose solution in PBS at 4°C overnight. Afterward, the medium was changed for PBS containing 7.5% gelatine and 15% sucrose and tissues were incubated at 37°C for 1 to 2 h. The impregnated tissue samples were embedded in the same 7.5% gelatin medium then rapidly frozen at −50°C in 2-methylbutane (MilliporeSigma). Twelve-μm thick cryosections were made and collected on poly-L-lysine (MilliporeSigma) coated slides. For blood smear preparation the blood was collected into sterile syringes containing heparin of 10 IU/mL concentration. Air dried blood smears were fixed in methanol for 10 min and stored at −20°C until further processing.

Immunocytochemistry and May-Grünwald Giemsa staining

The sections were rehydrated in PBS and incubated at room temperature with primary antibodies (Table 1) for 45 min, followed by biotinylated horse anti-mouse IgG (H+L) or biotinylated goat anti-rabbit IgG (H+L) (Vector Laboratories, Inc., Burlingame, CA) and avidin-biotinylated peroxidase complex (ABC; Vectastain Elite ABC kit, Vector Laboratories). Before ABC incubation the endogenous peroxidase activity was quenched by 3% hydrogen-peroxide (MilliporeSigma) in PBS. The binding sites of the primary antibodies were visualized by 4-chloro-1-naphthol (MilliporeSigma).

For immunofluorescence staining, sections were incubated first with primary antibodies followed by relevant secondary antibodies (Alexa-Flour-488-conjugated anti-mouse IgG (H+L), Alexa-Flour-488-conjugated anti-mouse IgG1 (ɣ1), Alexa-Flour-488-conjugated anti-mouse IgG2a (ɣ2a), Alexa-Flour-594-conjugated anti-mouse IgG2b (ɣ2b), Alexa-Flour-488-conjugated anti-rabbit IgG (H+L)). All Alexa conjugated secondary antibodies were obtained from ThermoFisher Scientific (dilution 1:200). Cell nuclei were stained with 4,6 diamidino-phenylindole (**DAPI**) for 20 min. The sections were covered by aqueous Poly/Mount (Polyscience, Inc., Warrington, PA).

The methanol fixed blood smears were stained with May-Grünwald Giemsa method and immunofluorescently labeled with anti-T and B cell specific antibodies (Table 1) as described before (Bódi et al., 2009). For light microscopy the binding sites of the 7H3 mAb was visualized by 3,3'-diaminobenzidine (**DAB**; Millipore-Sigma). Wholemount and section images were recorded with a Nikon SMZ25 fluorescence stereomicroscope and an Olympus IX70 fluorescence microscope. Three to 5 samples were analyzed per each time point. Image processing was performed using Nikon and Olympus proprietary software and ImageJ. Images were compiled using Adobe Photoshop 7.0.

Flow Cytometry

Cell suspensions from 6-wk-old chicken bursa were obtained by dissociation of the organs using a syringe plunger and a stainless-steel sieve post hatch. Leukocytes from bursa and blood were then obtained by density gradient centrifugation on Biocoll (1.077 g/mL, Biochrom, Berlin, Germany). Staining of cells for flow cytometric analysis was performed according to standard procedures (Seliger et al., 2011). For 2- and 3 color staining, cells were first incubated with the respective primary antibodies (7H3, anti-chCD45, anti-chB6, anti-CXCR4) followed by a mixture of isotype specific secondary antibodies (goat-anti-mouse-IgG1-FITC, goat-anti-mouse IgG2a-RPE, goat-anti-mouse-IgG3-AF647, all Southern Biotechnology Associates, Birmingham, AL). Flow cytometry was performed with a FACSCanto (Becton Dickinson, Heidelberg, Germany) and data were analyzed using FACSDiva (Becton Dickinson) and FlowJo (FlowJo LLC, OR) software.

Table 1. List of primary antibodies.

Antigen	Clone	Isotype	structure/cell identified	source of antibody
chB6 (Bu-1a/b)	BoA1	IgG1	B cells	Igyarto et al., 2008, Bio-Rad Hungary Ltd.
chB6 (Bu-1a/b)	AV20	IgG1	B cells	Southern Biotechnology Associates, Birmingham, USA
CXCR4	9D9	IgG2a	different leukocyte subpopulations	Escot el al., 2013
CD45	HISC7	IgG2a	hematopoietic cells	Cedi-Diagnistics B.V.
CD45	16-6	IgG2a	hematopoietic cells	Viertlboeck and Gobel, 2007
Laminin	3H11	IgG1 IgG1	basement membrane	Developmental Studies Hybridoma Bank, USA
CD3		polyclonal rabbit	CD3	Dako, Glostrup, Denmark
QH1		IgG1	hematopoietic and endothelial cell	Developmental Studies Hybridoma Bank, USA
NIC2		IgG1	dendritic cell	Nagy et al., 2001
BID3		IgG1	macrophage	Gumati et al., 2003
N-cadherin	6B3	IgG1	cell adhesion molecule	Developmental Studies Hybridoma Bank, USA

Functional Blocking of 7H3 Antigen

To determine the role of 7H3 antigen function, blocking experiments combined with chorioallantoic membrane (**CAM**) transplant method were performed as described previously (Heffron and Golden, 2000; Gerhart et al., 2007; Nagy et al., 2020). Briefly, E9 chicken BF primordium was dissected in cell culture medium. The RPMI-1640 cell culture medium was purchased from MilliporeSigma and supplemented with Glutamax Supplement (Gibco), 10% fetal calf serum albumin (**FCS**, MilliporeSigma), 0.01 M HEPES solution (MilliporeSigma); 1% MEM Non-essential Amino Acid Solution (MilliporeSigma); 1% MEM vitamin solution (Sigma), 0.0014 M 2-Mercaptoethanol (MilliporeSigma); 1% penicillin-streptomycin (MilliporeSigma). For functional studies the complete RMPI-1640 medium was supplemented with either 100 μg/mL 7H3 mAb (n = 8) or with 100 μg/mL 6B3 (n = 7), a chicken N-cadherin specific mAb as control (George-Weinstein et al., 1997). Before bursal mesenchyme injections, Fast Green dye at a final concentration of 0.01% was added to the medium. The injected BF primordium was transplanted onto the CAM of E9 chicken and further cultured for additional 7 d. CAM grafts together with the surrounding membranes was excised, fixed in 4% buffered formaldehyde and embedded in gelatin/sucrose for cryosectioning.

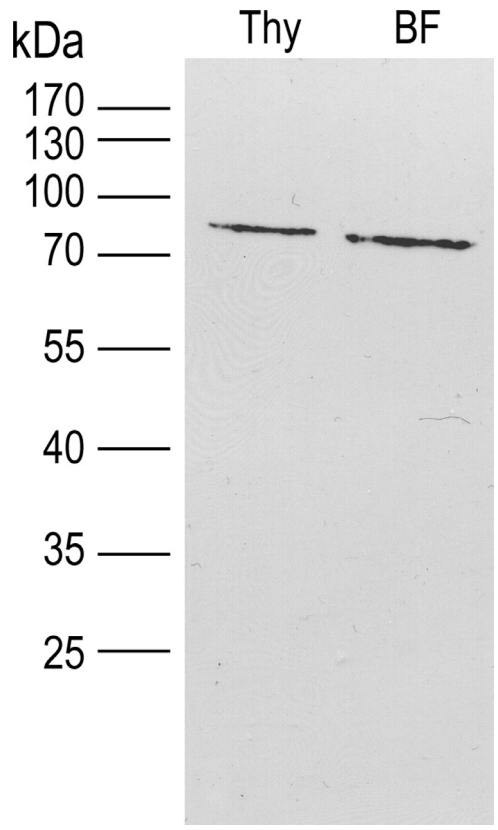

Figure 1. Western blot analysis of the antigen recognized by mAb 7H3. Cell lysates of 6-wk-old guinea fowl bursa of Fabricius (BF) and thymus (Thy) were electrophoresed in polyacrylamide gel under reducing conditions, where a single band of about 80 kDa was detected. The position of the molecular weight markers is indicated on the left in kDa.

RESULTS

Characterization and Tissue Distribution of the 7H3 Antigen in the Post-hatched Guinea Fowl Lymphoid Organs

Monoclonal antibodies were raised against 8-wk-old guinea fowl bursa cell suspension using standard hybridoma technique (Nagy et al., 2001). The antigenic specificity of the newly developed 7H3 mAb (isotype: IgG2b) was studied with Western blotting using lysate from 6-wk-old guinea fowl bursa of Fabricius and thymus. Under reducing condition, 7H3 mAb detected a single protein band with a molecular weight approximately 80 kDa (Figure 1). Immunohistochemical staining of the post-hatched guinea fowl lymphoid tissues revealed that 7H3 mAb identified round cells in all lymphoid tissues examined. More specifically, expression of the 7H3 antigen was restricted to the cell membrane of all the T and bursa specific B cells and did not show immunoreactivity with other cell types of the lymphomyeloid lineage. In non-lymphoid organs, such as in central nervous system and kidney, 7H3 positive (+) cells were detected only in the lumen of blood vessels. Although with less intense immunostaining the 7H3 mAb crossreacts with chicken and does not show cross-reactivity with quail, goose, duck, turkey or mouse tissues at any stage of development.

In 10-wk-old guinea fowl BF 7H3 immunoreactive lymphocytes were located exclusively in the cortex of the bursal follicles (Figure 2A). Most of the 7H3+ cells formed small groups close to the basement membrane underlining the cortico-medullary epithelium (Figure 2B). Since 7H3 immunolabelled cells display lymphocyte-like morphology, 7H3 mAb was compared with the T cell specific CD3 and B cell specific chB6 mAbs. Double immunofluorescence analysis demonstrates that cortical 7H3+ cells coexpress chB6 antigen (Figure 2C). Few 7H3+/CD3+ T cells occur in the interfollicular connective tissue and under the surface epithelium (data not shown). Immunocytochemical characterization was compared with other members of B cell specific antibodies, like anti-CXCR4, which specifically mark the cortical lymphocytes in chicken BF (Nagy et al., 2020). The staining pattern of CXCR4 on guinea fowl BF was identical with previously described (Nagy et al., 2020) staining pattern of chicken; heterogeneous CXCR4 expression was observed on cortical part of the follicles: B cells with high expression level of CXCR4 receptor ($CXCR4^{high}$) appeared in the peripheral part of the cortex, while $CXCR4^{low}$ and $CXCR4^{dim}$ B cells were present close to the cortico-medullary border (Figure 2D). 7H3+ cell groups in the cortex were found exclusively in those areas, where the CXCR4 expression was low or absent (Figure 2E). Moreover, 7H3+/$CXCR4^{dim}$ cells were accumulated around the capillaries of cortex (Figures 2B, 2C, 2E).

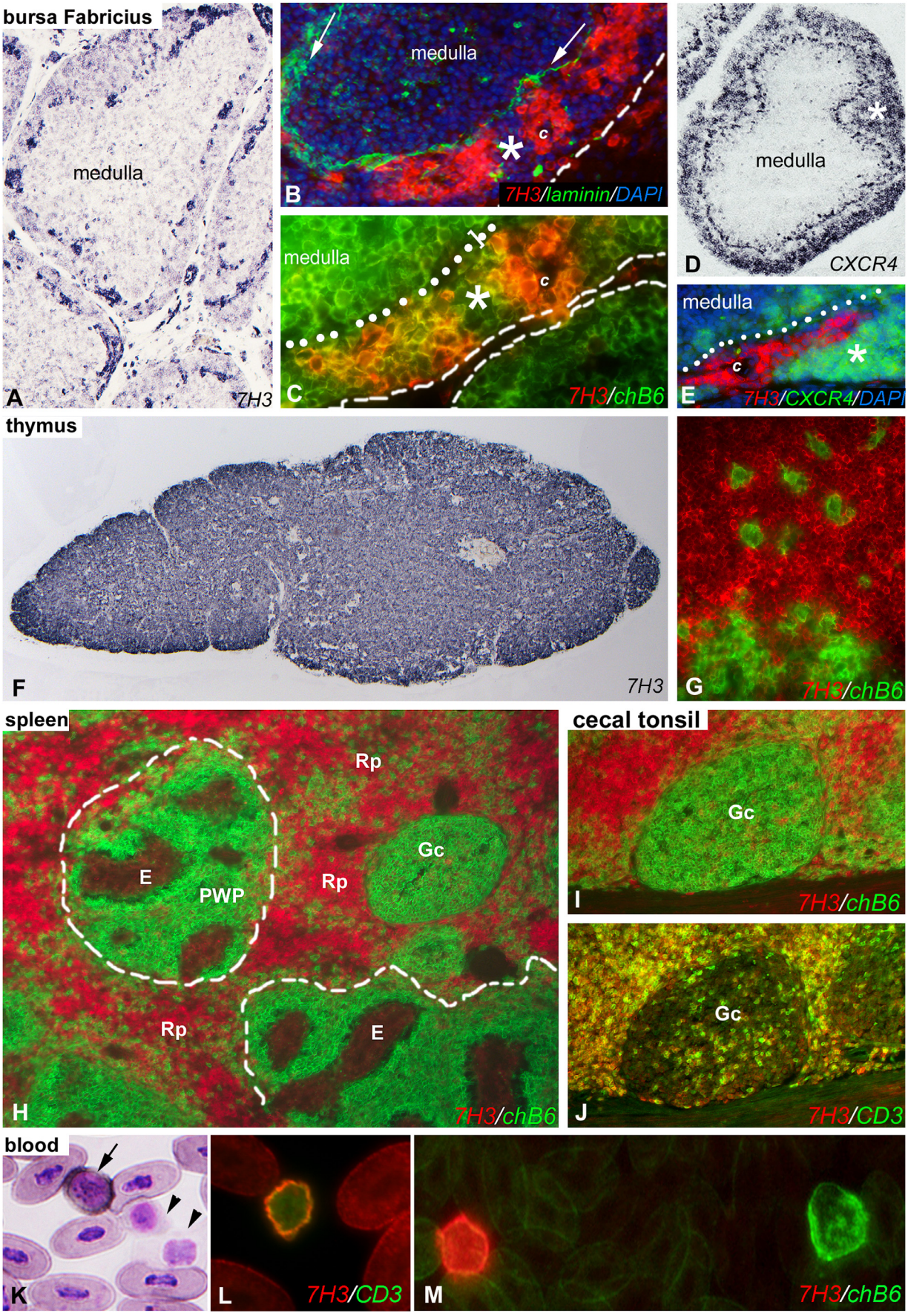

Figure 2. Expression of 7H3 antigen in post-hatched guinea fowl lymphoid organs. Bursa of Fabricius (A–E), thymus (F and G), spleen (H), cecal tonsil (I and J), and blood smear (K–M) of 10-wk-old guinea fowl. (A) A subpopulation of cortical cells with round shape are labeled with 7H3 mAb, while medullary cells of the bursal follicles are 7H3 negative. (B) 7H3 and laminin double immunofluorescence staining indicates that membrane positive 7H3 cells form small clusters in the cortex (*) along the cortico-medullary border of the bursal follicles. Arrows point to laminin+

In contrast to BF, 7H3 mAb strongly labeled the thymus (Figure 2F) and the T-dependent areas of secondary lymphoid organs, like spleen and cecal tonsils. All of the CD3+ T-lymphocytes were 7H3+ as well, but thymic chB6+ B cells, which were mainly located in the medulla, did not show 7H3 immunoreactivity (Figure 2G). The 7H3 staining pattern of the thymus was unrelated to the age of the guinea fowls. In the spleen sections, distribution of 7H3+ cells corresponded mainly to the T-dependent area and intense labeling was detected within the periarterial lymphatic sheath and the red pulp. Only a few 7H3+ cells scattered inside the B-dependent periellipsoidal white pulp and germinal centers (Figure 2H). The staining pattern of the cecal tonsil was virtually the same as that seen for the spleen; only the CD3+ T cells of the interfollicular area were immunoreactive with 7H3 mAb (Figure 2J). chB6+ B cells of cecal tonsil accumulating in germinal centers did not express 7H3 antigen, however few scattered 7H3 +/CD3+ cells were detected inside them (Figure 2I). After hatching no 7H3+/chB6+ B cells in the peripheral blood could be detected by double immunofluorescence analysis; in contrast, all circulating CD3+ T cells were labeled with 7H3 mAb (Figures 2K−2M). Cells of erythroid lineage and thrombocytes were 7H3-negative.

Ontogeny of the 7H3 Expressing Cells in Guinea Fowl

Expression of 7H3 antigen was first detected on the surface of round cells inside the newly formed yolk sac blood islands of the 2-day-old (Hamburger Hamilton stage HH12-HH13) guinea fowl embryo (data not shown). At the onset of embryonic circulation (stage HH15) 7H3+ cells occur in the intraembryonic vessels and some of them were attached to the covering endothelial cells (Figure 3A). In order to identify the phenotype of the early 7H3+ cells, markers of hematopoietic cells were used. The commonly used anti-chicken CD45 hematopoietic cell specific mAb does not cross-react with guinea fowl tissues while QH1 mAb originally developed to mark the quail endothelial and hematopoietic cells (Pardanaud et al., 1987) is less species-restricted and cross-reacts with guinea fowl tissues. Double immunofluorescence staining demonstrated that round shaped 7H3+ cells co-express the QH1 antigen, but QH1 immunoreactive endothelial cells lining the blood vessels did not show 7H3 immunoreactivity (Figure 3B). At HH20 stage (4-day-old), a well-developed 7H3+ cell cluster was found attached to the middle portion of the ventral wall of the dorsal aorta (Figure 3C). In contrast to chicken and quail, where intra-aortic clusters form two symmetric ridges on the ventrolateral side of the aorta (Jaffredo et al., 1998), guinea fowl hematopoietic 7H3+ intra-aortic cells form a single ridge located in the ventral side of the dorsal aorta. At HH25 (6-day-old) 7H3+ cells with round morphology occurred in the circulation and in the para-aortic mesenchyme (Figure 3D).

7H3 Expression During Ontogeny of the Guinea Fowl Bursa of Fabricius

To investigate a possible 7H3 expression at early stages of lympho-myeloid differentiation we have analyzed the hematopoietic cells in the guinea fowl BF primordium from E12 to E18. To follow the contribution of 7H3+ hematopoietic cells to developing lymphoid organs, immunocytochemistry was performed on cross-sections of guinea fowl BF at various developmental stages. Distribution of 7H3+ cells was compared with the staining pattern of chB6+ B cells (Igyártó et al., 2008), BID3+ macrophages (Gumati et al., 2003), and NIC2+ bursal secretory dendritic cells (**BSDC**) (Nagy et al., 2001). First 7H3+ cells emerge in the mesenchyme of the E12 BF, which precedes that of the chB6 + cells. At E14 the bursa primordium contains few isolated NIC2+, BID3+ and chB6+ cells in the mesenchyme far from the surface epithelium. On the contrary, many 7H3+ cells can be found in the mesenchyme, close to the surface epithelium (Figure 4A, B). Double immunofluorescence confirms that chB6+ B cell precursors co-express 7H3 antigen (Figure 4C), while BID3+ macrophages were not stained for 7H3 (Figure 4D). Few well-defined follicle buds filled with 7H3+ cells were present at ED16 (Figure 4E). At ED18 the majority of the follicular 7H3+ cells co-expresse the chB6 antigen, although individual cells with a 7H3+/chB6- or 7H3-/chB6+ phenotype also occur around the follicle buds (Figures 4F−4H). The 7H3 expression appeared restricted to the lymphoid lineage, as BSDC specific NIC2+ did not colocalize with the 7H3 antigen (Figure 4I). Around hatching, bursal follicles uniformly express the 7H3 antigen (Figure 4J,K). Two wk after

basement membrane of the cortico-medullary epithelial cells. "c" represents capillaries surrounded by 7H3+ cells. Dashed line mark the border of the follicle. (C) In the cortex (*) 7H3+ cells coexpress chB6, the pan-B cell specific antigen. Dotted line represents the cortico-medullary border. (D) Majority of cortical B cells are CXCR4+ but cortical cells next to the cortico-medullary border are $CXCR4^{low}$ or do not express CXCR4 antigen ($CXCR4^{dim}$). (E) Double immunofluorescence shows that 7H3+ cells are localized in $CXCR4^{low}$ or $CXCR4^{dim}$ cortical area (*). 7H3+ cells are grouped around cortical capillaries (C). (F) Cortical and medullary T-lymphocytes of the thymus are uniformly positive for 7H3 mAb. (G) chB6+ B cells in the medullary region of the thymus do not show immunoreactivity with 7H3 mAb. (H) 7H3+ cells are restricted to the red pulp (Rp) of the spleen while B-dependent (chB6 positive) areas such as periellipsoidal white pulp (PWP) and germinal centers (Gc) are lacking 7H3 expression. (I) In the cecal tonsils distribution of 7H3 expressing cells is restricted to the T-dependent interfollicular area. (J) CD3+ T lymphocytes are double labeled by 7H3 mAb and located mainly in the interfollicular area. Few CD3/7H3 double positive cells are scattered over the germinal centers. (K) Blood smear stained with May-Grünwald Giemsa followed by immunolabeling with 7H3 mAb reveals that there are 7H3+ (brown color; arrow) lymphocytes in the peripheral blood. 7H3-negative thrombocytes are labeled with arrowheads. (L and M) Double immunofluorescence staining demonstrates that all CD3+ T cells express 7H3 antigen; circulating chB6+ B cells are not labeled with 7H3 mAb. Abbreviations: E, ellipsoid; Gc, germinal centers; PWP, peri-ellipsoideal white pulp; Rp, red pulp.

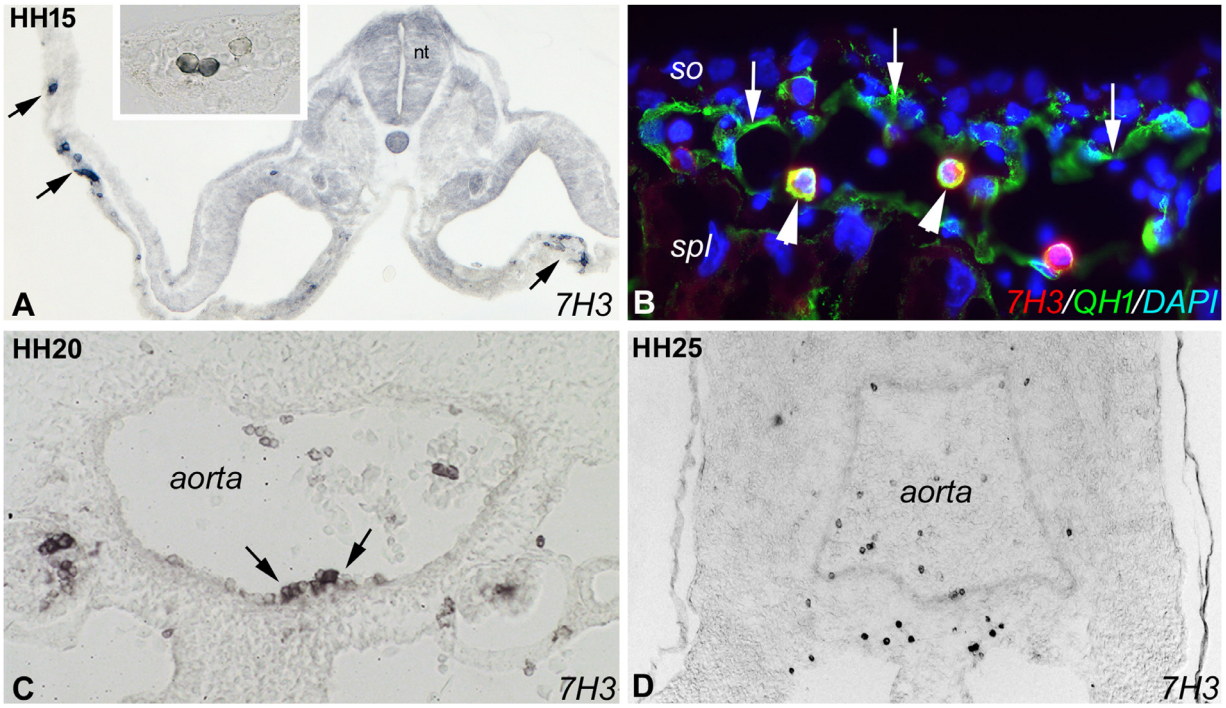

Figure 3. Distribution of 7H3+ hematopoietic cells in early guinea fowl embryo (E2 to E6 embryonic stage). (A). 7H3+ cells first appear in the yolk sac blood islands (arrows) and show a round morphology. Yolk sac blood-islands magnified in inset. (B) Double immunostaining with 7H3 and hematopoietic and endothelial cell specific QH1 mAbs confirms the hematopoietic origin of the 7H3+ cells. QH1+ endothelial cells (arrows) do not express 7H3 antigen. Arrowheads mark 7H3+/QH1+ hematopoietic cells. (C) 7H3 immunostaining of E3 (HH20 stage) embryo shows numerous round cells aggregated as intra-aortic cell cluster (arrows). (D) Section from the thoracic region of HH25 embryo stained with 7H3 mAb shows immunoreactive cells with round morphology present both in the aortic lumen and scattered in the para-aortic mesenchyme. Abbreviations: nt, neural tube; so, somatopleura, spl, splanchnopleura.

guinea fowl chicks hatching the 7H3 expression gradually diminishe from medullary B cells and it is restricted to the cortical cells of the bursal follicles (Figure 4L).

7H3 Antigen is Necessary for B Cell Migration During Chicken BF Development

Similar to the guinea fowl embryo, cells reacting with 7H3 mAb were detected among all types of chicken hematopoietic cells. At E13, BF hematopoietic cells with round morphology carried the 7H3 antigen (Figure 5A). Before hatching, all B cells in the follicle buds were labeled with 7H3 mAb while erythrocytes and ramified myeloid cells appeared negative (data not shown). Double labeling with 7H3 and CXCR4 indicated that the CXCR4+ B cells in the E16 chicken lymphoid follicles were 7H3+ (Figures 5 B–5D). Similar to our observations in the guinea fowl BF, after hatching, 7H3 antigen expression was also downregulated from chicken BF cells, except from a subpopulation of cortical cells (Figures 5E and 5F).

Flow cytometric analysis of bursal cell suspensions from 6-wk-old chicken confirmed that all 7H3+ cells were CD45+ hematopoietic cells (Figure 5G). Double staining with 7H3 and the B cell marker anti-chB6 demonstrated that highest expression of the 7H3 antigen was found on non-B cells (AV20neg; MFI 23,693 ± 2,941) and revealed two 7H3 positive B cell subpopulations (chB6^{high}/7H3^{low} (17.8 ± 5.6% of all cells; MFI 973 ± 148) and chB6med/7H3med (1.5 ± 0.2% of all cells; MFI 15,755 ± 3,022) (Figure 5H). As we have shown recently that bursal B cells downregulate expression of CXCR4 before they emigrate from the bursa, we analyzed the CXCR4 expression of 7H3 positive B cells. While the chB6^{high}/7H3^{low} subpopulation contained both CXCR4^{low} and CXCR4^{high} expressing cells, chB6med/7H3med cells show a homogenously low expression of CXCR4 (Figure 5I), demonstrating that 7H3 is expressed on that bursal B cell subpopulation from which the emigrating cells originate. In contrast to the heterogeneous 7H3 staining on bursal B cells, all blood B cells are 7H3+ and show a homogenous chB6^{high}/7H3^{med} phenotype (Figure 5J). As all blood B cells express 7H3, we can conclude that the fraction of recent bursal emigrants (**RBE**) among blood B cells is also 7H3+, confirming that bursal B cells start to express 7H3 before emigration.

As detailed immunocytochemistry had demonstrated that the 7H3 antigen is expressed on B cells colonizing BF follicle buds, we completed our study by testing whether the 7H3 mAb injected into the developing BF mesenchyme affects B cell migration. Culturing of chicken BF transplants, which were treated with 7H3 antibody, on a E9 chicken chorioallantoic membrane for 7 d, resulted in an inadequate follicle formation (Figures 6A and 6B). In contrast, administration of no additive cell culture medium or injection of an anti-N-cadherin control antibody, did not block lymphoid follicle formation (Figures 6C and 6D).

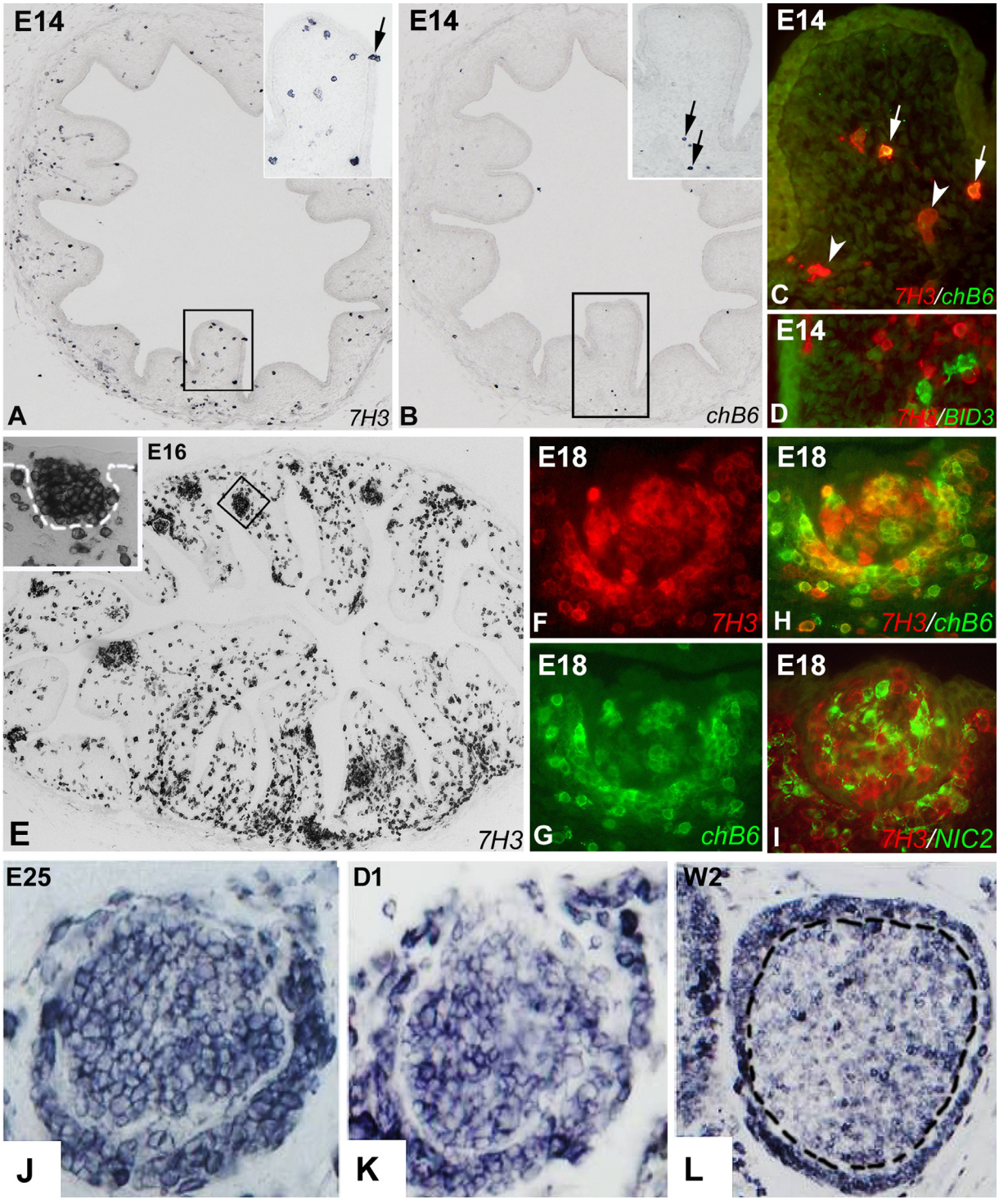

Figure 4. Detection of 7H3 expressing cells during development of the bursa of Fabricius. (Guinea fowl). (A–L) Sections of bursa of Fabricius of guinea fowl. (A–D) Sections of E14 bursa of Fabricius. (A) At this stage large number of 7H3+ cells colonized the bursa primordia and some of them entered to the surface epithelium (inset, arrow). (B) At this stage only few chB6+ B cell precursors (inset, arrows) are scattered in the bursa mesenchyme. (C) Double immunofluorescence staining of the E14 bursa demonstrates that all chB6+ cells express 7H3 antigen (arrow). 7H3+/chB6- cells are also present in the mesenchyme (arrowhead). (D) BID3 immunoreactive macrophages colonizing the bursal mesenchyme do not express 7H3 antigen. (E) In E16 bursa 7H3+ cells fill up the bursal follicle buds and many cells are present in the mesenchyme. Inset shows high magnification of a 7H3+ lymphoid follicle. The dashed line shows the basement membrane surrounding the forming follicle. (F and G) Sections of E18 bursa of Fabricius. Developing follicle bud stained with 7H3 (F) and chB6 mAbs (G). (H) Majority of 7H3+ cells coexpress chB6 antigen. (I) NIC2 mAb which specifically labels bursal secretory dendritic cells of guinea fowl do not express 7H3 antigen. (J) At E25 well developed 7H3+ lymphoid follicles are present. (K) One day after hatching 7H3 surface antigen is uniformly expressed on cortical and medullary bursal cells. (L) Two-wk-old bursa of Fabricius: cortical cells are heavily stained with 7H3 mAb, while the 7H3 immunoreactivity in medullary B cells is low or absent (dashed line marks the cortico-medullary border).

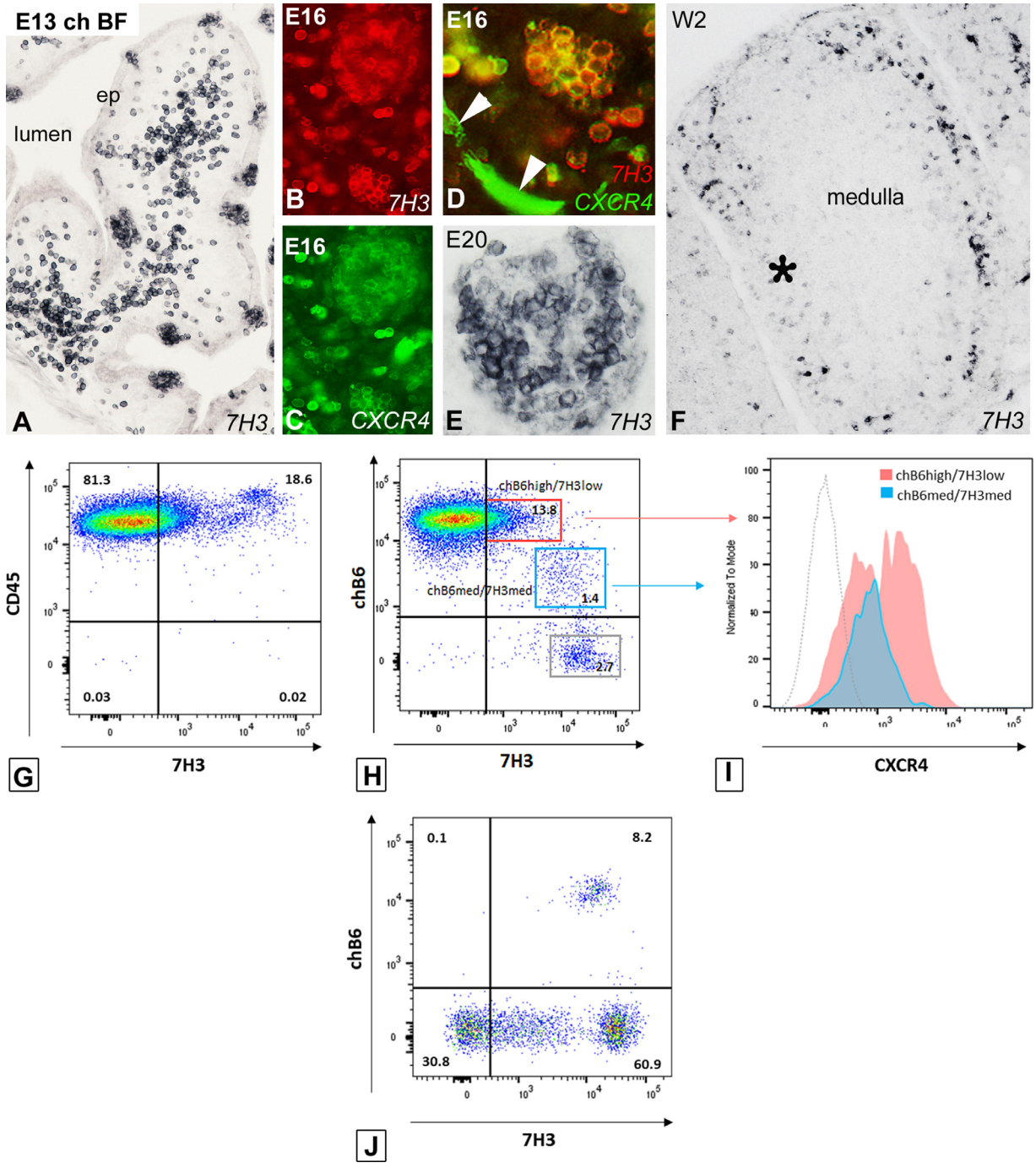

Figure 5. Immunocytochemistry and flow cytometric analysis of chicken bursa of Fabricius. (A–F) Sections of chicken bursa Fabricius stained with 7H3 mAb. (A) At E13 the developing follicle buds are filled with round 7H3+ cells. The mesenchymal core of the bursal folds and newly formed follicle buds are infiltrated with round-shaped 7H3+ cells. (B–D) E16; consecutive sections double stained with 7H3 and CXCR4. At this stage numerous 7H3+ cells are present in the follicle buds, and the bursa mesenchyme. (C) Similar to the 7H3 labeling, CXCR4+ cells are localized in the follicle buds and the mesenchyme. (D) CXCR4+ endothelial cells (arrowheads) do not express 7H3 molecule. (E) Before hatching (E20) most of the follicular B cells express 7H3 antigen. (F) Two wk after hatching 7H3+ cell are grouped in the follicular cortex (*) with only a few cells in the medulla. Flow cytometric analysis of bursal (G–I) and blood leukocytes (J), stained with the respective antibodies. Data were gated for viable, single cells. (I) shows the CXCR4 expression on $chB6^{high}/7H3^{low}$ and $chB6^{med}/7H3^{med}$ cell populations shown in (H) and an isotype control (dotted line).

As the effect of 7H3 inhibition on B cell migration could result from changes in B cell survival, we examined apoptosis with double immunofluorescence using 7H3 and anti-activated caspase-3 immunostaining. Only a small subset of B cells and mesenchymal cells expressed caspase-3 and their frequency was not increased compared with the no additive-injected or 6B3 mAb injected bursae (Figures 6E and 6F). Taken together, these results show that the expression of 7H3 molecule is associated with bursal B cell migration.

DISCUSSION

The avian bursa of Fabricius offers a unique opportunity to study the development, differentiation, and

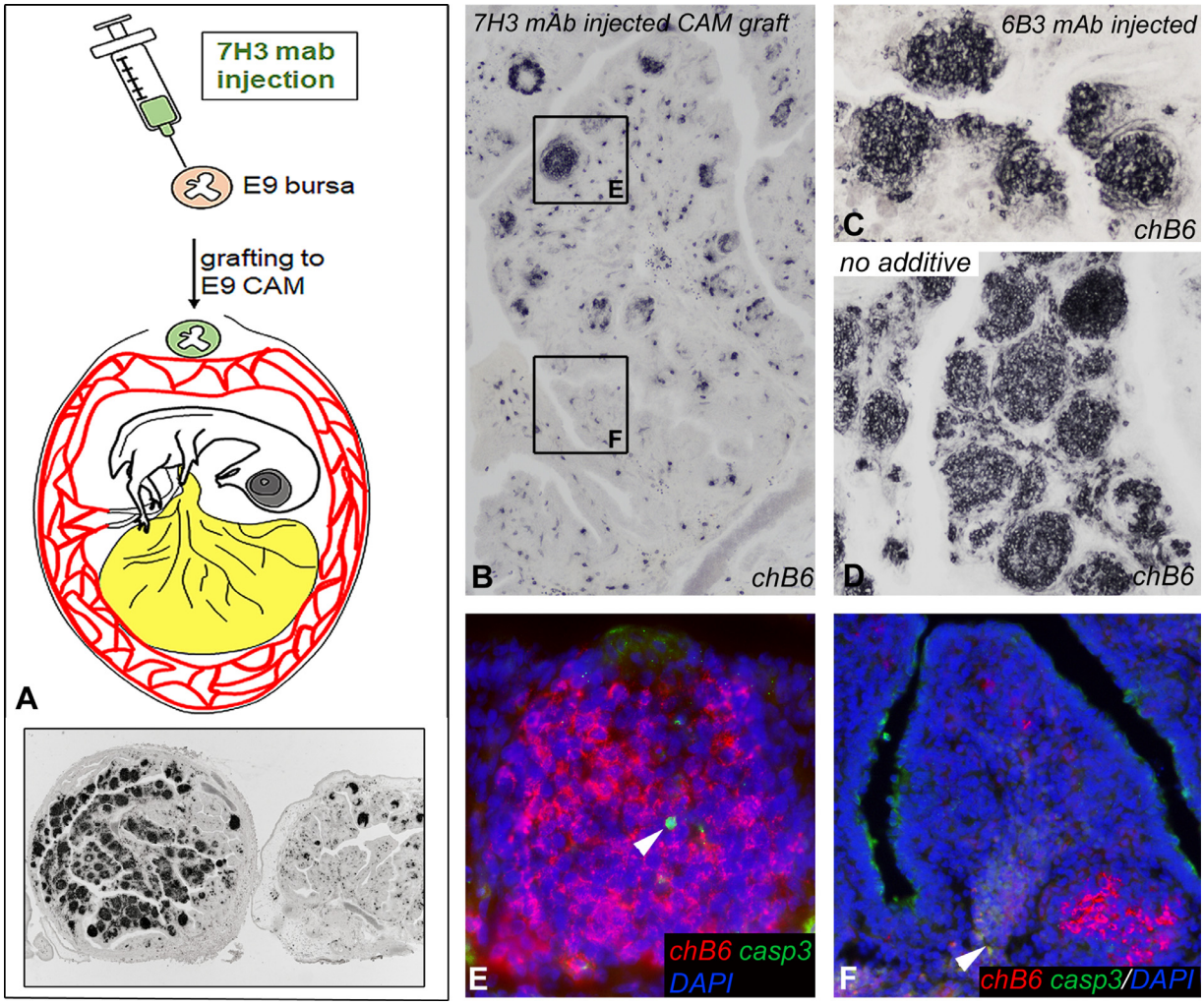

Figure 6. Function blocking injection of 7H3 mAb in chicken BF mesenchyme leads to abnormal B cell colonization. (A) E9 chicken BF were injected with 7H3 mAb (n = 8), 6B3 mAb (n = 7) or complete RPMI-1640 cell culture medium (no additive; n = 6) ex vivo and then transplanted onto the chorioallantoic membrane (CAM) of an E9 host chick embryo. (inset: comparison of bursal size of no additive and 7H3 injected BF 7 d after incubation on CAM). (B) 7H3 mAb treatment significantly decreased the number of lymphoid follicles. Only few chB6+ B cell precursors colonized the bursa primordium. (C and D) In contrast, injection of anti-N cadherin specific 6B3 mAb or no add cell culture medium did not alter the follicle formation. (E and F) Cross sections of the 7H3 mAb injected BF were stained for cleaved caspase-3 to detect apoptosis and chB6 to label B cells.

maturation of B cells (Glick et al., 1956; Ratcliffe, 2002). Multiple lines of evidence suggest that the bursa contains different B cell populations that can be distinguished by their expression of IgM, CD15, LT2, or CXCR4 antigens. Furthermore, morphological and experimental data demonstrate that B cells in bursa follicles present different stages of development. Despite the fact that B cell populations vary dynamically in the bursa, very little is known about the B cell subpopulations that have different developmental potentials. Over the past 3 decades, a number of monoclonal antibodies have been developed that are suitable for the selective labeling of subtypes of avian B lymphocytes. These include antibodies that specifically recognize different subpopulation of B cells, or cell surface molecules that can be associated with a particular stage of lymphoid differentiation. Antibodies that label avian B cells are of great help in understanding the developmental mechanisms that occur in bursa of Fabricius.

In this study, we have produced and characterized a novel guinea fowl and chicken B cell specific mouse monoclonal antibody. Our results indicate that besides T cells in thymus and T cells of secondary lymphoid organs, 7H3 mAb recognizes a BF specific B cell population clustered around the capillaries in the inner portion of the follicular cortex. This expression pattern differs considerably from that obtained by staining BF cells with other avian B cell specific antibodies. Expression of 38-40 kDa Ov antigen (mAb 11A9) (Houssaint et al., 1991) was found to be specific for all T cells and 3% of post-hatching B cells in chicken. Although the molecular weight is different from the 7H3 antigen, during embryonic development the Ov antigen is also present on most hematopoietic cells and similar to the 7H3 antigen disappears around hatching except for T cells. Chicken interleukin-2 receptor alpha chain (chCD25, molecular weight: 55 kDa) is expressed in chicken BF follicle cortex, spleen and small intestine (Lee et al., 2011). In contrast to 7H3, chCD25 mAb also stains multiple

macrophage cell lines (Lee et al., 2011). Similar to 7H3, LT2 antigen is uniformly expressed on cortical B cells of 1- to 3-wk-old chickens. However, the tissue distribution of LT2 in the peripheral organs does not match the results obtained with our 7H3 mAb; at post hatch, only a subset of T cells and 60% of peripheral blood B cells are LT2+ (Paramithiotis and Ratcliffe, 1994). Another mAb recently produced against chicken tumor necrosis factor-like ligand 1 (chTL1A; molecular weight: 32 kDa) stained chicken lymphoid cells located in the BF cortex, while in the thymus and cecal tonsils the chTL1A specific mAb recognized elongated cells (Lee et al., 2014). These data suggests that LT2 or anti-chTL1A and 7H3 mAbs stain different cell types in the cortex.

First 7H3+ cells were detected in the yolk sac blood islands of E2 (HH12) guinea fowl embryo. Furthermore, in HH15 stage 7H3+ cells were associated to the endothelial cells of newly formed extraembryonic vessels and co-express the hematopoietic cell specific QH1 antigen. Yolk sac blood islands are the sources of primitive hematopoietic cells (Minko et al., 2003; Sheng, 2010) involved in erythropoiesis, and generation of primitive macrophages, microglia and bursal secretory dendritic cell precursors (Cuadros et al., 1992; Nagai and Sheng, 2008; Sheng, 2010; Pardanaud and Eichmann, 2011; Dóra et al., 2017). However, 7H3 mAb does not react with CD45+ amoeboid or highly ramified myeloid precursor cells and QH1+ endothelial cells. In 3-day-old embryos (HH20-HH25) the 7H3 mAb marks cells in the intra-aortic hematopoietic cluster as well as cells of para-aortic foci. The cells of intra-aortic hematopoietic cluster are considered to be equivalent of definitive hematopoietic stem cells contributing the para-aortic foci which provides lymphoid-restricted progenitors to the BF (Dieterlen-Lièvre and Martin, 1981; Jaffredo et al., 2000; Yvernogeau and Robin, 2017, Nagai et al., 2018). The tissue distribution of the cells identified by 7H3 mAb in the early embryo was very similar to the distribution of those round shaped cells recognized by the hematopoietic cell specific QH1 and CD45 mAb (in guinea fowl and in chicken, respectively). Since virtually all the round shaped hematopoietic cells of the early embryo express 7H3 antigen, it is possible that this antigen specifically marks the precursors of the lymphoid lineage.

Blocking of CXCR4 signaling during BF development caused a drastic reduction of B cell follicles, demonstrating the importance of CXCL12-CXCR4 interaction for B cell immigration (Laparidou et al., 2020; Nagy et al., 2020). According to the comparable expression of 7H3 and CXCR4 molecules in the embryonic BF, we hypothesized that similar to CXCR4, the 7H3 molecule may play a role in migratory processes of the B cells. In order to study the function of the 7H3 antigen, we have used embryo manipulation methods where 7H3 mAb was injected in the precolonized BF mesenchyme which was further cultured on the chorioallantois membrane as described previously (Nagy et al., 2020). Failure of lymphoid follicle formation after blocking of 7H3 antigen strongly suggests that 7H3 molecule is necessary for migratory process of pre bursal B cells during the colonization of bursal anlage.

After hatching, expression of 7H3 antigen is gradually downregulated on BF lymphocytes and remains only on cortical B cells located in the vicinity of the capillaries. This change of the expression pattern of 7H3 antigen on B cells is obvious by the second week after hatching in chickens and by the sixth week in guinea fowl BF. In addition, we have observed that 7H3+ B cells were located in those parts of the follicular cortex, where the B cells show a low expression level of CXCR4. Downregulation of CXCR4 receptor is essential for the emigration of B cells from the cortex of the BF follicles to the periphery (Nagy et al., 2020). This accumulation of 7H3 expressing cells in the BF cortex around the capillaries coincides with the developmental time when the B cells emigrate to the peripheral lymphoid tissues. Strikingly, flow cytometry demonstrated that all bursal $CXCR4^{low}$ cells express the 7H3 antigen. In young birds, about 1% per hour of blood B cells have just left the bursa (Paramithiotis and Ratcliffe, 1993). As the more sensitive cell sorting technique identify peripheral chicken $CXCR4^{low}$ B cells expressing 7H3 antigen suggests that recently emigrated cortical cells retain 7H3 antigen and this subset of 7H3 immunoreactive cell cannot be identified by immunocytochemistry of the blood smears. Further biochemical and functional studies are needed in order to clarify any possible relationship of 7H3 antigen and CXCR4 as well as its contribution to B cell migration into secondary lymphoid organs.

In conclusion, we describe a new monoclonal antibody, 7H3, with specificity restricted reactivity to cortical B cell subpopulation in BF. Histological results from the tissue distribution, biochemical properties and functional assay strongly suggest that the molecule recognized by 7H3 is a potent player in lymphoid cell migration. While the 7H3 mAb has specificity for guinea fowl and chicken antigen, it does not cross-react with quail, turkey or mouse. Using this marker, the immune system of a game bird can also be studied and the results can be compared with those obtained from immunological studies of inbred chicken strains.

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DISCLOSURES

The authors declare no competing or financial interests.

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