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3C8, a new monoclonal antibody directed against a follicular dendritic cell line, HK

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= Abstract =

Background: Follicular dendritic cells (FDCs) play key roles during T cell-dependent humoral immune responses by allowing antigen-specific B cells to survive, proliferate, and differentiate within the FDC networks of secondary follicles, i.e., germinal centers (GC). **Methods**: A novel monoclonal antibody, 3C8, was generated by immunizing with an FDC line HK, in order to understand the molecular signals involved in the FDC-B cell interactions in the microenvironment of the GC. **Results**: The 3C8 antibody did not bind to mononuclear cells, including T cells, B cells, and monocytes. Murine L929 and human skin fibroblasts exhibited no or little reactivity to 3C8. However, 3C8 specifically recognized HK cells by flowcytometry. Furthermore, the antigen recognized by 3C8 was restricted to the GC of the human tonsil. Dendritic networks of the GC were intensely stained by 3C8, but cells outside the GC were not. **Conclusion**: Our results suggest that the antigen 3C8 may play some unique role on FDCs during the GC reactions.

Key Words: follicular dendritic cell, monoclonal antibody, germinal center

INTRODUCTION

Follicular dendritic cells (FDCs) are major micro-environmental components of secondary lymphoid follicles, and are known to play critical roles in the survival, proliferation, selection, and differentiation of germinal center (GC) B cells (1). FDCs display distinct morphological characteristics and retain unprocessed antigens in the form of immune complexes for a long time (2). Transfer experiments with bone marrow cells have shown that FDCs are not from the bone marrow (3). An experiment designed to investigate whether FDCs are derived from haematopoietic cells or the surrounding stromal components, demonstrated the host origin of FDCs induced in the spleens of SCID mice after the transfer of allogeneic lymphocytes (4). We recently suggested that antigen-activated B cells stimulate the maturation of FDC precursors, whose identity is unclear, via lymphotoxin-α and that mature FDCs, in turn, may provide potent proliferation signals to centroblasts (5). Therefore, the ontogeny of FDC is unknown.

We reported previously upon the establishment of an FDC line, HK, from human tonsils to overcome the practical difficulty in isolating pure FDCs and to mimic the GC reaction *in vitro* (6). HK cells indeed have the functional features of FDCs by delaying apoptosis, and by stimulating growth and differentiation of GC B cells. HK cells bind and prevent the apoptosis of GC B cells preferentially and have co-stimulatory effects on the proliferation of CD40-stimulated GC B cells (7,8).

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To provide further evidence for the relevance of HK cells to FDCs, we developed a new monoclonal antibody 3C8 by immunizing mice with HK cells. This antibody stains the dendritic network, but not mononuclear cells, in tonsil sections. The results demonstrate that HK cells share a common antigen with FDCs, and suggest that HK cells originated from FDCs.

MATERIALS AND METHODS

1. Antibodies used in this study

PE-conjugated anti-CD20 (L27), anti-CD3 (UCHT1), anti-CD14 (M5E2), and isotype controls were purchased from Becton Dickinson (San Jose, CA, U.S.A.). Unconjugated anti-CD44 (NKI-P1) was a kind gift from Dr. C. G. Figdor (University Hospital Nijmegen, Nijmegen, The Netherlands) and its isotype control was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

2. Culture of HK cells and skin fibroblasts

HK cells were established as described previously (6) and cultured in RPMI 1640 containing 10% FCS, 2mM L-glutamine (Life Technologies, Grand Island, NY, U.S.A.), and 40μg/ml of gentamicin (SoloPak Laboratories, Elk Grove Village, IL, U.S.A.). Human normal skin fibroblasts were purchased from ATCC (CCD-25SK, CRL-1474, Manassas, VA, U.S.A.) or were kindly provided by Dr. Jin-Ho Chung (SNU-20SK, Department of Dermatology, College of Medicine Seoul National University, Seoul, Korea) and cultured in RPMI containing 10% FCS, 2mM L-glutamine, and 40μg/ml gentamicin.

3. Preparation of human mononuclear cells, B cells, T cells, and monocytes

Tonsils were obtained from the Kangwon National University Hospital (Chunchon, Korea) from children undergoing tonsillectomy. Tonsillar mononuclear cells were prepared by centrifugation of cell suspensions on a Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). Human B cells, T cells, and monocytes were partially purified from heparinized peripheral blood voluntarily donated by healthy donors. Peripheral mononuclear cells were subjected to rosetting with sheep red blood cells. B cells were obtained from the non-rosetting fraction, after the depletion of monocytes by plastic adherence, which were collected separately. T cells were recovered from the rosetting fraction.

4. Production of 3C8 monoclonal antibody

Balb/c mice (Jackson Laboratory, Bar Harbor, ME, U.S.A.) were immunized with 1×10^7 HK cells intraperitoneally three times at 2-wk intervals. The splenocytes were fused with SP2/0 myeloma cells using polyethylene glycol 400 (Merck, Darmstadt, Germany). Hybridomas were cultured in DMEM supplemented with 20% FCS and HAT (Sigma Chemical Co.). Hybridoma screening was carried out by cell-based ELISA in 2 steps. In the first step, hybridoma supernatants were negatively selected for the binding to mononuclear cells, which included B cells, T cells, and monocytes. Supernatants that exhibited little or no reactivity to human skin fibroblasts were selected for the binding to HK cells in the second step. The resultant 3C8 hybridoma (IgG1) was cloned by limiting dilution.

5. Cell-based ELISA

Tonsillar mononuclear cells, HK cells, and skin fibroblasts were cultured in 96-well plates. Cells were fixed by 0.25% glutaraldehyde and washed three times with PBS. The plates were incubated with blocking buffer until use (PBS containing 1% FCS and 0.1% NaN_3). After adding primary antibodies, the plates were incubated at 37℃ for 2 h, and incubated with HRP-conjugated secondary Ab at 37℃ for 30 min. After rinsing with PBS 5 times, OPD was added in phosphate citrate buffer, and the reaction stopped by adding 4M H_2SO_4. The optical density of each well was measured with a plate reader (Vmax, Molecular Devices, Sunnyvale, CA, U.S.A.) at 490 nm.

6. Flow cytometry and Immunohistochemistry

Cells were stained for flowcytometric analysis as described previously (9), which was carried out on a FACScan (Becton Dickinson) with CellQuest software.

Frozen tonsil sections were thoroughly dried at room temperature and fixed in cold acetone for 10 min. Sections were stained with the primary Abs (i.e., 3C8, DRC-1, and isotype control) for 2 h at room temperature, using a Scytek Staining kit (Scytek Laboratories, Logan, UT, U.S.A.) according to the manufacturer's instructions. Developed slides were counterstained with Mayer's hematoxylin.

RESULTS

1. Production of 3C8 MAb

Since HK cells exhibit the functional features of FDCs, we postulated that FDCs and HK cells might share functionally important molecules and attempted to develop monoclonal antibodies against such potential molecules by immunizing mice with HK cells. Out of 300 hybridomas obtained, 27 were selected in the first screening step on the basis that these hybridomas produced antibodies, but did not react with tonsillar mononuclear cells. In the second screening step, which was designed to select any hybridoma that was not reactive with skin fibroblast but reactive with HK cells, only 1 hybridoma was obtained. The resultant 3C8 was of the IgG1 isotype. As shown in Fig. 1, 3C8 did not react with tonsillar mononuclear cells ($p > 0.05$) in cell-based ELISA. However, 3C8 strongly reacted with HK cells ($p < 0.01$). 3C8 did not display consistent results with human skin fibroblasts because it did not bind to the CCD-SK25 skin fibroblast cell line from ATCC (data not shown) but reacted with another cell line obtained from Seoul National University Hospital (Fig. 1).

2. 3C8 specifically binds to HK cells

To substantiate the cellular reactivity of 3C8 in a sensitive assay, flowcytometric analysis was performed. B cells, T cells, and monocytes were purified from peripheral blood. Consistent with the cell-based ELISA results, 3C8 did not bind to $CD20^+$ B cell, $CD3^+$ T cells, or $CD14^+$ monocytes (Fig. 2A ~ 2C). Moreover, the strong reactivity of 3C8 to HK cells was confirmed by flowcytometric analysis (Fig. 2D). However, 3C8 exhibited no reactivity to murine fibroblast line L929 and little reactivity to human skin fibroblasts (data not shown). These results suggest that the 3C8 antigen is specifically expressed by HK cells and may be important in the function of FDCs.

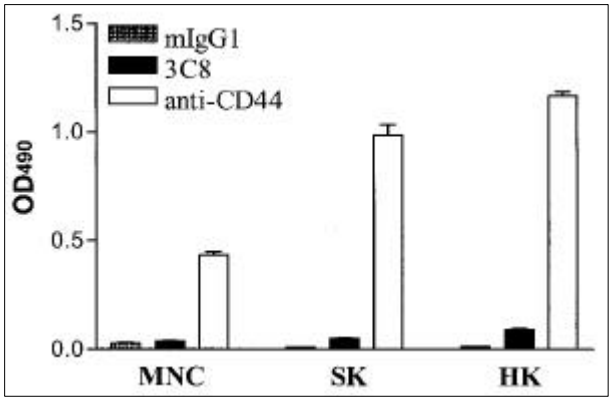

Fig. 1. Reactivity of 3C8 with tonsillar mononuclear cells (MNC), skin fibroblasts (SK), and HK cells. Cell-based ELISA was performed with cultured cells, as described in Materials and Methods. Mouse IgG1 antibody was included as an isotype-matched control and anti-CD44 antibody as a positive control. A representative result of three reproducible results is shown.

3. 3C8 specifically recognizes FDC networks within tonsillar germinal centers

To provide further evidence upon the relevance of HK cells to FDCs and to characterize the 3C8 antigen, wes tained tonsil sections with isotype control, 3C8, and DRC-1, which was already known to stain the GC (10). As shown in Fig. 3, 3C8 stained the same GC of serial tonsil sections as DRC-1, but 3C8 did not stain T cells, B cells, macrophages, or dendritic cells outside the GC. It was clear that 3C8 stains the dendritic network surrounding GC B cells, but not GC B cells themselves,

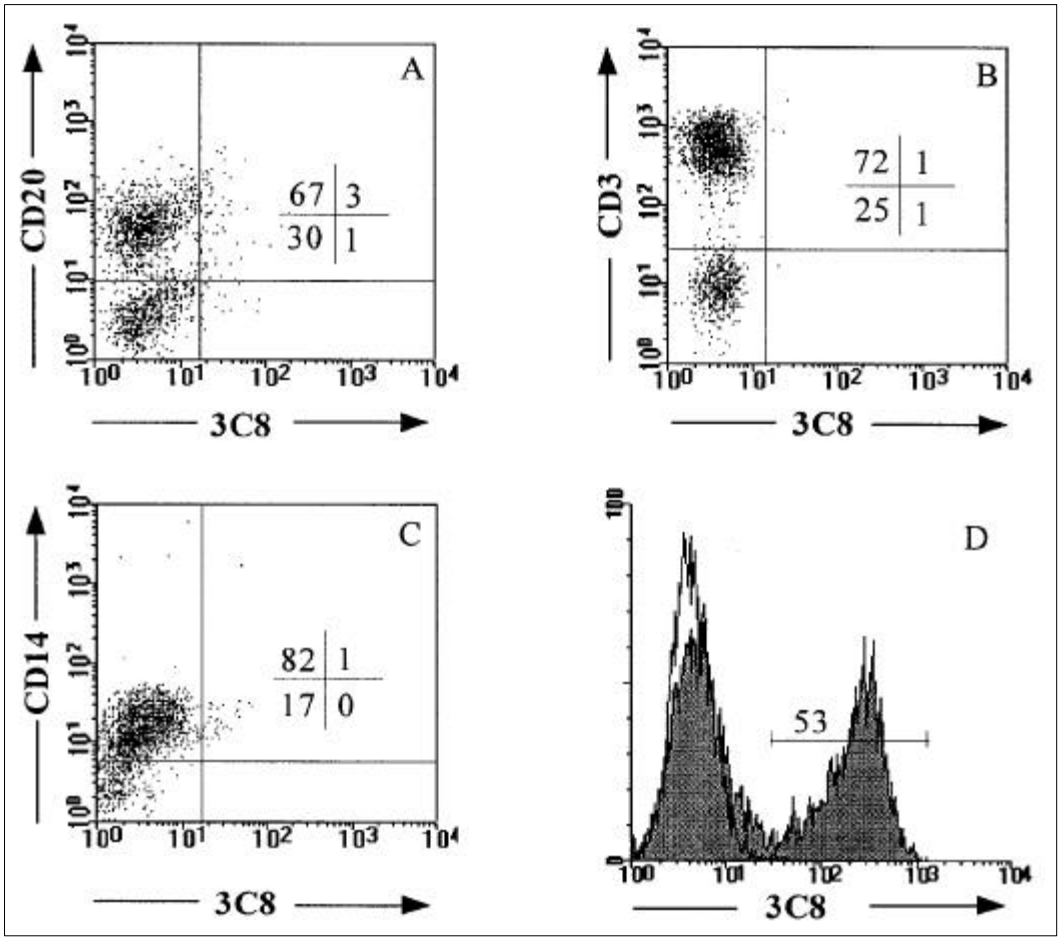

Fig. 2. Two-color flowcytometric analysis reveals the specific reactivity of 3C8 with HK cells. Human B cells (A), T cells (B), and monocytes (C) were purified from peripheral blood. Double staining was performed with the indicated antibody and 3C8. Histograms (D) indicate staining of HK cells with 3C8 and the isotype control. The results shown are from one of five reproducible experiments.

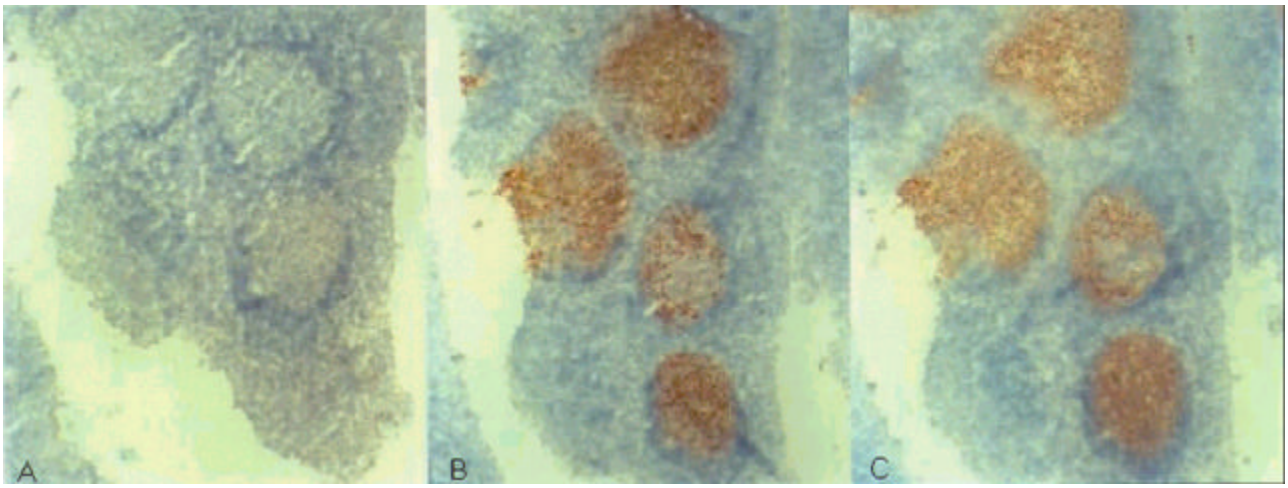

Fig. 3. Immunoenzymatic analysis of serial tonsil sections with 3C8. Human tonsil sections were stained with the isotype control (A), 3C8 (B), and DRC-1 (C). Original magnification, ×10.

which suggests that 3C8 may stain FDC network of the GC.

DISCUSSION

FDCs play pivotal roles in the survival, proliferation, and differentiation of GC B cells. For instance, the disruption of FDC-B cell clusters results in the apoptosis of B cells, and purified human FDCs enhance cytokine-dependent growth and Ig production in CD40-activated B cells (1,8). The aim of the current study was to develop a monoclonal antibody specific to an FDC line HK and eventually to FDC in order to understand the ontogeny of FDCs and the molecular signals involved in the complex GC reactions. The 3C8 antibody that was developed against HK cells did not react with T cells, B cells, monocytes, (Figure 2) and NK cells (data not shown). It did not bind to murine L929 fibroblasts, but displayed different reactivity to human fibroblasts. Human skin fibroblasts CCD-SK25 obtained from ATCC did not bind 3C8 antibody, whereas another skin fibroblasts SNU-20SK from the Seoul National University Hospital exhibited 3C8 binding reactivity comparable to that of HK cells. In addition, 3C8 antibody intensely stained tonsillar GC but not the extrafollicular areas.

The result that tonsillar GC was intensely stained by 3C8 is consistent with the finding that 3C8 did not react with mononuclear cells. Since the 3C8 staining was directed against the dendritic networks of the GC, the 3C8 antigen appears to be expressed on FDCs. It remains to be confirmed whether 3C8 antibody binds to purified FDCs. Liu et al. recently described the first characterized human FDC-specific molecule, 7D6, that turned out to be the long human CR2/CD21 isoform (CD21L)(11). Interestingly, DRC-1 and KiM4, which have been widely used as human FDC-specific antibodies, stained COS7 cells transfected with CD21L cDNA, indicating that 7D6, DRC-1, and KiM4 recognized the same target antigen. On the basis of the following results, 3C8 antigen appears to differ from CD21L. DRC-1 is cross-reactive between FDCs and B cells, whereas 3C8 is clearly negative for B cells. The staining of tonsil sections showed that the area stained with DRC-1 was absolutely restricted to the GC, while 3C8 reacts with minor fibroblast cells outside the GC (data not shown) as well as the major dendritic networks in the GC. In addition, the molecules recognized by DRC-1 and 3C8 antibodies have different molecular weights by SDS-PAGE analysis, i.e., 145 kD and 58 kD, respectively (manuscript in preparation). Further characterization of 3C8 is required to identify and to understand the biological function of this antigen.

Unlike the non-reactivity of the 3C8 antibody with CCD-25SK fibroblasts, which were used in the original screening step, 3C8 antibody reacted with SNU-20SK fibroblasts. Since 3C8 did not bind to murine fibroblasts, the antigen detected by 3C8 antibody seems to be specific to humans. However, further study is necessary to confirm that 3C8 antigen is indeed expressed in humans only and to determine the distribution of this antigen in human tissue. Whether the expression of the 3C8 antigen is limited to certain fibroblast cells or not, the 3C8 positivity of HK cells (this line may in fact be tonsillar fibroblasts that have the functional features of FDCs) and a skin fibroblast cell line sheds some clues on the origin of FDCs. It is currently accepted that FDCs originate from the surrounding reticular meshwork but not from bone marrow. Stromal cells such as fibroblasts may function not only as a tissue framework but provide important microenvironments in lymphoid tissue during immune response. If stromal fibroblasts undergo functional transition during immune response, it would be interesting to know what kind of signals trigger the transition. Regarding this aspect, a report by Matsumoto et al. and another by Lindhout et al. are worth mentioning. The former demonstrated that FDC organization and GC formation are controlled by both lymphotoxin-α-expressing bone marrow-derived cells and TNFR-I-expressing non-bone marrow-derived cells (12), while the latter reported that some FDC phenotypes are induced from fibroblast-like synoviocytes after stimulating with TNF-α and IL-1β (13).

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FOOTNOTES

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