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*J Immunol* 2003; 171:5975-5987; doi: 10.4049/jimmunol.171.11.5975

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Altered Regulation of FcγRII on Aged Follicular Dendritic Cells Correlates with Immunoreceptor Tyrosine-Based Inhibition Motif Signaling in B Cells and Reduced Germinal Center Formation

Yuksel Aydar,* Péter Balogh,* John G. Tew,† and Andras K. Szakal2*

Aging is associated with reduced trapping of Ag in the form of immune complexes (ICs) by follicular dendritic cells (FDCs). We postulated that this defect was due to altered regulation of IC trapping receptors. The level of FDC-M1, complement receptors 1 and 2, FcγRII, and FDC-M2 on FDCs was immunohistochemically quantitated in draining lymph nodes of actively immunized mice for 10 days after Ag challenge. Initially, FDC FcγRII levels were similar but by day 3 a drastic reduction in FDC-FcγRII expression was apparent in old mice. FDC-M2 labeling, reflecting IC trapping, was also reduced and correlated with a dramatic reduction in germinal center (GC) B cells as indicated by reduced GC size and number. Nevertheless, labeling of FDC reticula with FDC-M1 and anti-complement receptors 1 and 2 was preserved, indicating that FDCs were present. FDCs in active GCs normally express high levels of FcRs that are thought to bind Fc portions of Abs in ICs and minimize their binding to FcRs on B cells. Thus, cross-linking of B cell receptor and FcR via IC is minimized, thereby reducing signaling via the immunoreceptor tyrosine-based inhibition motif. Old FDCs taken at day 3, when they lack FcγRII, were incapable of preventing immunoreceptor tyrosine-based inhibition motif signaling in wild-type B cells but old FDCs stimulated B cells from FcγRIIB−/− mice to produce near normal levels of specific Ab. The present data support the concept that FcR are regulated abnormally on old FDCs. This abnormality correlates with a reduced IC retention and with a reduced capacity of FDCs to present ICs in a way that will activate GC B cells. The Journal of Immunology, 2003, 171: 5975–5987.

The follicular dendritic cells (FDCs)3 reside in the light zone of secondary lymphoid follicles, where their dendritic processes interdigitate and form a three-dimensional network, an FDC reticulum (1–4). FDCs function in capture and long-term retention of immune complexes (ICs) (5–7), the blocking of apoptosis, the survival of specific B cells (8, 9), and the promotion of the development of potent high-affinity (10) recall responses (11, 12). The capture and retention of ICs are mediated by an array of cell surface receptors that include FcγRII (13, 14) and complement receptors (complement receptors 1 and 2 (CR1/2)) (15–17).

Senescence leads to a pronounced reduction in the secondary humoral immune response, the appearance of atrophic FDCs (18) that trap and retain little ICs, produces few icososomes, and induces a reduced number of germinal center (GC) B cells (19). Although there is a marked age-related depression in GC development and specific Ab production, the ratio of the FDC reticulum to GC numbers remains 1:1 (18–21), emphasizing the dependence of GC development on FDCs even in aging.

FcγRII and CR2 (CD21) exist on both FDCs and B cells and are involved in important ligand-receptor interactions including FDC-FcγR-Fc in IC and FDC-CD21 ligand (CD21L)-B cell CD21. FDC-CD21L provides B cells with a critical costimulatory signal and FDC-FcγRII minimizes coligation of B cell receptor (BCR) and B cell-FcR (11, 13–17, 22). FcγRII is highly expressed on FDCs in secondary follicle GCs and the expression pattern of FcγRII on FDC reticula supports the development of a potent Ab response (11, 13, 23). The expression of FcγRII on FDCs in GCs is critical for FDCs to retain and mediate the conversion of ICs to a highly immunogenic form and for the generation of strong recall responses (11, 13). Furthermore, FcγRII promotes the maturation of the FDC reticulum (24); FcγRIIB−/− mice show a substantial delay in the development of FDC reticula (24). The ability of FDCs from FcγRIIB−/− mice to augment Ag-specific IgG production by wild-type B cells (11, 13) or when incubated with the anti-FcγRII mAb 2.4G2 is markedly depressed. Furthermore, it appears that engagement of Ig-Fc with numerous FcγRII on FDCs decreases the chances of signaling B cells via the immunoreceptor tyrosine-based inhibition motif (ITIM) induced by cross-linking BCR and B cell FcγRII via Ab-Ag complexes (J.G.T., unpublished data). Thus, FDCs minimize a negative signal to B cells.

Although the phenomenon of age-associated reduction in IC trapping in old mice is well known (18, 19), the cellular and molecular bases for this defect have not been defined. Numerous studies indicate the importance of FcγRII and CR1/2 in IC trapping.

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Received for publication October 24, 2002. Accepted for publication September 22, 2003.

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This work was supported by National Institutes of Health Grant AG-17063. Y.A. is the recipient of a scholarship from the Ministry of National Education in Turkey.

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*Abbreviations used in this paper: FDC, follicular dendritic cell; IC, immune complex; GC, germinal center; CD21L, CD21 ligand; CR1/2, complement receptors 1 and 2; ITIM, immunoreceptor tyrosine-based inhibition motif; PNA, peanut agglutinin; SHIP, Src homology 2-containing inositol phosphatase.
and mediation of costimulatory signals to B cells in humans as well as mice (3, 7, 9, 11, 13). However, potential age-related changes in the function and molecular interactions of the FcγRII and CR1/2 have not been determined.

Age-related changes in IC trapping, reductions in the FDC reticulum, GC numbers, and size prompted the hypothesis that in old mice reduced IC retention is due to a defect in expression of surface receptors that involve IC trapping. To test this hypothesis, lymph node sections of actively immunized mice were immunohistochemically quantitated for FcγRII and CR1/2 receptors, for FDC reticulum size (anti-FDC-M1), for the capacity to trap ICs (anti-FDC-M2), and for GCs (peanut agglutinin (PNA)). The results showed that aging reduced the expression of FcγRII on FDCs by day 3 after Ag challenge that correlated with the reduced trapping of ICs (FDC-M2 reactivity). In accordance with these results, old FDCs taken at day 3 could not prevent ITIM signaling and Src homology 2-domain-containing inositol phosphatase (SHIP) phosphorylation because of the dramatic age-related reduction of FcγRII expression.

Materials and Methods

Animals

BALB/c mice at 2–3 and 20–21 mo of age were purchased from the National Institute on Aging Contract Facility (Harlan, Indianapolis, IN) and maintained under specific pathogen-free conditions. The mice were housed in standard plastic shoebox cages with filter tops. Food and water were supplied ad libitum. The young mice were used between 2 and 3 mo of age and old mice were used between 21 and 22 mo of age. The mice were handled in accordance with Virginia Commonwealth University Animal Care and Use guidelines.

Immunizations

As described previously for active immunization (25, 26), mice received an initial 0.1-ml injection s.c. behind the nape of the neck consisting of 200 μg/ml aluminum potassium sulfate (A7167; Sigma-Aldrich, St. Louis, MO), precipitated OVA (A5503; Sigma-Aldrich), and 5 × 10^8 heat-killed Bordetella pertussis. Two weeks later, the mice were given a booster immunization of 50 μg of OVA i.p. and by s.c. injection 5 μg of OVA into each of the front legs and hind feet. Finally, all mice were challenged at least 2 wk after the last immunization by s.c. injection of 5 μg of OVA into each of the front legs and hind feet and used at 0, 1, 3, 5, and 10 days after the challenge.
Abs and other reagents

The rat mAbs against CR1/2 (CD21/35, clone 7G6), CD16/32 (clone 2.4G2), and biotinylated mouse anti-rat κ-chain mAb (MRK-1) were obtained from BD PharMingen (San Diego, CA). Low-Tox rabbit complement was purchased from Cedarlane Laboratories (Westbury, NY). The normal rat serum, phenylhydrazine-HCl, the PNA lectin conjugated with peroxidase (PNA-HRP), sodium orthovanadate, sodium fluoride, sodium deoxycholate, Igepal CA-630, PMSF, and the streptavidin-peroxidase (streptavidin-HRP) were obtained from Sigma-Aldrich. Rabbit anti-mouse SHIP-1/2 (SHIP-1α, 145 kDa; SHIP-1β, 135 kDa), HRP-conjugated anti-phosphotyrosine (4G10), and protein A agarose were obtained from Upstate Biotechnology (Lake Placid, NY). Peroxidase-conjugated affinity-purified goat anti-rabbit IgG (H + L) and affinity-purified F(ab')2 goat anti-mouse IgG (H + L) were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The FDC-M1 and FDC-M2 hybridoma cell lines were generously provided by Dr. M. Kosco-Vilbois (Serono, Switzerland), and their supernatant was used as primary Ab.

IC preparation and incubation of FDCs with IC and complement

The FDCs were prepared and incubated with ICs and complement as described previously by Aydar et al. (27). In brief, OVA and affinity-purified anti-OVA were used to make ICs for use in cultures. A final ratio of 1 ng/ml OVA to 6 ng/ml anti-OVA was used to make up the ICs in Ag

FIGURE 2. Size of the average FDC reticulum in the draining lymph nodes of young and old mice as assessed by FDC-M1 labeling. Young (■) and old (□) mice were actively immunized and challenged with OVA as described for the animals in Fig. 1. The averages were calculated using data from three to six mice per day. Note that FDC-M1-positive FDC reticula were essentially identical in both young and old mice and showed about a 30% increase in size during the first 10 days of GC response. The data represent the mean in square micrometers ± SEM.

FIGURE 3. Representative light micrographs illustrating the kinetics of immunohistochemical labeling of FDC reticula in draining axillary lymph nodes of actively immunized young (6–8 wk) and old (22 mo) mice with anti-CR1/2 (CD21/35, clone 7G6). The same relationships were apparent in brachial and popliteal lymph nodes. These sections are from the same mice described in Figs. 1 and 2. D, Day. Magnification, ×120.

CR1&2

D 0

D 1

D 3

D 5

D10

D 0

D 1

D 3

D 5

D10

YOUNG

OLD
rabbit complement to load FDCs with CD21L.

...tissue was subjected to a second 30-min digestion in a fresh aliquot of MD. After 30 min at 37°C in NaC O 2 incubator, the medium and released lymph nodes were placed in an enzyme mixture consisting of 1 ml of collagenase D (16 mg/ml, C-1088882; Roche, Basel, Switzerland), 0.5 ml containing 5 ml of DMEM with 20% FCS and placed at 4°C. The remaining...molar, mesenteric, and para-aortic) of normal young adult mice by using an enzymatic digestion. Isolation, the mice were exposed to whole body irradiation (600 rad, Cε137 source). The irradiation dose does not interfere with FDC accessory functions...positive GC size for PNA staining between young and old mice on days after Ag challenge. For each measurement, the objects of interest were selected by drawing an enclosed circle and filling it with the predefined threshold. Density measurement...�. BIOQUANT NOVA Advanced Image Analysis software (R&M Biometrics, Nashville, TN) was used to analyze the immunohistochemistry studies. BIOQUANT NOVA is the core measurement software for image analysis applications. It measures area, length, number, position, density, and a range of more specialized parameters.

Setting the threshold. A threshold is a set for red, green, and blue intensity values that are used to distinguish objects of interest in an image. The word “threshold” also refers to the colorful highlighting on the screen that results from setting a threshold range. Initially, we set an arbitrary threshold that was the best threshold possible for the objects of interest. The same threshold was used strictly throughout the measurements to enable a reliable comparison of average density for CR1/2, FcyRII, and FDC-M2 staining. FDC reticulum size for FDC-M1, CR1/2, FcyRII, and FDC-M2, and PNA-positive GC size for PNA staining between young and old mice on successive days after Ag challenge. For each measurement, the objects of interest were selected by drawing an enclosed circle and filling it with the predefined threshold.

Average density measurement. Density measurements in a color imaging system (like BIOQUANT NOVA) are done by examining three channels (red, green, and blue), which comprise a color image. All three color channels may be used when setting a threshold to define the objects of interest to be measured. But, the density measurement is made only on the channel designated in the density array’s comment. The default (and most commonly used) channel is the green channel since it most closely approximates the colors perceptible by the human eye. The average density analyses for FDC-M1, CR1/2, FcyRII, and FDC-M2 were done using average density. Average density looks at a group of pixels and measures their average color value. It is the most common way to compare the amount of light-absorbing stain present in one object to others. Computers work on a color scale from 0 to 255. Zero is black and 255 is the brightest color. Note that this is backwards from conventional “absorption” scales...region has larger data values than lighter regions. To make the data easily traceable for the readers, we reversed the relative density assignments by subtracting the measured values from the 255 so that 0 corresponds to white and 255 corresponds to black (darker regions have larger data values than lighter regions in our illustrations).

Area measurement. The area array records the area of selected objects in a single field or across multiple fields. The system measures the selected areas as square micrometers. When the density threshold settings and size settings are satisfactory, the areas are counted using “Object Mode for all Objects” comment. For the present studies, we measured FDC reticulum size for FDC-M1, CR1/2, FcyRII, and FDC-M2. We also measured PNA-positive GC size for PNA. FDC reticulum size, labeled with FDC-M1, CR1/2, FcyRII, or FDC-M2, was determined by using the automeasurement mode of BIOQUANT NOVA, filling the FDC reticulum with a predefined threshold that most closely approximates the borders of FDC reticulum. PNA-positive GC size was determined by using the manual measurement mode of BIOQUANT NOVA, which allows drawing of a circle around labeling the GC, then measures the inside of the circle.
Determination of average density, average FDC reticulum size, and average GC size

Draining lymph nodes (two axillaries, two brachial, and two popliteal) were sectioned and used to quantitate average density, average FDC reticulum size, and average GC size per follicle. The average density for FDC-M1, CR1/2, FcγRII, and FDC-M2 were done by adding up the densities of follicles in the draining lymph nodes and dividing the sum by the number of reticula. Similarly, the FDC reticulum sizes for FDC-M1, CR1/2, FcγRII, and FDC-M2 were measured by summing up the FDC reticulum size of follicles in the draining lymph nodes and dividing the sum by the number of FDC reticula. The average PNA-positive GC size was determined by adding up PNA-positive GC sizes in follicles in all draining lymph nodes and then dividing the sum by the number of GCs examined.

Cell culture and stimulation

A20 cells (mouse B lymphoma cell line) were obtained through American Type Culture Collection (Manassas, VA) and were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS (HyClone, Logan, UT), 20 mM HEKES, 2 mM glutamine, 50 μg/ml gentamicin, and MEM-nonsential amino acids. Cells were harvested and washed with cold HBSS and then the cells were equilibrated for 30 min at 37°C. To study the inhibition of SHIP phosphorylation by FDCs, 2 x 10⁷ A20 cells were cocultured with young or old FDCs for 30 min at 37°C and then the cells were stimulated with 10 μg/ml goat anti-mouse BCR Abs for 5 min at 37°C.

Preparation of cell lysates

The cocultured cells were lysed in cold 10 mM Tris-HCl buffer (pH 7.5) supplemented with 0.5% Nonidet P-40, 100 mM NaF, 1 mM Na₃VO₄, and protease and phosphatase inhibitors (100 mM aprotinin, 5 μM leupeptin, and 200 mM PMSF) for 30 min at 4°C, with agitation. The cell lysates were centrifuged at 12,000 g for 30 min at 4°C, and the soluble fractions were collected for immunoprecipitation of SHIP.

Immunoprecipitation of SHIP and Western blotting analysis

The cell lysates were incubated with anti-SHIP (2 μg/ml) overnight at 4°C. To precipitate the Ab-associated proteins, the lysates were incubated with 100 μl of protein A-coated beads (protein A agarose) overnight at 4°C and beads were recovered by centrifugation at 4000 × g for 5 min at 4°C with agitation. The cell lysates were centrifuged at 12,000 × g for 30 min at 4°C, and the soluble fractions were collected for immunoprecipitation of SHIP.
FIGURE 6. The density and size of average FDC reticulum labeling in the draining axillary lymph nodes of young and old mice as assessed by anti-FcγRII (CD16/32, clone 2.4G2). Young (■) and old (□) were actively immunized and challenged with OVA. These are the same lymph nodes (three to six mice per group) as those shown in the preceding figures. A. Note the striking decline in labeling intensity from day 1 in the reticula from aged FDCs. The data represent the mean ± SEM in color value of pixels and significant age-related differences are indicated by the asterisk (*, p < 0.05). B. Note that FcγRII-positive FDC reticulum size was significantly reduced in old mice compared with young and remained very small during the 3, 5, and 10 days of the GC response. The data represent the mean in square micrometers ± SEM and significant age-related differences are indicated by the asterisk (*, p < 0.0001).

Western blot data quantitation

The ECL signal was quantitated using a scanner and a densitometry program (Scion Image, Frederick, MD). The films were exposed to nitrocellulose membrane for 20 min at ambient temperature in a dark room and were developed using a Kodak XOMAT developer (Kodak, Rochester, NY). For loading controls, the membranes probed with HRP-conjugated anti-phosphotyrosine Ab were washed twice for 10 min in TBST after signal detection. Then the membranes were stripped with 0.1 M glycine, 50 mM KCl in distilled H2O at pH 2.2 for 15 min at room temperature to remove the anti-phosphotyrosine. Stripped membranes were incubated with rabbit anti-mouse SHIP (2 μg/ml) for overnight at 4°C and labeled with HRP-conjugated secondary Abs. Signals were detected using the ECL kit.

Results

In old mice, FDC reticula develop poorly when assessed by amount of Ag trapped, which is clearly reduced and contain reduced amount of immune complexes (18, 19). To understand the aberrant development of FDC reticula and their reduced capacity to trap IC in old mice, the present studies were designed to obtain a quantitative assessment of FDC-associated molecules involved in FDC reticulum development in the draining lymph nodes of young and old mice. The average density of molecules in an FDC reticulum was determined for FDC-M1, CR1/2, FcγRII, and FDC-M2 staining during the GC reaction in response to Ag challenges. The lymph nodes were evaluated at time 0 (pre-existing FDC reticula 3 wk after primary immunization) and then at days 1, 3, 5, and 10 after Ag injection. FDC reticulum size and PNA-positive GC size were expressed in square micrometers. Average density was expressed as average color value of the pixels in the region of the interest.

Kinetics of FDC-M1-positive FDC reticulum development

To determine the size of an average FDC reticulum, the FDC-specific mAb FDC-M1 was used. FDC-M1 is a murine follicular dendritic cell-reactive mAb that labels primarily the FDC reticula and some tingible body macrophages. Representative sections illustrating typical labeling patterns for FDC-M1 are presented in Fig. 1. The average reticulum size was quantitated using sections from three to six mice per group (Fig. 2). The pre-existing FDC-M1-positive FDC reticulum size was moderately large (~40,000 μm²) at day 0 (3 wk after primary immunization) in these actively OVA-immunized young and old mice (Fig. 2). FDC-M1-positive FDC reticulum size enlarged gradually at days 1 and 3 and peaked at day 5 (~70,000 μm²) in both young and old mice (Fig. 2). By day 10, FDC-M1-positive FDC reticulum size was reduced to pre-challenge level and became more compact both in young and old mice (Fig. 2). No significant size differences were found between young and old mice, suggesting the number of FDCs in the reticula of aged mice is not deficient.

Kinetics of CR1- and 2-positive FDC reticulum development

Since it is known that complement plays a significant role along with FcγRII in the trapping of ICs by FDCs, we examined the CR1/2 reactivity of FDCs in the reticula of draining lymph nodes using a mAb reactive with CR1/2. FDCs labeled intensely when compared with B cells (Fig. 3). The average density of CR1/2 staining, and the average size of CR1/2-positive FDC reticula were determined and results for old and young mice at days 0, 1, 3, 5, and 10 were indistinguishable (Fig. 4). The size of the pre-existing CR1/2-positive FDC reticula was moderately large (~38,000 μm²) in both young and old mice at day 0 (Fig. 4B), but it increased and peaked at day 5 (~68,000 μm²). By day 10, the CR1/2-positive FDC reticulum size slightly reduced and became more compact in both the young and old lymph nodes (Fig. 4B). Overall, there was no significant difference between the CR1/2-positive FDC reticulum size of young and old mice during the GC response and results were consistent with FDC-M1.

Kinetics of FcγRII-positive FDC reticulum development

We next examined whether age-related changes in the level of FcγRII expression were detectable using anti-FcγRII mAb (clone 2.4G2) (Fig. 5). The rat mAb 2.4G2 recognizes both mouse FcγRII and FcγRIII. However, histochemistry studies using FcγRIIB/-/- mice showed no labeling for the rat mAb 2.4G2 on FDCs, although FDC reticula were present in active GCs as indicated by a variety of other FDC markers and FcγRIII was present on other cells (13). The conclusion of this previous study was that FDCs have little if any FcγRIII and, thus, we attribute labeling of FDC reticula with 2.4G2 to be due to FcγRII-positive FDCs (13). Note the intense labeling of FDC reticula compared with B cell areas and a remarkable loss of labeling of the reticula in old mice after day 1 (Fig. 5). Morphometry showed that at 0 time the average density for FcγRII labeling was low for both young and old mice between (55 and 65...
The average density for FcγRII labeling, which reflects the amount of FcγRII on FDCs in the reticula, increased significantly by day 1 in both young and old mice and remained high in young mice between 194 and 173 pixels (Fig. 6A). In marked contrast, in old mice the average density rapidly dropped off and stayed low (between 137 and 51 pixels) during the rest of the FDC reticulum reaction (Fig. 6A).

The size of pre-existing FcγRII-positive FDC reticulum 3 wk after priming was small (7,000 μm²) (Fig. 6B) in comparison to 30,000–40,000 μm² for FDC-M1 and CR1/2 labeling, indicating that only a proportion of the FDC reticula labeled at day 0 both in young and old mice. Between days 0 and 1, there was a substantial 3-fold increase in FcγRII-positive FDC reticulum size in both young and old mice. An additional 2-fold increase occurred between days 1 and 3 in young mice (Fig. 6B). In contrast, in old mice the FDC reticulum size increased by 3-fold between days 0 and 1, but the FDC reticulum size then dramatically decreased from 20,000 μm² and leveled off at 5,000 μm². This represents >10-fold deficit in FcγRII on FDCs in the reticula of aged mice at days 3, 5, and 10.

**Kinetics of FDC-M2-positive FDC reticulum development**

The reactivity of mAb FDC-M2 with FDCs is dependent on trapped ICs capable of fixing complement. FDC-M2 reacts specifically with the C4 fragment of complement associated with the FDC ICs (30). Thus, FDC-M2 labeling closely reflects the location of ICs on FDCs.

The kinetics of the FDC-M2 labeling was studied to obtain a functional parameter that reflects Ag localization that can be correlated with FcγRII and CR1/2 labeling of FDCs, both of which are functional in IC trapping. Representative sections showing FDC-M2 labeling are shown in Fig. 7. The average densities of pre-existing FDC-M2-positive FDC reticula were rather high (100–110 pixels) at day 0 in both young and old mice (Fig. 8A). The average density for FDC-M2 labeling increased in young mice significantly by day 1 and remained high (between 175 and 210 pixels) at days 5 and 10. In contrast, in old mice the average density after a brief increase at day 1 (~150 pixels) rapidly dropped off and stayed low (at 60 pixels) after day 5 of the GC reaction (Fig. 8A).
The asterisk (*) in young mice was significantly larger (45,285 \pm 12,000 \mu m^2) than that of old mice (8,000 \mu m^2) and was comparable to FDC-M1 and CR1/2 labeling (Fig. 8B).

In young mice, the size of FDC-M2-positive FDC reticulum increased gradually and peaked at day 5 (72,623 \mu m^2) comparable to M1 and CR1/2 labeling. At day 10, FDC-M2-positive FDC reticulum size was slightly reduced and became more compact in young mice (Fig. 8B).

In old mice, the size of FDC-M2-positive FDC reticulum increased from 8,194 \mu m^2 at day 0 to 22,573 \mu m^2 at day 1, but declined and leveled off at \sim 8,000 \mu m^2, representing greater than \sim 12-fold reduction in FDC reticulum size at days 3, 5, and 10 (Fig. 8B).

**Kinetics of PNA-positive GC development**

In the preceding studies, the development of the FDC reticulum was monitored. We reasoned that the response of the GC B cells to Ag provided by the FDCs could be measured by monitoring the development of PNA-positive GCs, since B cells labeling with PNA are activated and proliferating cells. Representative sections (Fig. 9) illustrating the PNA labeling of developing GCs are shown for the same group of young and old axillary lymph nodes from day 0 through day 10 of the GC response. The average GC size in the draining lymph nodes of young and old mice was determined and the values of responder draining lymph nodes were then plotted against time (Fig. 10).

In young draining lymph nodes, the average pre-existing PNA-positive GC size at day 0 was small but much larger than an FDC reticulum. In young draining lymph nodes, there was a significant increase in mean GC size from day 1 to day 5 (Fig. 10). The size of de novo OVA challenge-induced GCs increased through day 5 in the young lymph nodes, but in old mice the size was only 15% of normal at days 5 and 10. The results indicate a general failure of Ag on old FDCs to stimulate GC B cells and are consistent with the lack of FcγRII and FDC-M2 labeling in old mice.

**Comparison of FDC reticula and GC numbers in the pool of draining lymph nodes at day 5**

Kinetic analysis of the average FDC reticulum or average GC in the pool of draining lymph nodes of young and old mice revealed some remarkable deficits in old mice. By day 5, the FDC reticula and GCs were well developed and in some cases the number of FDC reticula and GCs appeared to be reduced in old mice. Analysis of FDC reticula and GC number in the pool of draining lymph nodes (two axillaries, two brachial, and two popliteal) indicated between 55 and 65 FDC reticula and GCs in young mice (Fig. 11). Furthermore, the number of FDC-M1- and anti-CR1/2-positive FDC reticula were similar in young and old mice (Fig. 11). In marked contrast, the number of anti-FcγRII- and FDC-M2-positive FDC reticula was reduced by 70–80% in old mice and this correlated with a 70–80% reduction in PNA-positive GCs (Fig. 11).

**Comparison of ITIM signaling in B cells in the presence of young and old FDC**

Recent results suggest that FDCs can block ITIM signaling in A20 cells induced by co-cross-linking BCR and B cell FcγRII and that FDC FcγRII is critical to blocking this process (J.G.T., unpublished data). We reasoned that FDCs bind Ig-Fc and minimize co-cross-linking BCR to B cell FcγRII and subsequent activation of the ITIM. It followed, therefore, that the lack of FcγRII on old FDC would correlate with ITIM activation as indicated by SHIP phosphorylation. To test this, A20 cells were stimulated using anti-BCR, a well-known inducer of SHIP phosphorylation, as an analog for ICs (Fig. 12). In contrast with anti-BCR (a positive control), the F(ab')2 of anti-BCR cannot engage B cell FcγRII and represents a negative control. As expected, young FDCs blocked SHIP phosphorylation while old FDCs did not and were similar to control FDCs that lacked FcγRII or with FcγRII blocked by specific Ab (Fig. 12).

**Complement and IC-mediated FDC Ag stimulation**

In a recent study, old FDCs functioned as well as young FDCs when the primary B cell signal was delivered by LPS and when FDCs were incubated with ICs and complement to provide old FDCs with adequate CD21L to engage the B cell coreceptor complex (27). However, BCR and FcγRII are not coligated and ITIM signaling does not occur when LPS is the primary stimulator, thus a deficit in FcγRII on old FDCs would not be a problem. This prompted us to reason that increasing the CD21L concentration on old FDCs would not restore immune responses when specific B cells were stimulated with ICs given that old FDCs lack normal levels of FcγRII. Memory B and T lymphocytes from young mice were incubated with ICs and complement to keep these elements equivalent and to reduce the study to a comparison of old vs young FDCs. As predicted, the old FDCs with additional CD21L failed to
induce a normal response with young memory B and T lymphocytes (Fig. 13) and this result is consistent with the lack of FcγRII on old FDCs. Old FDCs and FcγRIIB<sup>−/−</sup> B lymphocytes

Based on previous (27) and present observations, we reasoned that if a major problem with old FDCs relates to an inability to express normal levels of FcγRII and bind Ig-Fc, then old FDCs should be able to stimulate B cells lacking FcγRII quite normally. It is known that FDCs from FcγRIIB<sup>−/−</sup> mice fail to stimulate wild-type B cells where co-cross-linking of BCR and FcγRII can occur, but that they stimulate B cells in the FcγRIIB<sup>−/−</sup> mice normally (13). Accordingly, we tested old FDCs for their capacity to stimulate Ab production by FcγRIIB<sup>−/−</sup> lymphocytes in a system where ITIM signaling via BCR and B cell FcγRII cannot occur. In the absence of the IC-derived inhibitory B cell signaling, old FDCs were able to induce a much improved Ab response and when given ICs with complement to provide CD21L, the responses were comparable to those of young FDCs (Fig. 14).

Discussion

Humoral responses exhibiting high levels of high affinity Ab to T-dependent Ags require not only T and B cells but also FDCs (31–35). In the present study, we compared the expression of surface receptors (i.e., FcγRII, and CR1/2) on young and old FDCs that is critical for IC retention and is involved in FDC-B cell interactions (5–7, 10–17). The experiments were designed to study the kinetics of receptor expression during the GC reaction and provided the first comparative analysis of the kinetics of expression of FcγRII, CR1/2, and the epitopes for FDC-M1 and FDC-M2 during recall responses in young and old mice. The mAb FDC-M1 was used to monitor FDC presence (the FDC reticulum) and FDC-M2, which reacts with the C4 fragment of complement associated with the FDC-ICs (30), was used to monitor Ag trapping/retention by FDCs in the FDC reticulum. Our goal was to understand why old FDCs trap less IC, exhibit depressed Ag-specific and costimulatory signals, and support only low level Ab production (3, 18, 20, 27). Results revealed that both the average density of FDC-M1 labeling and the area of FDC reticulum labeled with FDC-M1 were similar in young and old mice. The mAb FDC-M1 labeling and the area of FDC reticulum labeled with FDC-M1 were similar in young and old mice. Thus, the data suggested that reduced IC trapping in old mice was not due to a decrease in the number of FDCs or to reticulum development. However, FcγRII labeling on old FDCs was dramatically reduced in the 3- to 10-day period of the GC reaction. Not only was the average density of FcγRII labeling on old FDCs diminished but FDC reticula number and sizes were dramatically reduced, indicating that only some
and this was confirmed with a reduced FDC-M2 labeling that correlates with reduced IC trapping by old FDCs (18, 19) positive reticula in young and old mice. Analysis of FDC reticula and GC number was done when young and old animals are provided with identical Ag-Ab complexes over a wide range of Ag:Ab ratios (36) and Ab levels of 2–300 μg are sufficient to trap ICs well in young mice (37), and these levels are achieved in aged mice (20). More significantly, when young and old animals are provided with identical amounts of specific Ab by passive transfer, ICs form in old animals normally and can be found in the afferent lymph and the subcapsular sinuses of lymph nodes as they are in the young. However, in young animals ICs are retained on FDCs and numerous GCs develop. In marked contrast, in old animals a much smaller amount of IC is retained on the FDC reticulum and GCs are reduced in number and are much smaller (18, 19). Similarly, when young and old mice are injected with young memory B and T cells, to be certain memory B and T cells are in equal number, the recall responses in aged mice that depend on old FDCs are abnormally low (20). Thus, the problem with FDC function in aged mice does not appear to simply be a problem with Ab and IC formation or the presence of memory cells.

FDC-FcγRII appears to be needed to trap optimal amounts of ICs and then plays a role organizing these ICs in a stimulatory format for B cells (11, 13). Direct interaction of ICs with B cells is known to lead to ITIM signaling and a lack of B cell responsiveness (38–40). Recent data suggest that FDCs can block the ITIM signaling in A20 cells apparently by minimizing interaction...

FIGURE 10. Size of the average PNA-positive GCs in the draining lymph nodes of young and old mice as assessed by labeling with PNA labeling. Young (■) and old (□) mice were actively immunized and challenged with OVA. These are the same lymph nodes as those shown in the preceding figures. The averages were calculated using data from three to six mice per day. Note that the GC size was significantly reduced in old mice compared with young mice and remained very small during the 3, 5, and 10 days of the GC response. The data represent the mean in square micrometers ± SEM and significant age-related differences are indicated by the asterisk (*, p < 0.003).

FIGURE 11. Correlation between the number of FDC reticula expressing FcγRII, IC trapping (indicated by FDC-M2), and a reduced number of PNA-positive GCs in old mice. Analysis of FDC reticula and GC number was done using a pool of draining lymph nodes (two axillaries, two brachial, and two popliteal). Young (■) and old (□) represent results from mice that were actively immunized and challenged with OVA in each of the front legs and hind feet. The data represent the mean number of FDC reticula positive for FDC-M1, anti-CR1/2, anti-FcγRII, or FDC-M2, or the mean number of GC positive for PNA in pooled draining lymph nodes ± SEM and significant age-related differences are indicated by the p value. Some GCs in old mice appeared to fragment and closely associated fragments in a single follicle were counted as a single GC. These data were obtained from mice taken at day 5 but the same relationship exists at days 3 and 10. Y, Young; O, Old.

FIGURE 12. Old FDCs fail to inhibit SHIP phosphorylation in A20 cells stimulated with anti-BCR. A20 cells were cocultured with young and old FDCs for 30 min at 37°C to allow FDC-A20 cell interactions (for in vitro GCs to form) and then stimulated with anti-mouse BCR, a well-known inducer of SHIP phosphorylation. Cells were then lysed, the lysate was immunoprecipitated with anti-SHIP, resolved using SDS-PAGE, transferred to a nitrocellulose membrane, and probed using anti-phosphotyrosine Ab. Note that incubation of A20 cells with young FDCs reduced ITIM signaling as indicated by reduced SHIP phosphorylation compared with A20 cells stimulated with anti-BCR. On the other hand, incubation of A20 cells with old FDCs, FcγRIIB−/− FDCs, or FDCs with FcγRII blocked with 2.4G2 failed to block SHIP phosphorylation in A20 cells stimulated with anti-BCR. This result is representative of three experiments of this type.
of Ig-Fc in ICs with A20 cell FcγRII and thus promoting B cell activation. Activation of the ITIM in cells results in phosphorylation of SHIP (38, 41). SHIP is the main ligand for phosphorylated ITIM and that SHIP phosphorylation correlates with ITIM signaling (42–46). Based on these observations, we hypothesized that old FDCs lacking FcγRII would not block SHIP phosphorylation in cells stimulated by anti-BCR. The capacity of old FDCs, taken 3 days after Ag challenge, to block SHIP phosphorylation in A20 cells induced by anti-BCR was shown to be impaired (Fig. 12). The data suggest that Ag handling and costimulatory activity of old FDCs become defective and their capacity to prevent ITIM signaling deteriorates with aging. It appears that this is a consequence of reduced expression of FcγRII on old FDCs that fails to block the inhibitory effect of ICs by binding of Fc in the IC. This ultimately results in B cell inactivation and significantly depressed secondary Ab responses.

Recently, we reported that incubation of old FDCs with C and ICs repaired the age-related defect when the primary B cell signal was delivered by LPS and costimulatory signals were provide by aged FDC (27). In this model, the critical FDC costimulatory signal was delivered by CD21L and additional CD21L was provided by incubating the aged FDCs with ICs and complement. The additional CD21L dramatically improved the ability of old FDCs to augment Ab production by LPS-stimulated B cells (27). We sought to determine whether an increased level of CD21L would remedy the age-related deficit in handling specific Ag. Old FDCs were treated with OVA-anti-OVA and complement and then tested in a recall response using memory T and B cells from OVA-immune mice. However, the treatment of old FDCs with complement and IC did not repair the depressed Ag-handling capacity of old FDCs and anti-OVA production remained depressed (Fig. 13). We reason that the lack of FDC-FcγRII on aged FDCs cannot prevent coligation of B cell FcγRII and BCR (13) and lack of FcγRII on FDCs may also interfere with the ability of FDCs to arrange for the formation of iccosomes, which are the IC-coated small spherical structures on which Ag is delivered from FDCs to B cells for endocytosis and processing (19, 20, 31, 47). Thus, it is not surprising that while additional CD21L may augment LPS-stimulated B cells it does not restore normal Ag handling and the suppression of B cell activation.


**FIGURE 13.** Incubation of old FDCs with ICs and complement from young mice did not restore stimulatory activity. Note that young FDCs (y) stimulate OVA-specific Ab production by memory lymphocytes (Ly) but old (o) FDCs could support only a lower anti-OVA response. Incubation of old FDCs with active C (aC) and IC did not restore the Ag-handling capacity of old FDCs and was not different from IC plus heat-inactivated complement (inaC). These data are typical of three experiments of this type and results are depicted as the mean SEM of total IgG produced (p < 0.005).

**FIGURE 14.** Old FDCs stimulated FcγRIIB/−/− B cells to produce near normal levels of specific Ab. Wild-type old FDCs were cocultured with young wild-type OVA-specific lymphocytes (Ly) or with young OVA-specific FcγRIIB/−/− lymphocytes in the presence of IC with or without complement. The depressed Ab response typical for old FDCs was not apparent with FcγRIIB/−/− B lymphocytes because ITIM signaling induced by cross-linking of BCR and B cell FcγRII cannot occur. Addition of complement also improved the Ab response by providing more CD21L for costimulation. KO, Knockout.
induced by ITIM signaling in specific B cells. However, when we used FcγRIIB−/− lymphocytes where ITIM signaling via BCR and B cell FcγRII cannot occur, old FDCs given ICs with complement were able to induce Ab responses that were comparable to those of young FDCs (Fig. 14). This experiment provides additional independent support for the concept that old FDCs lacking normal levels of FcγRII do not inhibit ITIM signaling and fail to stimulate wild-type B cells normally.

In conclusion, our data may be summarized in a simple working model illustrated in Fig. 14. Based on immunocytochemical data, there is an exceptionally higher density of FcγRII on old FDCs than on B cells. The FcγRII on young FDCs apparently engages essentially all Ig-Fc on the Ag-Ab complexes. Thus, very little Ig-Fc is left that would be able to bind to B cell FcγRII receptors and activate ITIM signaling. By contrast, old FDCs are deficient in FcγRII and do not engage all (or most) Ig-Fc on the ICs. This would allow B cell-BCR and FcγRII receptors to be cross-linked and ITIM activated. This ultimately results in B cell inactivation and depressed Ab production. N. Nucleus, ITAM, immunoreceptor tyrosine-based activation motif.

References


