Follicular Dendritic Cells Produce IL-15 That Enhances Germinal Center B Cell Proliferation in Membrane-Bound Form[1]

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Factors that control the survival and proliferation of Ag-stimulated B cells within the germinal center (GC) are crucial for humoral immune responses with high affinity Abs against infectious agents. The follicular dendritic cell (FDC) is known as a key cellular component of the GC microenvironment for GC-B cell survival and proliferation. In this study, we report that IL-15 is produced by human FDC in vivo and by an FDC cell line, FDC/HK cells, in vitro. IL-15 is captured by IL-15Rα on the surface of FDC/HK cells. The surface IL-15 is functionally active and augments GC-B cell proliferation. Because GC-B cells have the signal-transducing components (IL-2/15R$\beta\gamma$), but not a receptor for binding of soluble IL-15 (IL-15Rα), IL-15 signaling is possibly transduced by transpresentation from FDCs to GC-B cells via cell-cell contact. Together, these results suggest that IL-15 from FDC, in membrane-bound form, plays an important role in supporting GC-B cell proliferation, proposing a new target for immune modulation as well as treatment of B cell tumors of GC origin. *The Journal of Immunology,* **2004, 173: 6676–6683.**

For humoral immune responses, the germinal center (GC)[4] provides a specialized microenvironment, in which Ag-activated B cells are able to proliferate and differentiate and to provide protection through the production of Abs with optimal affinity against invading microorganisms (1–3). Factors that control the vigorous proliferation of GC-B cells are crucial for the expansion of a few initial clones as well as somatic hypermutation, a process through which a sufficient pool of diverse high affinity BCRs is obtained. Simultaneously, to ensure that the immune responses are not directed toward self Ags, factors controlling the selection process within GC are also critical (4–6). The signals received through BCR, known to be important for these GC reactions, have been investigated extensively (7–10). The cofactors from the GC microenvironment, however, are not so clearly understood, but have become increasingly recognized as critical factors for the GC reactions because they can alter the strength or the threshold of responses, possibly resulting in alteration of the specificity and intensity of immune responses (11–15).

A major producer of GC microenvironmental factors is the follicular dendritic cell (FDC), which is present in lymphoid follicles and belongs to stromal cells of these organs (9, 12, 16–18). FDCs are initially known to retain Ags on their surface for a long time and present those native Ags to GC-B cells (19, 20). For the last 10 years, investigations on FDCs have focused on their extraordinary capacities to support GC-B cell survival and proliferation via both direct cell-cell contact and secreted soluble factors (21–24). The physical interaction between FDCs and GC-B cells is able to prevent apoptosis of GC-B cells in vitro, a response that cannot be achieved by CD40 ligation and/or BCR cross-linking alone (25–27). Adhesion molecules such as ICAM-1 and VCAM-1 on FDC are important for this protective FDC-B cell binding (26, 28). FDCs also costimulate GC-B cell proliferation through their expression of molecules such as CD44 and 8D6 protein (12, 29). These factors, however, have not been shown to replace the FDC effect completely (17, 23, 27). FDCs are essential for GC-B cells to survive and proliferate in vitro upon stimulation with cytokines such as IL-2, IL-4, and IL-10 (6, 30).

IL-15 was identified originally as an IL-2-like T cell growth factor (31–33), and has similar biologic properties to IL-2 in vitro such as augmentation of T cell proliferation and activation (34). IL-15 mRNA is expressed ubiquitously by a large variety of tissues, but not primary T cells (32, 35, 36). However, its translation and secretion of protein are regulated tightly by multiple complex mechanisms (37). IL-15 binds to lymphocytes via a heterotrimeric IL-15R comprised of IL-15Rα (37), IL-2Rβ (38), and IL-2Rγ (39). The specific binding to IL-15 is conferred by IL-15Rα to deliver IL-15 signal (35), or via direct cell-cell contact as a membrane-bound ligand (40, 41). Although IL-15 stimulates T and NK cells, IL-15 also plays important functions in B cells and malignancies of B cell origin (42–48). Moreover, IL-15 is produced by synoviocytes (49), which have intrinsic properties of FDCs (50). Hence, we investigated the function of the IL-15 on GC-B cells and FDCs in our unique in vitro experimental model that mimics the in vivo GC reaction (6, 30, 51).

We show that IL-15 is produced by FDCs. IL-15 is presented on the surface of FDC/HK cells, being captured by IL-15Rα and transpresented to GC-B cells. GC-B cells do not express IL-15Rα, but do express the signal transduction complex IL-2/15 Rβ and Rγ. IL-15 presented on the membrane of FDC/HK cells is biologically active and augments GC-B cell proliferation.

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[4] Abbreviations used in this paper: GC, germinal center; DAPI, 4′,6′-diamidino-2-phenylindole; FDC, follicular dendritic cell; hIL, human IL; MFI, mean fluorescent intensity; L, ligand.

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Materials and Methods

Antibodies

Anti-IL-15 mAb (M110, M111, and M112: IgG1) were generated as follows: BALB/c mice were boosted twice with 10 μg of human (h) IL-15-flag in RIBI adjuvant (Ribi, Hamilton, MT). Three months after the last boost, one animal was boosted i.v. with 3 μg of hIL-15 in PBS. Three days later, the spleen was removed and fused with Ag8.653 using 50% polyethylene glycol (Sigma-Aldrich, St. Louis, MO). The fused cells were plated into 96-well plates in DMEM containing hypoxanthine/aminopterin/thymidine supplement (Sigma-Aldrich). Hybridoma supernatants were screened by Ab capture assay. Briefly, 96-well plates were coated with 10 μg/ml goat anti-mouse Ig overnight. After blocking with 3% BSA, 50 μl of cell supernatant was added to each well. After 1 h, plates were washed with PBS with 0.05% Tween 20. Iodinated hIL-15 was added to plates at a previously determined concentration, for 1 h. After washing, plates were exposed to phosphor imager plates for 3 h. Positive cells were cloned out twice, using similar screen to detect positives. A CTLL-2 cell proliferation assay was also performed to determine IL-15-blocking activity. Specificity of these mAbs has been tested and used previously (U.S. patent 5,795,966) (43, 52). Mouse IgG1 (MOPC 21) for isotype control was purchased from Sigma-Aldrich. Anti-IL-15 mAb (MAB247, mouse IgG1), goat polyclonal anti-IL-15, and goat normal control Ig were purchased from R&D Systems (Minneapolis, MN). PE-conjugated anti-CD20 mAb and FITC-conjugated goat anti-mouse Ab were purchased from BD Pharmingen (San Diego, CA). DRC-1 mAb (mouse IgG1) was purchased from DakoCytomation (Carpinteria, CA). Alexa 594-conjugated goat anti-mouse Ab was purchased from Molecular Probes (Eugene, OR). FITC-conjugated donkey anti-goat Ab was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cytokines and reagents

The culture medium used was IMDM (Irvine Scientific, Santa Ana, CA) and RPMI 1640 (Sigma-Aldrich) supplemented with 10% FCS (Invitrogen Life Technologies, Grand Island, NY), 2 mM glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin (Irvine Scientific). Cytokines used were IL-2 (Hoffmann-LaRoche, Nutley, NJ) and IL-4 (a generous gift from Schering-Plough, Union, NJ). Recombinant trimeric human CD40 ligand (L) and IL-15 were prepared, as described previously (32, 42, 53). Percoll and Ficoll were purchased from Pharmacia Biotech (Uppsala, Sweden), and BSA from Sigma-Aldrich.

Immunofluorescence staining of FDC clusters

Human tonsillar FDCs were isolated, as described previously (54). Isolated cells were cytospinned on glass slides at 700 rpm for 5 min (Cytospin 2; Thermo Shandon, Pittsburgh, PA). The cytospin slides were fixed in cold acetone (-20°C) for 5 min and stored at -70°C until required. Slides were hydrated with PBS for 10 min at room temperature, then incubated with blocking solution (DakoCytomation) for 1 h at 25°C in a humidified chamber. Slides were stained with optimal amount of goat anti-IL-15 Ab or control goat Ig overnight at 4°C. Then the slides were washed three times and incubated with FITC-conjugated anti-goat Ig for 1 h at room temperature. For costaining, DRC-1 mAb (see Fig. 1, *A* and *C*) or PE-conjugated anti-CD20 mAb (for Fig. 1*B*) was added together with primary Abs. DRC-1 staining was visualized by secondary Alexa-594-conjugated anti-mouse Ab staining. For single FDC staining (Fig. 1*D*), slides were incubated in 4′,6′-diamidino-2-phenylindole (DAPI) solution (Molecular Probes) for nuclear counter staining, then stained with mouse anti-IL-15 or control mAb, followed by FITC-conjugated goat anti-mouse Ab. Slides were washed and mounted with anti-fade fluorescent mounting medium (Molecular Probes). Images were collected on a deconvolution microscope (Axiovert 200M; Carl Zeiss Microimaging, Thornwood, NY). Images were processed using the slidebook software (version 1.6.587; Intelligent Imaging Innovations, Denver, CO) and Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

Flow cytometric analysis

FDC/HK cells were cultured in 10% FCS RPMI 1640 medium, as described previously (54). FDC/HK cells of passages 4–9 were used for the experiments. For FACS analysis, FDC/HK cells were collected with enzyme-free cell dissociation solution (Specialty Media, Philipsburg, NJ). All FACS staining for surface IL-15 detection was performed with modification to previously described procedures for amplification (6). Briefly, cells were washed in cold FACS buffer (0.05% FCS, 0.01% NaN_3 in PBS) and subsequently incubated with the appropriate concentration of anti-IL-15 mAb (B247) for 15 min at 4°C. After washing with cold FACS buffer, the amplification procedures using Flow-Amp kit (Flow-Amp Systems, Cleveland, OH) were followed, according to the manufacturer's instruction. For competition study, anti-IL-15 Ab was incubated with 300 ng/ml rIL-15 for 30 min at 4°C before FACS staining. Samples were analyzed with FACSCalibur (BD Biosciences, San Jose, CA) and CellQuest-Pro programs. Specific mean fluorescence intensity (MFI) was obtained by subtraction of fluorescence value from that of corresponding control.

Acid stripping and binding of IL-15

Acid stripping of previously bound IL-15 was performed, as described earlier (40, 48). Briefly, FDC/HK cells were washed twice with cold PBS, and then incubated with glycine buffer (25 mM glycine, 150 mM NaCl, pH 3) for 10 min at 4°C. Cells were then collected and washed twice with cold PBS and subjected to FACS staining. For binding experiment, FDC/HK or GC-B cells were collected and washed with cold PBS twice, and then incubated with a saturating dose of IL-15 (100 ng/ml) for 30 min at 4°C, washed with cold PBS, and then stained for FACS analysis.

CTLL-2 cell assay

CTLL-2 cells (American Type Culture Collection, Manasas, VA) were maintained in RPMI 1640 medium containing 10% FCS, IL-2 (30 U/ml), and 2-ME (5×10^{-5} M; Sigma-Aldrich). Serially diluted numbers of FDC/HK cells (from 2×10^4 cells/well to none/well) were cultured in 96-well plates for 1 day in 5% CO_2 incubator. The plates were then washed and fixed in 1% paraformaldehyde in PBS for 1 h at 4°C, followed by extensive washing in cold PBS. CTLL-2 cells (5×10^3 cells/well) in maintaining medium were added in triplicate to the 96-well plates coated with fixed FDC/HK cells and cultured with anti-IL-15 mAb or isotype control mAb. After 20 h of culture, cells were pulsed with 0.5 μCi of [^{3}H]TdR (20 Ci/mM; PerkinElmer Life Sciences, Boston, MA) for additional 4 h. The cultures were harvested onto glass fiber filter, and [^{3}H]TdR incorporation was measured by a liquid scintillation counter (Rackbeta; LKB Instruments, Houston, TX). Results are expressed as the mean cpm $\pm$ SEM of triplicate cultures.

RT-PCR

To examine the expression of mRNA for IL-15Rα, IL-2Rα, IL-2Rβ, and IL-2Rγ, total RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA). One-microgram aliquot of RNA was transcribed using random oligo-dT and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). Complementary DNA was amplified in a 25-μl reaction mixture containing 200 μM each dNTP, 500 nM primers, and 2.5 U of *Taq* polymerase. Amplification of each cDNA sample was conducted under condition, as follows: denaturation at 94°C for 50 s, annealing at 57°C for 50 s, and extension at 72°C for 50 s. Human GAPDH was used to ensure equal sample loading. A mock PCR was performed to serve as a negative control. Amplified PCR products were separated on 1.5% agarose gel and visualized by ethidium bromide staining. Primers used are as follows: for IL-15Rα, 5′-GTCAAGAGCTACAGCTTGTAC-3′ and 5′CATAGGTGGTGAGAGCA GTTTTC-3′; for IL-2Rα, 5′-AAGCTCTGCCACTCGGAACACAAC-3′ and 5′-TGATCAGCAGGAAAACACAGC-3′; for IL-2Rβ, 5′-ACCTCTTGGG CATCTGCAGC-3′ and 5′-CTCTCCAGCACTTCTAGTGG-3′; for IL-2Rγ, 5′-CCAGAAGTGCAGCCACTATC-3′ and 5′-GTGGATTGGGTGGCT CCAT-3′; and for GAPDH, 5′-CCCTCCAAAATCAAGTGGGG-3′ and 5′-CGCCACAGTTTCCCGGAGGG-3′.

Preparation and culture of human tonsillar GC-B cells

GC-B cells were purified from tonsillar B cells by MACS (Miltenyi Biotec, Auburn, CA), as described previously (6). The purity was greater than 95%, as assessed by the expression of CD20 and CD38. GC-B cells (2×10^5 cells/well) were cultured in 24-well plates in the presence of irradiated FDC/HK cells (2×10^4 cells/well; 5000 rad), CD40L (100 ng/ml), IL-2 (30 U/ml), and IL-4 (50 U/ml). IL-2 was included to increase sensitivity, except for the experiment for Fig. 5*B*, because the overall recoveries of cultures were very low without IL-2 (6). For blocking experiment, anti-IL-15 or isotype control mAb (10 μg/ml, if not indicated otherwise) was incubated for 30 min before adding GC-B cells. Some of blocking and corresponding control mAbs contained $<0.00002\%$ of sodium azide at working concentration, which is 100-fold lower than the concentration of sodium azide, which started to show toxicity in our in vitro culture system (data not shown). For addition experiment (see Fig. 5*B*), IL-15 (1–100 ng/ml) was added 30 min before adding GC-B cells. For cell division experiment, GC-B cells were labeled with CFSE (Sigma-Aldrich; 5 μM in PBS) at 37°C for 10 min. FCS was added to stop staining, and then labeled cells were washed with culture medium. After culture, the CFSE intensity was

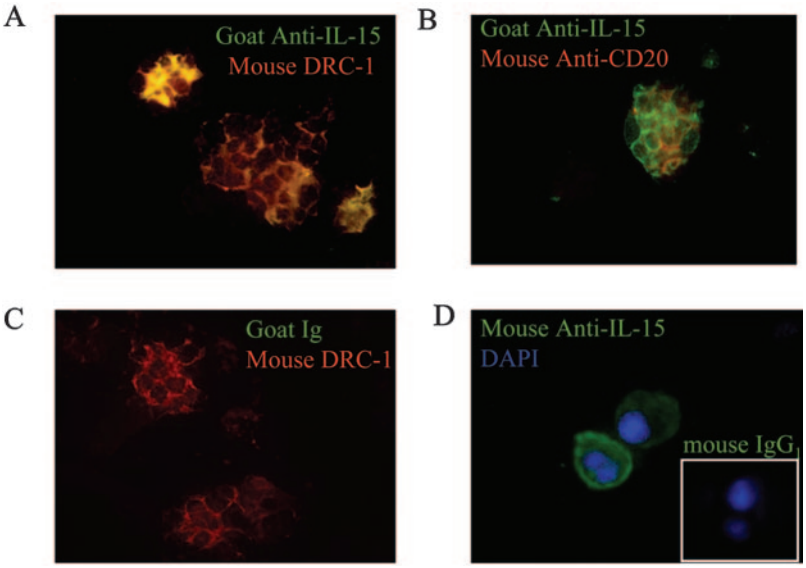

FIGURE 1. IL-15 is expressed in human tonsillar FDCs, not in B cells. Cytospin preparations of human tonsillar FDC clusters were stained with goat polyclonal anti-IL-15 Ab (*A* and *B*, green), mouse anti-IL-15 mAb (*D*, green), and corresponding control Abs (*C* and *D*, *inset*, green). Slides were costained with DRC-1 mAb for FDCs (*A* and *C*, red), anti-CD20 mAb for B cells (*B*, red), and DAPI for nucleus (*D*, blue). Original magnification ×400.

measured by FACSCalibur and analyzed by ModFit LT software 3.0 (Verity Software House, Topsham, ME). Recovered viable cells were counted by trypan blue exclusion.

Results

IL-15 was produced by FDC, but not by B cells

To identify the cellular source of IL-15 in the GC, we examined in vivo expression of IL-15 by staining freshly isolated FDC-B cell clusters with specific Abs to IL-15 (Fig. 1).

FDC clusters were cellular aggregates consisting of a typical FDC with large cytoplasm and more than 10 B cells (29) (Fig. 1, *A–C*). IL-15 was expressed in the FDC clusters, suggesting the presence of IL-15 in vivo (Fig. 1, *A* and *B*). To determine the cellular source of IL-15 in FDC clusters, FDC-specific marker DRC-1 mAb or B cell-specific marker anti-CD20 mAb was costained with goat anti-IL-15 Ab, respectively (29, 55). Anti-IL-15 Ab (green) costained with DRC-1 mAb (red; costaining, yellow; Fig. 1*A*), but not with anti-CD20 mAb (red; Fig. 1*B*), suggesting that DRC-1-positive FDCs, not B cells, produce IL-15. The staining was specific for IL-15 because there was no costaining in samples costained with the goat control and DRC-1 Abs (Fig. 1*C*). Some FDCs (10–20%) were not clustered with B cells, but can be identified by their abundant cytoplasm and frequent double nuclei (16) (Fig. 1*D*). These single FDCs also expressed IL-15 as stained by a murine anti-IL-15 mAb (MAB247), confirming the above result. Similarly, there was no green staining, but only blue nuclear staining in samples stained with mouse control mAb and DAPI (Fig. 1*D*, *inset*).

IL-15 was present on the surface of FDC/HK cells bound to IL-15Rα

Because it was practically impossible to obtain a sufficient number of freshly isolated FDCs without contaminating B cells, we next investigated the production of IL-15 by primary FDC cell line, FDC/HK, which was shown to share many of FDC characteristics, including the capacity to support GC-B cell survival and proliferation (12, 23). We were not able to detect IL-15 in the culture supernatant of FDC/HK cells (2×10^5 cells/ml) by ELISA (assay sensitivity ≥19 pg/ml; data not shown), so we explored the surface expression of IL-15, as reported previously (53–56). We used a highly sensitive surface FACS-staining method using tyramine amplification method (Flow-Amp) to detect IL-15. As shown in Fig. 2*A*, IL-15 was detected on FDC/HK cells, whereas GC-B cells were negative (Fig. 2*A*). These results are consistent with the previous immunofluorescence staining data on FDC-B cell clusters. The specific staining of IL-15 on FDC/HK was verified by competing with soluble IL-15. When anti-IL-15 mAb was preincubated with excess amount of IL-15, the staining of IL-15 on the surface of FDC/HK cells was completely reduced to that of isotype control. These results were reproduced in three separate experiments.

There are two conflicting explanations for the mechanisms of surface IL-15: one is the presence of an alternative membrane type IL-15 molecule (52), and another is through the rebinding of secreted IL-15 (40, 41). To explore the mechanism, we tried to remove IL-15 on the FDC/HK cell surface by acid treatment, as described previously (40). IL-15 was completely removed from the surface of FDC/HK cells after treatment with glycine buffer (pH

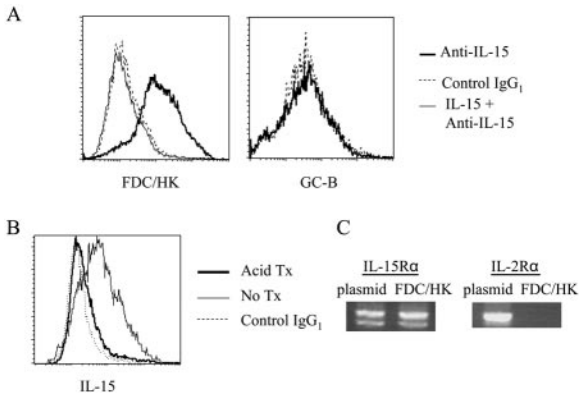

FIGURE 2. FDC/HK cells express IL-15 on their surface bound to IL-15Rα. *A*, Surface expression of IL-15 by FACS. Surface FACS staining with specific or control mAb was amplified with Flow-Amp kit (bold and broken line, respectively). Competition experiments were performed to confirm specificity by incubating specific mAb with IL-15 (300 ng) for 30 min on ice before staining cells (solid line). *B*, Disappearance of surface IL-15 after acid stripping. FDC/HK cells were incubated in cold glycine buffer (pH 3.0) for 10 min on ice and then stained with specific Ab or isotype control Ab (acid treatment, bold line; no treatment, solid line; isotype control, broken line). *C*, Expression of IL-15Rα mRNA in FDC/HK cells. RT-PCR for IL-15Rα and IL-2Rα (an internal control) was performed with the same amount of FDC/HK cell mRNA under the same conditions.

3.0) to the staining level with the control mAb (Fig. 2*C*). This result suggests rebinding of secreted IL-15 rather than an alternative membrane type protein.

Because IL-15Rα binds to IL-15 with high affinity (35), we examined the presence of IL-15Rα in FDC/HK cells. In RT-PCR experiments, the specific band for the IL-15Rα was amplified from the cDNA of FDC/HK cells as well as positive control plasmid, whereas that for the IL-2 Rα was not amplified, which was included to serve as an internal negative control (Fig. 2*C*). This result indicates that FDC/HK cells express mRNA for IL-15Rα.

Membrane-bound IL-15 on the FDC/HK surface is biologically active

To examine the biological activity of surface-bound IL-15 on FDC/HK cells, the IL-2- and IL-15-dependent CTLL-2 cell assay was used. Although soluble IL-15 was not detectable by ELISA, we fixed FDC/HK cells with 1% paraformaldehyde to exclude the false positive results by soluble IL-15. Incorporation of tritiated thymidine by CTLL-2 cells increased in proportion to the number of fixed FDC/HK cells present in cultures (Fig. 3*A*). At the ratio of 4:1 of FDC/HK cells to responding CTLL-2 cells, the value of cpm was almost 3 times higher than negative controls (21,000 to 7,500). The relatively higher background proliferation of CTLL-2 cells in control wells without fixed FDC/HK cells (7,500 cpm) can be attributed to suboptimal dose of IL-2 added to increase the sensitivity of the assay. The result is consistent with the previous report that the rebound IL-15 is functionally active on the cell surface (53–56). To examine the possible effect of soluble IL-15 released from the FDC/HK cells, the culture supernatant from the highest FDC/HK cell concentration (2×10^4 cells/well) was added to the same culture. There was no significant difference in cpm values between cultures with control medium and with FDC/HK cell culture supernatant, indicating the absence of IL-15 in the culture supernatant, which is consistent with the ELISA results.

To confirm that the stimulatory effect on CTLL-2 cells was mediated by IL-15, specific blocking mAb to IL-15 and isotype control mAb were added to the culture. As shown in Fig. 3*B*, the addition of anti-IL-15 mAb blocked completely the proliferation of CTLL-2 cells enhanced by fixed FDC/HK cells, whereas the control mAb had no effect.

GC-B cells express receptor components for IL-15 signal transduction, but not for high affinity binding

Production of IL-15 by FDC implied that IL-15 possibly had a biologic function in the GC reaction, most likely on GC-B cells. We thus examined the expression profile of specific receptors required for IL-15 signaling in GC-B cells (Fig. 4*A*). The expression of IL-15Rα mRNA, a receptor component for high affinity binding, was virtually negligible in RT-PCR, showing a similar faint band to that of IL-2Rα in freshly isolated GC-B cells (a negative control). In contrast, expressions of IL-2Rβ and IL-2Rγ mRNAs, the major components of signal transduction, were evident in GC-B cells whether freshly isolated or cultured, suggesting the presence of signaling receptor components for IL-15 or IL-2 in GC-B cells both in vivo and in vitro.

The absence of IL-15Rα mRNA was also confirmed by the failure to detect IL-15Rα protein in FACS staining of GC-B cells (data not shown) and the lack of IL-15 binding (Fig. 4*B*). In contrast to FDC/HK cells that exhibited intense binding of IL-15, no significant binding of IL-15 was detected on the surface of GC-B cells after incubation with excess IL-15, demonstrating the absence of IL-15Rα on the surface. Because soluble IL-15 needs IL-15Rα to transduce its mitogenic signal (56), the result suggests that GC-B cells cannot respond to soluble IL-15. This conclusion is consistent with the observation that soluble IL-15 in the absence of FDC/HK cells showed no noticeable difference in GC-B cell recovery (data not shown).

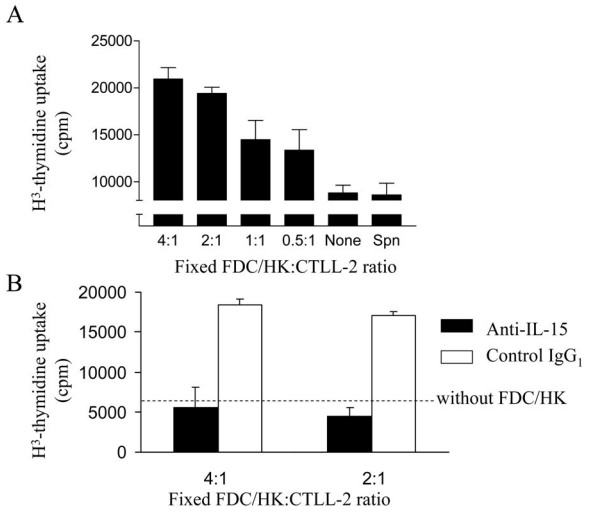

FIGURE 3. Membrane-bound IL-15 on the FDC/HK surface is biologically active. Different numbers of FDC/HK cells (2-fold dilution from 2×10^4 to none/well) were cultured in 96-well plates for 1 days and fixed with 1% paraformaldehyde. CTLL-2 cells (5×10^3 cells/well) were cultured for 1 day on FDC/HK cell-coated 96-well plates in triplicate in RPMI 1640 medium containing 10% FCS, 1 U/ml IL-2, and 2-ME. Cells were pulsed with 0.5 μCi of [^{3}H]TdR (20 Ci/mM) for last 4 h. [^{3}H]TdR incorporation was measured by a liquid scintillation counter. Results are expressed as the mean cpm $\pm$ SEM of triplicate cultures. *A*, Proliferation of CTLL-2 cells in various numbers of FDC/HK cells coated to the fixed number of CTLL-2 cells (none, 10% FCS RPMI 1640 medium control without coated FDC/HK; spn, FDC/HK culture supernatant). *B*, Inhibition of enhanced CTLL-2 cell proliferation by specific anti-IL-15 mAb (10 μg/ml). Broken line represents the cpm value of cultured CTLL-2 cells without FDC/HK cells or Ab. These results were reproduced in two independent experiments.

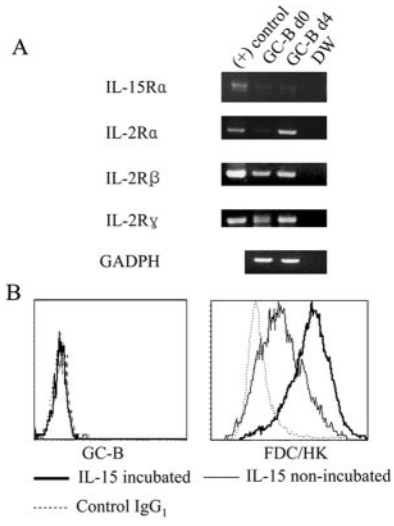

FIGURE 4. Expression of IL-15Rs in GC-B cell. *A*, RT-PCR was performed with mRNAs from freshly isolated or cultured GC-B cells, as described in *Materials and Methods*. (+) control, Plasmid containing respective genes; GC-B d0, freshly isolated GC-B cells; GC-B d4, 4-day-cultured GC-B cells; DW, distilled water to serve as a negative control. *B*, FACS profiles of IL-15-binding assay. Freshly isolated GC-B and FDC/HK cells were incubated with a saturating dose of IL-15 (100 ng) for 30 min on ice, and then stained with anti-IL-15 mAb (bold line)

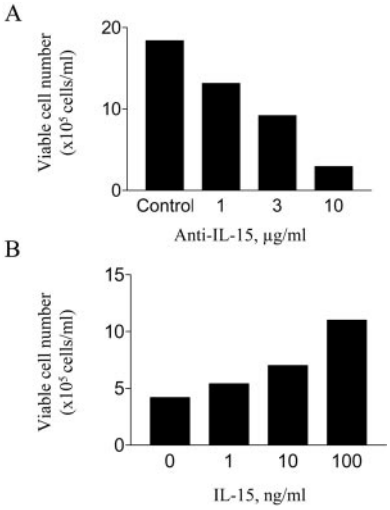

FIGURE 5. IL-15 on FDC/HK cells increases GC-B cell recovery when cultured with FDC/HK cells and cytokines. *A*, Viable cell recovery was decreased corresponding to the amount of added anti-IL-15 mAb. GC-B cells (2×10^5 cells/well) were cultured in 24-well plates with FDC/HK cells (2×10^4 cells/well, 5000 rad), CD40L (100 ng/ml), IL-2 (30 U/ml), and IL-4 (50 U/ml) with the indicated amount of specific mAb for 10 days. Cells were harvested at day 10 and counted by trypan blue exclusion. *B*, The viable cell numbers were increased proportionally to the amount of added IL-15. Indicated amount of IL-15 was added to the GC-B cell cultures. IL-2 was not included in this experiment. Representative results from four separate experiments were presented.

IL-15 on FDC/HK cells increases GC-B cell proliferation

In recent reports, IL-15 in surface-bound form is shown to deliver signals effectively in the absence of IL-15Rα on the target cells (40, 41). Therefore, despite the absence of IL-15Rα in GC-B cells, observations that IL-15 is expressed on the surface of FDC/HK cells and that GC-B cells expressed receptor components for signal transduction prompted us to investigate whether IL-15 on FDC/HK has a functional role in GC-B cell survival and proliferation.

GC-B cells were cultured with FDC/HK cells and cytokines, as described in *Materials and Methods*. When different amounts of anti-IL-15 mAb were added, GC-B cell proliferation was remarkably inhibited in a dose-dependent manner (Fig. 4*A*), suggesting that IL-15 on FDC/HK cells has biologic effect in supporting GC-B cell proliferation. At day 10, the number of viable GC-B cells in the culture containing anti-IL-15 mAb (10 μg/ml) was 17% of that of cultures containing isotype control mAb. However, blocking of IL-15 did not affect differentiation of GC-B cells in our in vitro culture system (data not shown). This result was reproduced in four separate experiments. Similar inhibitions were also observed in the experiments using other mAbs to IL-15 (clones M111, M112, and MAB247; data not shown).

Because IL-2 shares the receptor components for signal transduction with IL-15 and was present in above experiment to increase the sensitivity of assay, we modified our culture condition by omitting IL-2 to exclude possible indirect effect by IL-2 to verify the effect of IL-15 in the depletion experiment. As shown in Fig. 4*B*, the amount of surface IL-15 on FDC/HK cells was increased further by the incubation with exogenous IL-15. Hence, coated FDC/HK cells were incubated with different amount of IL-15 (1–100 ng) before GC-B cell cultures to augment IL-15 effect. The MFI of surface IL-15 by FACS were increased in proportion to the IL-15 added (data not shown, for 100 ng; Fig. 4*B*, *right panel*). The cell number recovered at culture day 10 was increased in a dose-dependent manner (Fig. 5*B*). In the presence of 100 ng/ml IL-15, the number of viable GC-B cells increased two and a half times more than the control culture. Given that GC-B cells do not express IL-15Rα, these results strongly suggested that surface IL-15 on FDC/HK enhanced GC-B cell proliferation. This result was reproduced in four separate experiments.

IL-15 on FDCs augments GC-B cell proliferation rather than protection from apoptosis

To investigate the augmenting effect of IL-15 on FDC/HK cells, division history of cultured GC-B cells in the presence or absence of IL-15 was analyzed by labeling cells with CFSE.

The recovery of viable CFSE-labeled GC-B cells in control culture with isotype control mAb in the presence of IL-2, IL-4, and CD40L at day 6 was increased to 172.5% of initial seeded cells (2×10^5/well to 3.45×10^5/well; Fig. 6*A*; control Ig), indicating that the CFSE (5 μM) did not interfere with cell proliferation compared with previous data (30). The significant increase in the recovery of control cells also demonstrated proliferation of GC-B cells in our culture system instead of just survival of certain subpopulations from the initial population. As shown in Fig. 6*A*, recovery increased by 49% in cultures with additional IL-15 compared with that of control cultures (5.15×10^5/well vs 3.45×10^5/well). In blocking experiments, the recovery of the cultured GC-B cells with anti-IL-15 decreased remarkably to 38%, compared with cultures containing control mAb (1.25×10^5/well vs 3.45×10^5/well). The recovery of GC-B cells in the culture with blocking anti-IL-15 mAb was decreased by 75% compared with that of cultures with a saturating dose of IL-15 (1.25×10^5/well vs 5.15×10^5/well), demonstrating the critical effect of IL-15 on GC-B cell growth.

Division profiles determined by CFSE intensity revealed that most of GC-B cells had divided more than four times by day 6 (Fig. 6*B*). In the presence of IL-2, IL-4, and CD40L with FDC/HK cells, the greatest proportion of harvested cells was found in the fifth and sixth division (76.6 and 18.9%, respectively), showing an almost synchronized response in proliferation to added mitogenic

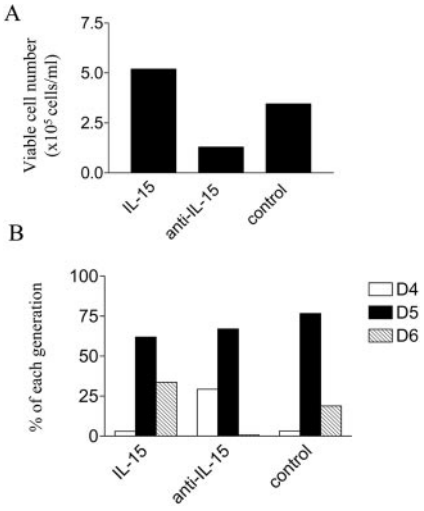

FIGURE 6. IL-15 enhances GC-B cell proliferation in vitro. Isolated GC-B cells were labeled with CFSE (5 μM) and then were cultured for 6 days with IL-15 (100 ng/ml), anti-IL-15 (10 μg/ml), or control mAb in the presence of FDC/HK cells and cytokine combinations. Harvested cells were counted and subjected to FACS analysis to measure the CFSE intensity. Results were analyzed with ModFit software. *A*, Comparison of viable cell numbers. *B*, Comparison of CFSE profiles of the recovered cells by percentage in each division (D, division).

signals. The effects of additional IL-15 on the distribution of cultured GC-B cells across divisions revealed a decrease in the fifth division population (from 76.6 to 61.9%) and a corresponding increase in the sixth division population (from 18.9 to 33.6%). Furthermore, when endogenous IL-15 was blocked by specific anti-IL-15 mAb, hardly any cells were found in sixth division, while the greatest percentage of cells was found in the fourth and fifth divisions (28.5 and 68%, respectively). These results suggest that the addition of IL-15 enhanced cell division slightly and the blocking of IL-15 retarded the majority of GC-B cells by at least one round of cell division behind that of the controls. Therefore, the overall increase in the recovery of viable cells in the presence of IL-15 can be mainly attributed to the proproliferative effect rather than antiapoptotic effect. However, the possibility of antiapoptotic effects on GC-B cells cannot be ruled out completely because the 14.7% difference in the most divided subpopulation between cultures with and without additional IL-15 does not appear to be sufficient to explain the ~30% difference in recovery between them.

The amount of IL-15 on the FDC/HK surface is increased by coculturing with GC-B cells or by the addition of TNF-α

Because the GC is formed by cellular interactions between B cells, T cells, and FDCs (3), we examined whether the amounts of IL-15 on the surface of FDC/HK cells can be modified by various GC factors.

Because FDC/HK cells are known to express CD40 (54), we first compared surface IL-15 levels after incubating FDC/HK cells in the presence or absence of CD40L. The IL-15 levels of FDC/HK cells cultured with GC-B cells and cytokines were also compared (Fig. 7). Compared with culture without cytokines (media), the surface-bound IL-15 on FDC/HK cells was not increased by incubating them with IL-2, IL-4, and CD40L (24L), unless GC-B cells were included in the culture. The amount of surface IL-15 on FDC/HK cells was dramatically increased when FDC/HK cells were cultured with GC-B cells (24L + GCB, MFI: 290 from 139). Hence, signals exclusively from GC-B cells, but not from CD40L and cytokines, modulated the amount of IL-15 on FDC/HK cells. Because TNF-α has been reported to be an essential GC-B cell factor for FDCs in network formation in vivo (16, 57, 58), we examined the effect of TNF-α. The increase in surface IL-15 induced by TNF-α was remarkable (MFI: 438). This result suggests that factors produced by GC-B cells increase the level of surface IL-15 on FDC.

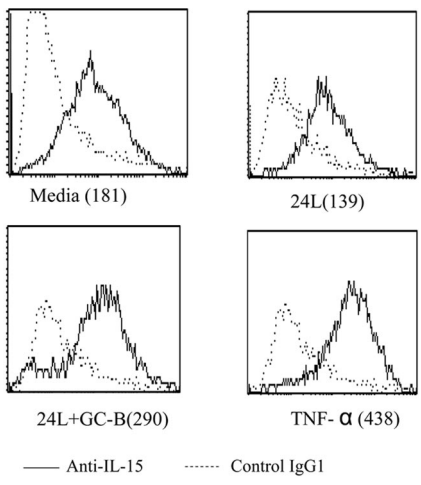

FIGURE 7. IL-15 levels on the surface of FDC/HK are enhanced by GC-B cells or TNF-α. FDC/HK cells were incubated for 3 days in 10% FCS IMDM medium with various induction conditions, as follows: media alone (media), IL-2, IL-4, and CD40L (24L); IL-2, IL-4, and CD40L with GC-B cells (24L + GC-B); TNF-α (10 ng/ml). Harvested cells were stained for FACS analysis. Numbers in the parentheses represent MFI of each sample, which is calculated by subtracting control value from that of specific mAb (broken line and solid line, respectively).

Discussion

The function of IL-15 in B cells has not been studied extensively, because, in part, in genetically modified mice, either eliminating IL-15 or forced expression model does not reveal evident differences in B cell responses compared with wild-type mice (59–61). Accumulating evidences, however, indicate that the biological roles of IL-15 may not solely be confined to T or NK cells, but to B cells. IL-15 is shown to enhance proliferation and Ig secretion of human peripheral B cells (42, 44, 62), to inhibit apoptosis induced by anti-IgM (63), and to induce proliferation of malignant B cells (43, 47). To our knowledge, however, there have been few reports about the function of IL-15 on GC-B cells. We, therefore, investigated the biologic function of IL-15 in GC reaction.

We first examined the cellular source of IL-15 within the GC. Although IL-15 mRNA and small amounts of soluble IL-15 have been reported to be produced by in vitro cultured FDC (64), it is important to confirm the production of IL-15 by FDC at the protein level: IL-15 mRNA is almost ubiquitously expressed, and the production and secretion of protein are mainly controlled by complex and inefficient posttranslational mechanisms (33, 34). Our data reveal that FDCs produce IL-15, as shown by the immunofluorescence staining of freshly isolated FDC clusters. This in vivo observation was confirmed by showing that a FDC cell line, FDC/HK cells, indeed produced IL-15. IL-15 protein was detected on the surface of FDC/HK cells. The specificity of membrane-bound IL-15 was confirmed by competition FACS analysis and by the blocking experiment of CTLL-2 bioassay. However, IL-15 was not detected by ELISA in the FDC/HK culture supernatant, and it was further confirmed with the CTLL-2 assay. The presence of membrane-bound IL-15 on FDCs in place of secreted protein is not uncommon. Various cell types, such as monocytes, fibroblasts, epithelial cells, and leukemic progenitors, have been reported to express membrane-bound IL-15 in the absence of detectable soluble protein (52, 65–67). In fact, IL-15 protein is rarely detected in culture supernatant or in tissues (68), due in part to the poor translation and short half-life of the cytokine. It is also possible that detection is difficult because IL-15 is usually bound to IL-15Rα. The membrane form is known to be biologically active, more potent than the soluble form (52, 66). The bioactivity of IL-15 on the FDC surface was also demonstrated by the CTLL-2 assay.

There are three possible mechanisms that could result in surface IL-15 expression: binding of secreted IL-15 by IL-15Rα (40), binding to a novel receptor as reported for mast cells (69), and expression of a new variant of IL-15 protein that contains a membrane-spanning region (52, 67). The complete loss of IL-15 staining after acid treatment and enhanced binding after incubation with exogenous IL-15 strongly suggest a receptor-anchored mechanism rather than the presence of an alternative membrane form of IL-15. Although the possibility that failure to detect IL-15 after acid treatment resulted from denaturation of transmembrane form cannot be ruled out completely, expression of specific mRNA for IL-15Rα in FDC/HK cells favors the first hypothesis.

Second, we investigated the biologic relevance of IL-15 signaling in the GC by examining the effect of IL-15 on GC-B cell proliferation by the removal or addition of IL-15. As shown in Fig. 5, GC-B cell growth was decreased significantly in the presence of anti-IL-15-blocking mAb and was enhanced when IL-15 was

added. Recovery of GC-B cells in the culture containing a saturating dose of IL-15 (100 ng/ml) was 4-fold higher than that of the culture where the activity of endogenous IL-15 was depleted by blocking mAb. The significance of IL-15 signaling in the GC was further supported by the observation that the amount of membrane-bound IL-15 was significantly modulated by GC-B cell factors. Given the dynamic nature of GC reactions, the presence of a positive feedback mechanism between GC-B cells and FDCs is especially plausible. To meet the urgent need for the production of high affinity protective Abs, a vigorous expansion of a few precursors having appropriate BCR in the initial phase of GC reaction requires a positive feedback amplification of proproliferative stimuli such as IL-15. Taken together, these results strongly suggest the presence of IL-15 signaling mechanism in the GC.

This enhancement of GC-B cell proliferation by IL-15 on FDC/HK can be attributed to indirect effect on FDCs. IL-15 on dendritic cells is reported to increase costimulatory molecules by autocrine loop (70, 71); thus, blocking IL-15 may lead to alteration of other FDC molecules, which can affect the proliferation and survival of GC-B cells. GC-B cells are entirely dependent on the combination of variety of soluble and cell surface factors from HK cells for their survival and proliferation; it is very difficult to investigate the effect of only the membrane-bound IL-15 of HK cells on GC-B cells using conventional experimental systems for membrane-bound molecules such as transwell experiments or paraformaldehyde fixation, which result in massive apoptosis of GC-B cells.

Although the indirect effect via changes in molecule of FDCs cannot be ruled out, direct effect of IL-15 has plausible implication in regard to GC reaction. Recent data suggest that IL-15Rα not only acts as a component of IL-15R complex for the high affinity binding, but can also act to present IL-15 to adjacent cells in vitro and in vivo (40, 41, 65, 67, 72). In the GC reaction, effective delivery of survival and proliferative signals only to appropriate clones having higher affinity BCR is essential so as not to expand overwhelming numbers of clones with low affinity BCR. Therefore, provision of cosignals, e.g., IL-15, from the microenvironment in a membrane-bound form may be advantageous in ensuring selective proliferation and prevention of nonspecific activation via bystander effects. It is also conceivable that direct IL-15 effect on GC-B cells and indirect effect through FDC by IL-15 autocrine loop may work together to accelerate GC reaction in vivo.

Vigorous proliferation of centroblasts within the stromal environment created by FDCs is a hallmark of GC reaction (2, 3). However, factors that are responsible for this rapid cell division have not been identified. Many cytokines have been reported to augment human GC-B cell proliferation in vitro, one of the most potent being IL-2 (6, 73). In the absence of IL-2, GC-B cell recovery has been shown to be decreased by at least 3-fold (6). However, activated T cells that produce IL-2 are not common in the dark zone of the GC (74), and centroblasts do not express IL-2Rα, which is essential for binding IL-2 with high affinity (75). In this study, we showed that IL-15 is present on FDC in the GC in vivo and that endogenous IL-15 from FDC/HK cells supported GC-B cell proliferation in vitro as comparable as or more than exogenous IL-2 alone when endogenous IL-15 was removed by blocking Ab (4.2×10^5 in Fig. 5*A*, *left first bar* vs 2.9×10^5 in Fig. 5*B*, *right end bar*). Moreover, GC-B cells proliferate in the presence of IL-15, dividing faster than the cells cultured without IL-15. Together, these results imply that IL-15 signaling may be one of the mechanisms responsible for the rapid proliferation of centroblasts in the GC in vivo.

In summary, IL-15 from FDC, in a membrane-bound form, plays an important role in supporting GC-B cell proliferation, proposing a new target for immune modulation as well as treatment of B cell lymphoma because IL-15 presentation by FDC may be an important trigger in the initiation of lymphomagenesis.

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