Wnt5a Is Secreted by Follicular Dendritic Cells To Protect Germinal Center B Cells via Wnt/Ca²⁺/NFAT/NF-κB–B Cell Lymphoma 6 Signaling

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Wnt5a Is Secreted by Follicular Dendritic Cells To Protect Germinal Center B Cells via Wnt/Ca\(^{2+}\)/NFAT/NF-κB–B Cell Lymphoma 6 Signaling

Jungtae Kim,* Dong Wook Kim,* Wookyoung Chang,* Jongseon Choe, † Jihun Kim,‡ Chan-Sik Park, ‡ Kyuyoung Song, § and Inchul Lee‡

Follicular dendritic cells (FDCs) protect germinal center (GC) B cells from rapid apoptosis to allow their survival and maturation. In this article, we show that FDCs normally produce and secrete Wnt5a to protect GC B cells. Wnt5a production is upregulated by polyI:C. Purified Wnt5a protects GC B cells from apoptosis in a dose-dependent manner. GC B cells are protected by FDC coculture or conditioned medium, and the protection is inhibited significantly by anti-Wnt5a Ab, suggesting a major role of Wnt5a in the FDC-mediated GC B cell protection. A calcium chelator BAPTA-AM blocks the Wnt5a-mediated GC B cell protection, implying a role of Wnt5a in the FDC-mediated GC B cell protection. In vivo, supporting a critical biological role in the development and regulation of B cell immune system (11). CD40-CD154 signaling activates NF-κB, which has been implicated in the protection of centrocytes from apoptosis (11–13). NF-κB is often activated constitutively in a subtype of diffuse large B cell lymphomas (14).

B cell lymphoma 6 (BCL-6) is a transcriptional regulator highly expressed in GC B cells (15, 16). BCL-6 plays a major role in the survival of GC B cells, particularly of centroblasts in which NF-κB is not activated (1, 2). The abnormal activation of BCL-6 by chromosomal translocation is frequently associated with a subtype of diffuse large B cell lymphoma (14). BCL-6 suppresses the expression of proapoptotic p53 (17) and the DNA-damage sensor ataxia telangiectasia and Rad3 related (18). BCL-6 also suppresses the transcription of positive regulatory domain-containing 1 (Prdm1), which encodes B lymphocyte-induced maturation protein 1 (BLIMP1). BLIMP1, in turn, suppresses BCL-6 transcription reciprocally, leading to plasma cell differentiation (15, 16). BCL-6 transcription in GC B cells is also suppressed by IFN regulatory factor 4 (IRF4) (19). NF-κB upregulates IRF4 expression, and thus suppresses BCL-6 transcription. However, BCL-6 is mostly activated by posttranslational modifications, whereas the transcriptional regulation appears to have a limited biological role (16).

Wnt signaling is an essential transcriptional regulatory mechanism that is well conserved in multicellular organisms for critical biological functions including normal development and stem cell regulation (reviewed in Refs. 20–23). The signaling is initiated by binding of Wnts to complex Frizzled receptors. Canonical Wnt/β-catenin signaling is characterized by a Wnt-dependent inhibition of glycogen synthase kinase-3β, leading to stabilization and nuclear translocation of β-catenin as a transcriptional factor through interactions with T-cell factor/lymphoid enhancer factor.
Noncanonical β-catenin–independent signaling, which is typically initiated by Wnt5a, does not require β-catenin for the downstream regulation (24–26).

Two major routes of β-catenin–independent signaling have been described including the Wnt/Ca2+ and Wnt/PCP (planar cell polarity) pathways (24, 25). In the Wnt/Ca2+ pathway, cytoplasmic free calcium regulates calcium-dependent downstream signaling as second messenger. Wnt/PCP signaling activates small GTPases, heterotrimeric G proteins, and JNK. It is required for the generation of uniform orientation of a population of cells within a single epithelial plane in Drosophila and vertebral gastrulation (24–26).

Recently, β-catenin–independent Wnt signaling has been implicated in the inflammatory regulation. In synovial cells of rheumatoid arthritis, the expression of Wnt5a and frizzled 5 is enhanced significantly (27), and the blockade of the signaling inhibits synovial cell activation (28). Wnt5a is expressed by activated macrophages and endothelial cells (29, 30). Wnt5a then activates endothelial cells, suggesting a paracrine and/or autocrine activation (30). Wnt5a is detectable in the sera of patients with severe sepsis (31).

The inflammatory regulation by Wnt5a suggests a potential role of Wnt5a in the immune regulation. Wnt signaling is highly dependent on the cell context (32). Wnt/β-catenin signaling has been implicated in the B cell regulation (33, 34), whereas the role of noncanonical Wnt signaling has been controversial. Wnt5a has been reported to regulate B cell proliferation negatively in Wnt5a targeted mice (35), but it increases B cell lymphopoiesis in bone marrow cell culture (36). The role of Wnt5a on the regulation of GC B cells has not been reported. In this article, we show that FDCs express Wnt5a actively and Wnt5a protects isolated GC B cells from apoptosis via β-catenin–independent signaling.

Materials and Methods

Reagents

Purified mouse recombinant Wnt5a protein was purchased from Millipore (Billerica, MA). It was purified from conditioned media using blue Sepharose, gel filtration, and heparin affinity chromatography. The endotoxin level in the lot used was negligible, <0.15 EU/μg (=0.015 ng/μg Wnt5a). Purified human TNF-α protein, BAPTA-AM, SP600125, and A23187 were purchased from Sigma (St. Louis, MO), and polyI:C was from InvivoGen (San Diego, CA). Goat anti-Wnt5a antiserum was purchased from R&D Systems (Minneapolis, MN). Mouse mAbs against RelA, RelB, NF-κB p50, p52, and β-catenin, and rabbit anti-JNK Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAbs against CD20, CD38, CD45RA, and CD45RO were purchased from BD Biosciences (San Jose, CA). Mouse mAb 1B10 (37) was purchased from Oxford Biotechnology (Kidlington, U.K.). Mouse mAb against β-actin was purchased from Sigma. Mouse mAb against p-JNK, target site T183/Y185 of JNK1 and JNK2, was purchased from Cell Signaling Technology (Danvers, MA). Mouse mAb 3C8 was prepared as described previously (38).

FIGURE 1. FDCs express and secrete Wnt5a actively. A, Real-time PCR of Wnt5a expression in FDCs, HEK293, THP-1, Ramos, GC B cells, and human blood monocytes. Measurements are done in triplicate using SYBR green real-time PCR and β-actin as endogenous control. Relative fold differences compared with FDCs are presented as means ± SD. B, Real-time PCR of BCL-6 in FDCs, HEK293, THP-1, Ramos, and GC B cells. Measurements are done in triplicate and relative fold differences are presented compared with FDCs as means ± SD. C, Western blotting of cytoplasmic proteins from murine control L cells, L-Wnt5a cell expressing Wnt5a, and FDCs using β-actin as loading control. Wnt5a is shown at 45 kDa. Relative densitometric expression folds compared with FDCs are presented graphically. Statistical significance is given as ***p < 0.001. D, Western blotting of Wnt5a in the culture media of L-Wnt5a and FDCs. Cells of the same number are cultured for 24 h, and 20 μg total proteins are loaded per lane. A nonspecific band from culture media is shown above Wnt5a. Relative densitometric folds are presented in comparison with FDCs.
Grand Island, NY) supplemented with 10% heat-inactivated FBS, 2 mM

glucose, and penicillin/streptomycin. Cultures were incubated at 37˚C with saturated humidity

The purity was 95%, as assessed by flow cytometry (FACSCalibur; BD Biosciences) for the expression of CD20 and CD38.

The purity was previously set forth by the Institutional Review Board of the Clinical Research Institute, the Asan Medical Center. GC B cells were purified from tonsillar B cells by MACS procedure (Miltenyi Biotech, Auburn, CA), as described previously (8). The purity was >95%, as assessed by flow cytometry (FACSCalibur; BD Biosciences) for the expression of CD20 and CD38. Primary human FDCs and tonsillar mononuclear cells were established from the adherent cells as described previously (37–41). The purity was analyzed using flow cytometry (Supplemental Fig. 1). FDCs exhibited minimal or no expression of CD45RA and CD45RO but displayed FDC-specific markers 3C8 and 1B10 (37, 38), indicating that FDCs did not originate from hematopoietic stem cells.

GC B and FDC preparation

GC B cells and FDCs were prepared from human tonsils. All samples were obtained with written informed consent in accordance with the guidelines set forth by the Institutional Review Board of the Clinical Research Institute, the Asan Medical Center. GC B cells were purified from tonsillar B cells by MACS procedure (Miltenyi Biotech, Auburn, CA), as described previously (8). The purity was >95%, as assessed by flow cytometry (FACSCalibur; BD Biosciences) for the expression of CD20 and CD38. Primary human FDCs and tonsillar mononuclear cells were established from the adherent cells as described previously (37–41). The purity was analyzed using flow cytometry (Supplemental Fig. 1). FDCs exhibited minimal or no expression of CD45RA and CD45RO but displayed FDC-specific markers 3C8 and 1B10 (37, 38), indicating that FDCs did not originate from hematopoietic stem cells.

GC B and FDCs were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated FBS, 2 mM t-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin G, and 100 µg/ml streptomycin. Cultures were incubated at 37˚C with saturated humidity and 5% CO2. Experiments were done using FDCs of passages 7–9, except for the analysis of high passage cells. PBMCs were obtained from healthy volunteers using Ficoll-Hypaque centrifugation.

Cell lines

Human Burkitt B lymphoma line Ramos was cultured in RPMI 1640, 0.5 × 105 cells/ml, supplemented with 10% FBS and penicillin/streptomycin, and subcultured every 2 d. THP-1 cells were also cultured in RPMI 1640 supplemented with 10% FBS and penicillin/streptomycin. L-Wnt5a cells and control L cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM (Life Technologies) with 4 mM t-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and 4.5 g/l glucose supplemented with 10% FBS and 0.6 mg/ml G-418. HEK293 cells were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin.

GC B cell protection assay

For the protection assay, 2 × 107/well GC B cells were seeded in 24-well plates with or without 2 × 105/well FDCs. All assays of a given set were performed simultaneously using GC B cells from a single donor. Cells were incubated in RPMI 1640 supplemented with 10% FBS for 24 h or without FBS for 6 h. GC B cells were then harvested, and viable cells were counted in a hemocytometer chamber with trypan blue staining. Alternatively, GC B cells were incubated in the FDC-conditioned media for 24 h.

### Table I. PCR primers for real-time PCR

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#### FIGURE 2.

Regulation of Wnt5a expression in FDCs and lymphoid tissue. A. Real-time PCR of Wnt5a expression in FDCs treated using 10 and 100 ng/ml TNF-α for 6 h compared with untreated control. Experiments are repeated in triplicate and relative fold differences are presented as means ± SD. B. Real-time PCR of Wnt5a expression in FDCs treated using 1 and 5 µg/ml polyI:C for 6 h. Experiments are repeated in triplicate. Statistical significance is given as *p < 0.05, **p < 0.01, ***p < 0.001. C. Western blotting for Wnt5a protein expression in whole cell proteins of FDCs treated using 5 µg/ml polyI:C for 6 h. Relative densitometric staining compared with untreated control are presented using β-actin as loading control. D. Immunohistochemical staining for Wnt5a in a hyperplastic lymph node removed from chin of a 10-y-old boy for soft tissue inflammation. In the GC, scattered FDC-like pleomorphic cells are immunostained intensely (arrows). Scale bar, 100 µm. E. Immunohistochemical staining for Wnt5a in a normal perigastric lymph node removed from a 64-y-old woman for gastric cancer surgery. No metastasis is present. F. Real-time PCR of Wnt5a expression in FDCs of different passages. The relative expression of passage 15 cells is compared with passage 8 cells using β-actin as endogenous control. Experiments are repeated in triplicate and relative fold differences are presented as means ± SD.

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To investigate the role of Wnt5a in the FDC coculture or FDC-conditioned media, 2 ng/ml monoclonal anti-Wnt5a Ab was applied as indicated. For a control Ab, isotype-matched anti-p65 Ab was used similarly. To analyze the effect of exogenous Wnt5a, 10 or 50 ng/ml Wnt5a was applied to GC B cells without FDCs. For the inhibitor assay, 1 μM BAPTA-AM or 10 μM SP600125 was added to GC B cells incubated with or without 50 ng/ml Wnt5a.

Quantitative RT-PCR

Quantitative real-time PCR was done as described previously (30, 41). After cell harvest, total RNA was extracted using TRIzol reagent (Invitrogen), and cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen). Quantitative PCR was performed using a continuous fluorescence detecting thermal cycler ABI 7900HT Fast Real-Time PCR System (ABI, Foster city, CA) and a SYBR green real-time PCR master mix (Toyobo, Osaka, Japan). Measurements were done in triplicate using ECL method (Amersham Biosciences).

Western blotting

Whole or fractionated cell protein samples were analyzed. Nuclear and cytoplasmic fractions were separated using a fractionation kit from BioVision (Mountain View, CA). Samples were solubilized in lysis buffer, and loaded, 20 μg/lane, on 12% SDS-PAGE. Proteins were blotted onto nitrocellulose membrane and probed using primary Abs and appropriate second Abs (Amersham Biosciences, Piscataway, NJ). Blots were washed and visualized using ECL method (Amersham Biosciences).

Immunohistochemical staining

Five human lymph nodes and a hyperplastic tonsil were investigated. They included two hyperplastic lymph nodes from 10- and 17-y-old subjects, and three normal lymph nodes from 40- to 64-y-old subjects taken from tumor surgeries. No tumor was present in lymph nodes. Immunohistochemical staining was done using a Benchmark autostainer (Ventana Medical Systems, Tucson, AZ), as described previously (30). Slides were not heated over 65˚C without Ag retrieval pretreatment. Anti-Wnt5a antiserum was applied at 1:200 dilution. This study was approved by the Institutional Review Board of Asan Medical Center and was done following the guidelines.

Statistical methods

All data and measurements were presented as the mean ± SD. The statistical significance was determined using ANOVA test.

Results

FDCs produce and secrete Wnt5a actively

We investigated the expression of Wnt5a in FDCs, isolated GC B cells, blood monocytes, monotypic THP-1 cells, GC B-like Ramos cells, and HEK293 cells using real-time PCR. FDCs expressed Wnt5a much more than GC B, THP-1, Ramos, and HEK293 cells did (Fig. 1A). PCR primers are summarized in Table I. Wnt5a expression was not detectable in peripheral blood monocytes. GC B and Ramos cells instead expressed BCL-6 much more than FDCs did (Fig. 1B). FDCs did not express Wnt3a, which typically induces canonical β-catenin–dependent signaling (data not shown).

The expression of Wnt5a protein was investigated using Western blotting of whole cell proteins of FDCs. L-Wnt5a cell, a murine cell line stably transfected with Wnt5a for producing Wnt5a-conditioned media, and control L cell were used as positive and negative controls, respectively. FDCs expressed Wnt5a protein comparable with the expression in L-Wnt5a cells using β-actin as loading control (Fig. 1C). We then investigated Wnt5a secreted in the 24-h culture media of the same number of FDCs and L-Wnt5a. Upon Western blotting, Wnt5a was present in the culture media of FDCs comparable with the L-Wnt5a cell media (Fig. 1D), indicating that FDCs produced and secreted Wnt5a actively.

Regulation of Wnt5a production in FDCs

It has been reported that Wnt5a is induced by the activation of macrophages and endothelial cells (29–31). We then investigated the regulation of Wnt5a expression in FDCs by inflammatory mediators using real-time PCR. Poly(I:C) and TNF-α enhanced the expression of TC1, a proinflammatory mediator (41), in 6 h (Supplemental Fig. 2A, 2B), supporting that FDCs are activated by the mediators. TNF-α also upregulated VCAM1 dose dependently (Supplemental Fig. 2C). However, Wnt5a was not upregulated by TNF-α up to 100 ng/ml (Fig. 2A). In contrast, poly:C upregulated Wnt5a transcription in a dose-dependent manner (Fig. 2B). Upon Western blotting, Wnt5a protein was also upregulated compared to control FDC-conditioned media (Fig. 2C).
with untreated control (Fig. 2C). Together, our data suggested that the expression of Wnt5a was not regulated by nonspecific inflammatory activation of FDCs, but a complex regulatory mechanism including TLR3-mediated signaling.

**Wnt5a expression in lymphoid tissue in vivo**

The expression of Wnt5a in human lymphoid tissue in vivo was investigated using immunohistochemical staining. The lymphoid tissue was diffusely immunostained for Wnt5a. GCs tended to be immunostained stronger than the mantle zone and parafollicular area (Fig. 2D, 2E). The expression of Wnt5a varied considerably among subjects. In hyperplastic lymph nodes and tonsils, Wnt5a was expressed strongly in FDC-like pleomorphic cells in GCs (Fig. 2D). Normal lymph nodes showed relatively weak Wnt5a expression in scattered FDC-like cells (Fig. 2E).

**Low-passage FDCs express more Wnt5a than high-passage cells**

We then investigated the expression of Wnt5a in purified FDCs of different passages using real-time PCR. Wnt5a expression was much higher, four to five times, in FDCs of passage 8 than of passage 15 (Fig. 2F). The passage 15 cells grew slowly, showing cytological characteristics of replicative senescence (data not shown).

**Wnt5a protects isolated GC B cells from apoptosis**

We then questioned whether Wnt5a was implicated in the FDC-mediated GC B cell protection. First, isolated GC B cell death was investigated under a serum-deprived condition. Because 2 × 10^7/well isolated GC B cells were incubated without FBS, they underwent apoptosis rapidly, leaving 1% of the seeded population alive in 6 h (Fig. 3A, column 4). In comparison, coculture of 2 × 10^7/well FDCs protected GC B cells significantly (p < 0.001, column 1). To analyze the role of Wnt5a in FDC coculture, cells were incubated with 2 ng/ml monoclonal anti-Wnt5a Ab or isotype-matched control Ab similarly. Anti-Wnt5a Ab inhibited the FDC-mediated GC B cell protection significantly (column 2), whereas control Ab did not (column 3). The survival of GC B cells with FDC and anti-Wnt5a Ab (column 2) was not significantly different from that of GC B cells alone (p > 0.2), suggesting that Wnt5a played a significant role in the GC B cell protection mediated by FDC coculture. Exogenous Wnt5a up to 50 ng/ml protected GC B cells in a dose-dependent manner (columns 4–6).

Similar results were obtained in repeated experiments using 24-h culture supplemented with 10% FBS (Fig. 3B). Most GC B cells incubated alone underwent apoptosis in 24 h (column 7). FDC coculture protected GC B cells (p < 0.001, column 1), and the protection was significantly inhibited by anti-Wnt5a Ab but not by control Ab (columns 2, 3). Exogenous Wnt5a protected GC B cells in a dose-dependent manner (columns 7–9). FDC-conditioned media protected GC B cells significantly (columns 4, 7), and the protection was inhibited by anti-Wnt5a Ab but not by control Ab (columns 4–6). Anti-Wnt5a Ab inhibited the GC B cell protection by FDC coculture and conditioned media so that the survival of GC B cells (columns 2, 5) was not significantly different from GC B cell alone control (column 7). Our data together supported that Wnt5a secreted by FDCs was a major survival factor for GC B cells. FDC coculture protected GC B cells more efficiently than FDC-conditioned media did under our experimental condition (p < 0.05, columns 1, 4), suggesting that a direct physical contact might also have a role in the GC B cell protection.

**Wnt5a protects GC B cells via Wnt/Ca²⁺ signaling**

To investigate the signaling pathway induced by Wnt5a, we treated GC B cells using a cell membrane-permeable calcium chelator BAPTA-AM or JNK inhibitor SP600125. Under the serum-deprived condition in 6 h, 50 ng/ml Wnt5a protected GC B cells from apoptosis (Fig. 3C, columns 1, 2). The Wnt5a-induced GC B cell protection was blocked completely by 1 μM BAPTA-AM (p < 0.001, columns 3, 4), whereas 10 μM SP600125 did not affect the protection (columns 5, 6). Our data together suggested a major role of Wnt/Ca²⁺ signaling in the Wnt5a-induced GC B cell survival.

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**FIGURE 4.** Downstream regulation by Wnt5a in GC B cells. A, Real-time PCR measurements of CD40 expression in GC B cells stimulated with 50 ng/ml Wnt5a for 1 and 4 h compared with the untreated controls. Independent experiments are performed in triplicate, and relative fold differences are presented as means ± SD. B, Western blotting of CD40 in the cytoplasmic fraction of GC B-like Ramos cells treated using 50 ng/ml Wnt5a for 0.5 and 1 h. Relative densitometric expression folds compared with the untreated control are presented using β-actin as loading control. C–E, Real-time PCR measurements of IRF4, BCL-6, and PRDM1 expression in GC B cells treated with 50 ng/ml Wnt5a for 1 and 4 h compared with the untreated controls. Experiments are repeated in triplicate and relative fold differences are presented as means ± SD. Statistical difference is given as *p < 0.05, **p < 0.01, ***p < 0.001.
We also analyzed whether JNK was activated by Wnt5a in Ramos cells using phosphorylated JNK-specific Ab. JNK phosphorylation was not induced but rather inhibited by Wnt5a treatment for an hour (Supplemental Fig. 3).

**Wnt5a does not upregulate reported GC B cell survival factors in FDCs**

So far, several FDC-secreted GC B cell survival factors have been reported (6–9). We then investigated whether Wnt5a regulated the transcription of them. BAFF, IL-6, CXCL13, and IL-15 were not upregulated by Wnt5a treatment for 6 h (data not shown). FDCs did not express detectable CD154 (data not shown).

To investigate whether Wnt5a induced inflammatory activation of FDCs, the expression of COX-2 and TC1 was measured in FDCs treated with Wnt5a up to 100 ng/ml. Wnt5a did not upregulate but rather downregulated COX-2 and TC1 transcription in 6 h (Supplemental Fig. 4A, 4B), supporting that Wnt5a did not activate FDCs themselves.

**Wnt5a upregulates CD40 and IRF4 mRNA expression in GC B cells**

Wnt signaling regulates downstream genes promptly. We investigated the Wnt5a-induced transcriptional regulation of downstream genes in GC B cells. Upon real-time PCR measurement, 50 ng/ml Wnt5a upregulated the expression of CD40 more than three times the baseline level in an hour (Fig. 4A), supporting prompt activation of GC B cells. The expression of CD40 protein was also investigated in Ramos cells treated with Wnt5a for an hour. Wnt5a enhanced the CD40 expression continuously for an hour upon Western blotting using β-actin loading control (Fig. 4B).

IRF4 is a B cell-specific transcriptional regulator critical for GC formation (42). Wnt5a upregulated IRF4 promptly (Fig. 4C).

**FIGURE 5.** Activation of transcriptional regulators by Wnt/Ca²⁺ signaling. A. Western blotting of the nuclear fraction of Ramos cells stimulated using 50 ng/ml Wnt5a for 0.5 and 1 h without (left column) or with 10 μM CsA pretreatment for 10 min (right column). Relative densitometric folds of NFATc2 and BCL-6 expressions are presented between Wnt5a and CsA-Wnt5a treatments using β-actin as loading controls. B. The nuclear fraction of Ramos cells treated using 0.1 mM A23187, a calcium ionophore, are analyzed by Western blotting similarly without (left column) or with 10 μM CsA (right column). Relative densitometric folds of BCL-6 between A23187 and CsA-A23187 treatments are presented using β-actin as loading controls. Statistical difference is given as **p < 0.01, ***p < 0.001.
Wnt5a downregulated BCL6 mildly (Fig. 4D) but upregulated PRDM1 (Fig. 4E), suggesting an IRF4-mediated transcriptional regulation (19).

**Wnt5a activates NFAT, NF-kB, and BCL-6 in Ramos cells**

We then investigated the activation of transcriptional regulators by Wnt5a in GC B-like Ramos cells. NFATc1 and NFATc2 have been implicated in the immune regulation (43, 44). Nuclear NFATc1 and NFATc2 were upregulated robustly by 50 ng/ml Wnt5a in 30 min compared with untreated controls (Fig. 5A, left column). RelB, a major mediator of noncanonical NF-kB signaling in lymphoid cells (45), was upregulated promptly and then downregulated in an hour. RelA(p65) and NF-kB1(p50) were also upregulated mildly in 30 min, suggesting activation of both noncanonical and canonical NF-kB pathways. BCL-6 was also activated mildly. NF-kB2(p52) and TP53 did not change significantly.

**NFAT is a major transcriptional regulator in Wnt/Ca^{2+} signaling**

The robust Wnt5a-induced activation suggested a major role of NFAT in the regulation of GC B cells. To investigate whether NFAT regulated NF-kB and BCL-6, Ramos cells were pretreated using 10 μM cyclosporine A (CsA), an inhibitor of calcineurin that activates NFAT, for 10 min before Wnt5a treatment. CsA blocked the Wnt5a-induced nuclear translocation of NFATc1 (Fig. 5A, right column). NFATc2 activation was also inhibited significantly. CsA also blocked the Wnt5a-induced activation of RelB, RelA, and BCL-6 (Fig. 5A, right column), suggesting that the Wnt5a-induced activation was mediated by NFAT.

Our GC B cell protection data suggested a major role of Wnt/Ca^{2+} signaling (Fig. 3C). To investigate the role of Ca^{2+} signaling, we treated Ramos cells using 0.1 μM A23187, a calcium ionophore. Nuclear NFATc1, NFATc2, RelB, and RelA were upregulated robustly in 30 min and then reduced significantly in an hour (Fig. 5B, left column), similarly to the Wnt5a-induced regulation (Fig. 5A, left column), supporting a role of Wnt/Ca^{2+} signaling in the activation. BCL-6 was also activated mildly.

CsA also blocked the Ca^{2+}-induced nuclear translocation of NFATc1, NFATc2, RelB, and RelA (Fig. 5B, right column). BCL-6 activation was inhibited mildly by CsA in 30 min upon densitometric measurements using β-actin as loading control. Together, our data supported a major role of NFAT in Wnt/Ca^{2+} signaling of GC B cells (Fig. 6).

**Discussion**

GC B cells are unique in that they proliferate extremely fast and are particularly prone to apoptosis. GC B cells are protected by FDCs. Given the critical importance in B cell immunity, the protection of GC B cells has been investigated intensely. We have shown that FDCs produce and secrete Wnt5a actively. Our gain- and loss-of-function data together support that Wnt5a is a novel survival factor for GC B cells provided by FDCs. GC B cells are protected by purified Wnt5a in a dose-dependent manner. The GC B cell survival by FDC coculture and conditioned media is inhibited by anti-Wnt5a Ab similarly, supporting a major role of Wnt5a in the FDC-mediated GC B cell survival.

Our data support that FDCs produce and secrete Wnt5a in lymphoid in vivo. Other cell types such as activated macrophages and endothelial cells may also contribute Wnt5a under inflammatory conditions. Given the close spatial proximity between FDCs and GC B cells, however, Wnt5a secreted by FDCs may reach GC B cells readily for a prompt and efficient regulation (Fig. 6). The role of direct physical contact for the GC B cell protection needs to be investigated further.

Wnt5a activates NFAT, NF-kB, and BCL-6 promptly via Wnt/Ca^{2+} signaling in GC B cells (Fig. 6). Calcium ionophore activates NFAT robustly in Ramos cells. NF-kB and BCL-6 are also activated promptly by calcium influx, suggesting a possible direct activation by Ca^{2+} signaling. NF-kB is known to be activated by calcium signaling via protein kinase C-dependent modification (45), whereas the calcium-dependent activation of BCL-6 has not been reported.

Our data support a major role of NFAT in Wnt/Ca^{2+} signaling in GC B cells, suggesting a hierarchy of transcriptional regulators. NFAT may intensify Wnt/Ca^{2+} signaling by activating NF-kB and BCL-6 for the protection of GC B cells. It is not known how NFAT activates NF-kB and BCL-6; however, the prompt inhibition by CsA suggests activation via posttranslational modifications. Wnt5a does not enhance but rather inhibits the transcription of BCL-6 mildly in GC B cells.

NFAT is a well-known target for such effective immunosuppressants as CsA and FK506. It is now clear that NFAT regulates not only T cells but also other immune cells including dendritic cells, B cells, and megakaryocytes (44). However, the role of NFAT in GC B cell regulation has not been characterized well. Our data suggest another potential mode of action of the immunosuppressants via GC B cell regulation. It is of interest that calcineurin-deficient mice develop larger GCs but have reduced plasma cell development and Ag-specific Ab production in T cell-dependent immune response (46). Wnt/Ca^{2+}/NFAT signaling has been implicated in the survival of Bcr-Abl^{+} chronic myeloid leukemia and acute lymphoblastic leukemia cells (47), suggesting a possible role in the survival of various hematopoietic lineages.

The upregulation of CD40 by Wnt/Ca^{2+}/NFAT signaling would enhance CD40 signaling. The enhancement of CD40 signaling by NFAT would protect GC B cells via NF-kB activation (10–13). NF-kB upregulates IRF4 and BLIMP-1, which repress BCL-6 expression. Our data show that Wnt5a/Ca^{2+}/NFAT signaling activates BCL-6 in Ramos cells, supporting an activation via protein modification. The role of CD40 signaling in the Wnt5a-induced BCL-6 regulation needs further investigation.

Our data suggest that Wnt5a is a novel survival factor for GC B cells. Wnt5a signaling might be a target for the regulation of B cell-dependent immunity.
Disclosures

The authors have no financial conflicts of interest.

References