Interaction Between Toll-Like Receptors and Natural Killer Cells in the Destruction of Bile Ducts in Primary Biliary Cirrhosis

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Primary biliary cirrhosis (PBC) is characterized by chronic nonsuppurative destructive cholangitis (CNSDC) associated with destruction of small bile ducts. Although there have been significant advances in the dissection of the adaptive immune response against the mitochondrial autoantigens, there are increasing data that suggest a contribution of innate immune mechanisms in inducing chronic biliary pathology. We have taken advantage of our ability to isolate subpopulations of liver mononuclear cells (LMC) and examined herein the role of Toll-like receptors (TLRs), their ligands, and natural killer (NK) cells in modulating cytotoxic activity against biliary epithelial cells (BECs). In particular, we demonstrate that Toll-like receptor 4 ligand (TLR4-L)-stimulated NK cells destroy autologous BECs in the presence of interferon alpha (IFN-α) synthesized by TLR 3 ligand (TLR3-L)-stimulated monocytes (Mo). Indeed, IFN-α production by hepatic Mo is significantly increased in patients with PBC compared to disease controls. There were also marked increases in the cytotoxic activity of hepatic NK cells from PBC patients compared to NK cells from controls but only when the NK cells were prepared following ligation of both TLR3-L- and TLR4-L-stimulated LMC. These functional data are supported by the immunohistochemical observation of an increased presence of CD56-positive NK cells scattered around destroyed small bile ducts more frequently in liver tissues from PBC patients than controls. *Conclusion:* These data highlight critical differences in the varied roles of Mo and NK cells following TLR3-L and TLR4-L stimulation. (HEPATOLOGY 2011;53:1270-1281)

See Editorial on Page 1076

The cholangitis of primary biliary cirrhosis (PBC) has been called an orchestrated immune attack, including involvement of autoantibodies, $CD4^+$, and $CD8^+$ T cells.[1,2] This concept has led to the thesis that a multilineage response against the immunodominant autoantigen PDC-E2 is an essential component of disease pathogenesis.[3] It is unclear whether the natural history of PBC is "entirely" secondary to adaptive autoimmune responses; epidemiologic analysis has suggested a role of transient exposure

Abbreviations: BEC, biliary epithelial cells; CNSDC, chronic nonsuppurative destructive cholangitis; IFN, interferon; LMN, liver mononuclear cells; mAb, monoclonal antibody; mDC, myeloid dendritic cells; Mo, monocytes; NK cells, natural killer cells; NKT cells, natural killer T cells; PBC, primary biliary cirrhosis; pDC, plasmacytoid dendritic cells; PSC, primary sclerosing cholangitis; TLR, Toll-like receptor; TLR-L, Toll-like receptor ligand; TRAIL, TNF-related apoptosis inducing ligand.

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Received July 22, 2010; accepted January 4, 2011.

Supported a by Grant-in-Aid for Scientific Research(C) (Kakenhi 22590739) and National Institutes of Health grant DK39588.

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DOI 10.1002/hep.24194

Potential conflict of interest: Nothing to report.

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to environmental agents in the etiology of PBC.[4] The data presented herein suggest that innate immune mechanisms contribute to the pathology characteristic of PBC by either accelerating disease or by specific chronic destruction of small bile duct epithelial cells.[5] Indeed, one paradox in PBC has been the relative lack of a therapeutic response to the various immunosuppressive drugs that have been administered to PBC patients, despite the observation that PBC is a model autoimmune disease.[6] A more detailed analysis of the effector mechanisms involved in the pathogenesis of human PBC has led us to suggest that in addition to the documented adaptive autoimmune responses there is also a direct role of innate immune responses in the biliary pathology of PBC.[2,5,7-9]

The studies described herein take advantage of our ability to culture primary human biliary epithelial cells (BEC) *in vitro* as well as to isolate subpopulations of liver infiltrating mononuclear cells.[8,10,11] Although there are significant numbers of natural killer (NK) cells present around small bile ducts, especially during the early stages of PBC,[12] we note that there are NK cells present throughout the disease course. Importantly, we focused on these NK cells and report herein that such NK cells are highly cytotoxic for autologous BEC following ligation of the Toll-like receptor 4 (TLR4) expressed by NK cells in the presence of interferon-α (IFN-α). Furthermore, this function of NK cells is dependent on the activation of monocytes (Mo) by way of TLR3. We submit that activation of Mo and their crosstalk with NK cells contribute to the pathology of PBC. The data supporting this view are the basis of the present report.

Patients and Methods

Subjects and Protocol. A total of 22 explanted liver tissues constitute the present study. Eight of these 22 liver tissues were from patients with PBC, three from patients with hepatitis B virus infection, eight with hepatitis C virus infection, and three with alcoholic liver disease. The term control diseases in this report refers to patients with diseases other than PBC. All patients had endstage liver cirrhosis without detectable signs of other acute liver injury from an unrelated cause. The diagnosis of PBC was based on established criteria[2] and sera from each of these patients had readily detectable high titers of antimitochondrial antibodies.[2] The immunohistochemical studies reported herein were performed on fresh tissue samples from wedge biopsies of 47 patients including 11 normal controls with metastatic liver disease, 14 patients with PBC, 16 with hepatitis C, and six with primary sclerosing cholangitis (PSC). All of the tissues from patients used herein for immunohistological studies were classified as early stage without detectable signs of cirrhosis. Samples were obtained and studied after informed consent of the donor and all experimental protocols were approved by the Research Ethics Committee of Kyushu University and the University of California at Davis. The isolation, verification of purity, and the specific protocols used are described below.

Isolation of Intrahepatic BECs and Liver-Infiltrating Mononuclear Cells (LMCs). The liver mononuclear cell populations were isolated as described in detail by our laboratory.[7] Briefly, liver specimens were first digested with 1 mg/mL of collagenase type I. Cells from the digested tissue were purified using a Ficoll-hypaque gradient to obtain LMC.[9] The LMC were allowed to adhere by incubating the cells overnight in tissue culture plates and an enriched population of adherent cells harvested. This adherent cell population was maintained in tissue culture until the cells reached full confluence, usually by day 14, and the nonadherent cell population aspirated, washed, and cryopreserved in media containing 7.5% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen.

BECs were separated from adherent cells using CD326 (EpCAM) conjugated MicroBeads (Miltenyi Biotec) specific for epithelial cells. Cells were then resuspended in media consisting of a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% fetal calf serum (FCS), epithelial growth factor (10 ng/mL), cholera toxin (10 ng/mL), hydrocortisone (0.4 μg/mL), triiodothyronine (1.3 μg/L), transferrin (5 μg/mL), insulin (5 μg/mL), adenine (24.3 μg/mL), and 10 ng/mL hepatocyte growth factor (R&D systems, Minneapolis, MN) and cultured.[7] The purity of the cells was verified by immunohistochemical examination of an aliquot of these cells for the expression of cytokeratins 7 and 19 using appropriate antibodies (Dako, Glostrup, Denmark) and only cultures that were >90% positive for these cytokeratins and >95% viable (as determined by trypan blue) were used for the studies reported herein. The cultures used in the studies herein were between four to six passages to exclude the possibility for potential loss of phenotype after prolonged *in vitro* culture.

Isolation of T Cells, Mo, NK Cells, Myeloid Dendritic Cells (mDC), Plasmacytoid DC (pDC), and Natural Killer T (NKT) Cells. As reported,[8] the T cells used for the studies were isolated from LMC using a Pan T cell isolation kit II (Miltenyi Biotec).[8]

Similarly the highly enriched population of Mo and NK cells used were purified using Mo and NK cell isolation kits, respectively (Miltenyi Biotec).[8] The purity of the CD3+ T cells, Mo, and NK cells used were >90% as determined by flow cytometric analysis of an aliquot from each isolation. In efforts to ensure the purity of the cell population being studied, the population of T cells, Mo, or NK cells were each harvested separately. In addition, the same assay was performed following depletion of each of the three cell lineages from LMCs in efforts to confirm that the data obtained were indeed the function of the lineage being studied. The mDCs (BDCA-1+), pDC (BDCA-2+), and NKT cells were isolated using the mDC, pDC, and NKT cell isolation kits (Miltenyi Biotec), respectively, which included two magnetic separation steps. The purity of BDCA-1+ mDCs and the CD3+ CD56+ NKT cells were each >80% as determined by flow cytometric analysis of an aliquot of the cell preparation used for the study. An enriched population of mDC and NKT cells were harvested separately and, once again, the same assay was performed following depletion of the specific cell population in efforts to confirm that the function identified was due to the specific cell lineage being studied.

Cytotoxicity Assay Against Autologous BEC. The cytotoxic activity of LMC was assessed using an 8-hour ^{51}Cr release assay using autologous BEC as target cells.[7] Briefly, the detached BECs were labeled with 2 μCi/mL ^{51}Cr (Amersham) overnight, washed 3× in media and 5 × 10^3 ^{51}Cr-labeled cells dispensed into individual wells of a 96-well round-bottom plate. The nonstimulated, the interleukin (IL)-2, or TLR-activated LMCs were added to triplicate wells at an effector to target cell ratio of 20:1 in a total volume of 200 μL of complete RPMI medium. The IL-2-stimulated effector LMCs used for the assay were stimulated for 3 days with IL-2 (100 units/mL) and the TLR-activated LMC comprised of a series of cell cultures incubated with a single or mixture of TLR ligands each at a predetermined optimal concentration of 2-10 μg/mL of the appropriate TLR-L prior to their addition to the target cells. The TLR ligands used included TLR2 ligand (lipoteichoic acid, LTA: TLR2-L), TLR3 ligand (polyinosine-polycytidylic acid, poly (I:C): TLR3-L), TLR4 ligand (lipopolysaccharide, LPS: TLR4-L), TLR5 ligand (Flagellin: TLR5-L), TLR7/8 ligand (CL097: TLR7/8-L), TLR9 ligand type A (ODN2216, CpG type A: TLR9-LA), and TLR9 ligand type B(ODN2006, CpG type B: TLR9-LB). The combination of TLR ligands used for activation of LMC included (1) TLR2-L + the ligands for either TLR3, 4, 5, 7/8, 9-LA, or 9-LB; (2) TLR3-L + the ligands for either TLR4, 5, 7/8, 9-LA, or 9-LB; (3) TLR4-L + ligands for either TLR5, 7/8, 9-LA, or 9-LB; (4) the TLR5-L + the ligands for either 7/8, 9-LA, or 9-LB; (5) TLR7/8-L + the ligands of either 9-LA to TLR9-LB; (6) TLR9-LA + TLR9-LB. The TLR ligands were purchased from Invitrogen (San Diego, CA). Controls consisted of triplicate wells containing target cells cultured in media alone and target cells that were incubated with 10% Triton X-100 to determine spontaneous and maximal ^{51}Cr release, respectively. Following incubation of the cocultures of the effector with target cells for 8 hours, 100 μL of supernatant fluid was collected from each well and counted and the percentage of specific ^{51}Cr release calculated as (cpm of experimental release − cpm of spontaneous release) / (cpm of maximal release − cpm of spontaneous release) × 100). Experiments using the combination of TLR3-L and TLR4-L were performed on aliquots of samples at least three times from each of the patients. As further controls, polymyxin B and chloroquine were used as specific inhibitors of LPS and poly I:C, respectively, for assays involving TLR4 and TLR3-induced activation. Although polymyxin B was added at the time of TLR4 activation, chloroquine was added 2 hours prior to the activation of the TLR3 pathway for the cytotoxicity assay.

Hepatic Mo, T cells, and NK cells were isolated from LMC following in vitro activation with TLR3-L and TLR4-L for 3 days. Subsequently, highly enriched populations of Mo, T cells, NK cells, and LMC depleted of Mo, T cells, and NK cells were assessed for their cytotoxic activity against autologous BEC at an effector-to-target cell ratio of 5:1. Thence enriched populations of NK cells and LMC were stimulated with several combinations of TLR3-L and TLR4-L in the presence of a variety of supernatant fluids prepared as described above. The combinations included (1) activation of the appropriate cell cultures with TLR3-L and TLR4-L in the presence of supernatant of unfractionated LMC; (2) the activation of the appropriate cell cultures with TLR3-L in the presence of supernatant of TLR4-L-activated LMC; (3) activation of the appropriate cell cultures with TLR4-L in the presence of supernatant of TLR3-L-activated LMC; and (4) activation of the appropriate cell cultures with supernatants of TLR3-L and TLR4-L-stimulated LMC. The stimulated NK and LMC were then assessed for cytotoxicity against autologous BEC. Finally, unfractionated LMC and highly enriched populations of mDC, Mo, NKT, or LMC depleted of mDC, Mo. or NKT cells were cultured at 1 × 10^5/200 μL in 96-well plates for 48 hours in the presence of either TLR3-L or supernatant fluids obtained from cultures of NK

Table 1. Primer Sequences Used for Real-Time Polymerase Chain Reaction Analyses

	PCR Product Size	Forward Primer	Reverse Primer
NKG 2D	200	GTCTCAAAATGCCAGCCTTC	TCGAGGCATAGAGTGCACAG
NKp46	258	ATGGGGCTGTTGAATACCAG	TCTCTCCCGAGATCACTTCG
CD94	279	CCACGGAGTAACATCCCATC	GAAGCTGCAGTGAACCATGA
NKG2A	174	TCCATGGGTGACAATGAATG	CTGCAAATGCAAACGCTTTA
FasL	257	TCTACCAGCCAGATGCACAC	CAAGATTGACCCCGGAAGTA
TRAIL	143	GGCAACTCCGTCAGCTCGTTA	GGTCCCAGTTATGTGAGCTGCTA
Granzyme B	257	TCCCTGTGAAAAGACCCATC	TTCGCACTTTCGATCTTCCT

cells stimulated with TLR4-L. The cultures were then assessed for cytotoxicity against autologous BEC.

In efforts to study the influence of IFN-α, an additional cytotoxicity assay was performed in which highly enriched populations of NK cells were stimulated with TLR4-L in the presence or absence of recombinant IFN-α. In parallel, the supernatant fluids from TLR3-L-stimulated Mo in the presence or absence of anti-IFN-α antibody (Abcam) were studied. Similarly, in nested experiments, anti-TNF-related apoptosis inducing ligand (TRAIL) monoclonal antibody (mAb) (R&D Systems, final concentration: 1 μg/mL), anti Fas-L mAb (R&D Systems, final concentration: 1 μg/mL), or Granzyme B inhibitor (BioVision, final concentration: 10 μM) were used in the same cytotoxicity assay in attempts to identify the effector molecules involved. Importantly, each of these experiments was performed on samples from all PBC patients and control liver disease patients at least three times.

IL-12, IL-15, IL-18, and IFN-α Production from Mo. In efforts to identify the nature of the cytokines that were involved in promoting NK cell effector function, supernatants from the TLR3-L-stimulated hepatic Mo cultured for 3 days were analyzed for levels of IL-12, IL-15, IL-18, and IFN-α. These cytokines were selected based on previously published data that reported their involvement in NK cell functional activity.[13] Assays were performed using a sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems), using a combination of unlabeled and biotin- or enzyme-coupled monoclonal antibody to each cytokine. Data reported herein represent results obtained from each of the experiments performed on samples from all patients at least three times.

Isolation and Quantitation of Messenger RNA (mRNA) for Select Markers. Aliquots of NK cells from PBC patients and disease controls were cultured in media alone (unstimulated) or cultured in the presence of TLR4-L, IFN-α, or the combination of TLR4-L and IFN-α for 24 hours. Total RNA was isolated from the cultured NK cells using RNAeasy columns (Qiagen, Valencia, CA) and quantitative analyses carried out utilizing a real-time polymerase chain reaction (PCR) assay using SYBR Green PCR Master Mix (Invitrogen) and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Tokyo, Japan). The relative levels of NKG2D and NKp46 (activating receptors), CD94 and NKG2A (inhibitory receptors), and FasL, TRAIL, and Granzyme B (effector function markers) were determined using the primers noted in Table 1. Data are expressed as the fold-change in levels of mRNA versus unstimulated NK cells.

Immunohistochemical Staining of Human Liver Specimens for CD56 Expression. Deparaffinized and rehydrated sections and frozen sections of liver tissues from 11 normal controls with a diagnosis of metastatic liver disease, 14 patients with PBC, 16 with hepatitis C, and six with PSC were used for the detection of CD56-expressing cells using standard immunostaining. Endogenous peroxidase was blocked using normal goat serum diluted 1:10 (Vector Laboratories, Burlingame, CA) for 20 minutes; CD56 was diluted 1:100 (Dako) and immunostaining was performed on coded sections and the data interpreted by a "blinded" pathologist.

Statistical Analysis. All experiments were performed in triplicate and data points shown are the mean values of results of these triplicates. Comparisons between the points for certain datasets are expressed as mean ± standard deviation (SD), and the significance of differences was determined by Student's *t* test. All analyses were two-tailed and *P*-values <0.05 were considered significant. Statistical analyses were performed using Intercooled Stata 8.0 (StataCorp, College Station, TX).

Results

Autologous BEC Killing Assay by LMC. As noted in Fig. 1A and as expected, LMC when cocultured with autologous BEC demonstrated no detectable cytotoxicity (0.5 ± 4.3%). However, following incubation of LMCs with IL-2 (100 μ/mL) a marked increase in cytotoxic activity against autologous BEC was observed (48.3 ± 9.7%). It is well known that innate immune effector cells can be activated *in vitro*

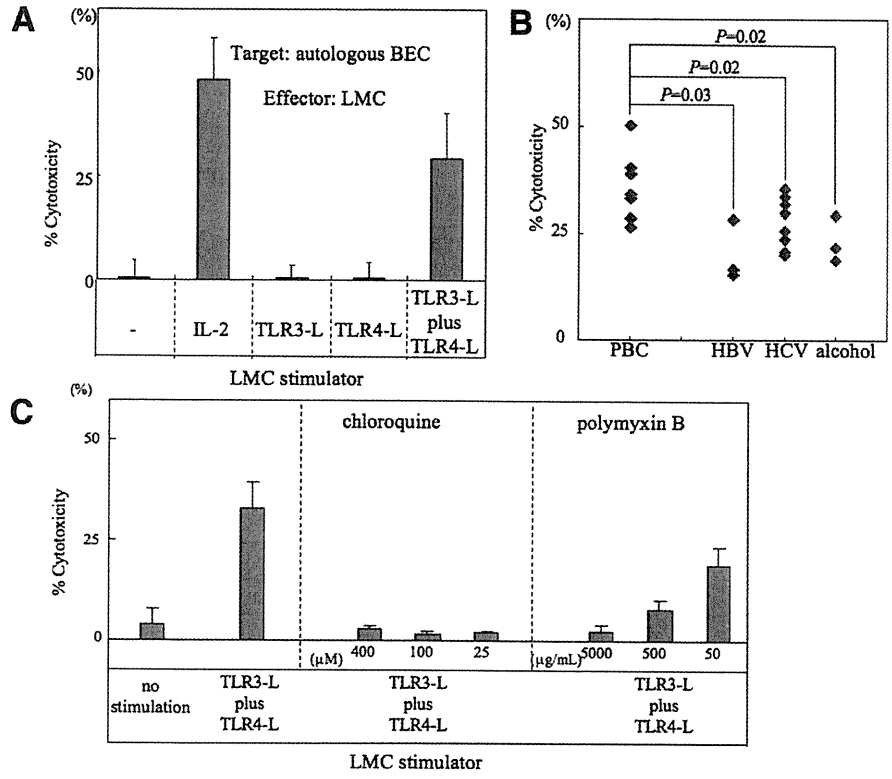

Fig. 1. (A) *In vitro* activation requirements of LMC for cytotoxicity against BEC. LMC isolated from eight patients with PBC and 14 control patients were cultured *in vitro* with either IL-2, TLR3-L alone, TLR4-L alone, or a mixture of TLR3-L+TLR4-L for 3 days and then washed and assayed for cytotoxicity against autologous BEC using the standard ^{51}Cr release assay. LMC cultured in media alone served as a negative control. The assay was performed in triplicate for each activation agent and expressed as mean ± SD. Representative data from one PBC patient are shown. (B) The net cytotoxicity for LMC against BEC was performed. There were statistical differences in the degree of net cytotoxicity induced by TLR3-L and TLR4-L activation of LMC in cells from PBC when compared to other control liver diseases. (C) The use of inhibitors of the TLR3 and TLR4 signaling pathways on the cytotoxicity of activated LMC against autologous BEC. LMC from eight PBC patients and 14 control patients were activated *in vitro* with TLR3-L+TLR4-L in the presence of various concentrations of either chloroquine (TLR3 pathway inhibitor) or polymyxin B (TLR4 pathway inhibitor) and tested for cytotoxicity against autologous BEC. The left panel shows the control cytotoxicity data of LMC cultured in media alone or following activation with TLR3-L and TLR4-L. The middle and right panels reflect data obtained on aliquots of the same LMC activated using TLR3-L and TLR4-L but cultured in the presence of chloroquine or polymyxin B, respectively. Each culture was performed in triplicate and the data shown are mean ± SD. The data shown are from one PBC patient but are representative.

by way of a number of TLR pathways besides IL-2. Thus, we studied a variety of TLR ligands either individually or in various combinations as outlined in Materials and Methods. First, whereas LMC did not demonstrate any detectable cytotoxicity against autologous BEC following ligation of any single TLR ligand (for example, the CTL activity following TLR3-L ligation was 0.5 ± 3.1% and following TLR4 ligation was 0.6 ± 3.9%) (Fig. 1A; Supporting Fig. 1A), use of the combination of TLR3-L and TLR4-L led to significant cytotoxicity against autologous BEC (CTL activity; 29.3 ± 11.1%). Importantly, LMC did not induce significant cytotoxicity against autologous BEC using any other combination of TLR ligands (Supporting Fig. 1B). To exclude the possibility that the cytotoxicity noted using the combination of TLR3-L+TLR4-L was not due to the direct effect of the TLR ligands on BEC instead of LMC, we cocultured BEC with TLR3-L and TLR4-L in a similar cytotoxic assay described above. However, no detectable cytotoxic activity was found (data not shown).

Studies were then carried out to evaluate the differences if any in the cytotoxicity of BEC following TLR3-L and TLR4-L stimulation of LMC from PBC as compared with LMC isolated from other disease controls. The net cytotoxicity of LMCs from PBC patients (n = 8) against BEC was 36.4 ± 7.5. In the case of LMCs from HBV (n = 3), HCV (n = 8), and alcohol-related cirrhosis (n = 3) controls, the net cytotoxicity was 20.2 ± 7.1, 27.7 ± 5.9, and 23.4 ± 5.5, respectively, as shown in Fig. 1B. There were statistical differences in the degree of net cytotoxicity induced by TLR3-L+TLR4-L activation of LMC in cells from PBC when compared to similarly activated LMCs

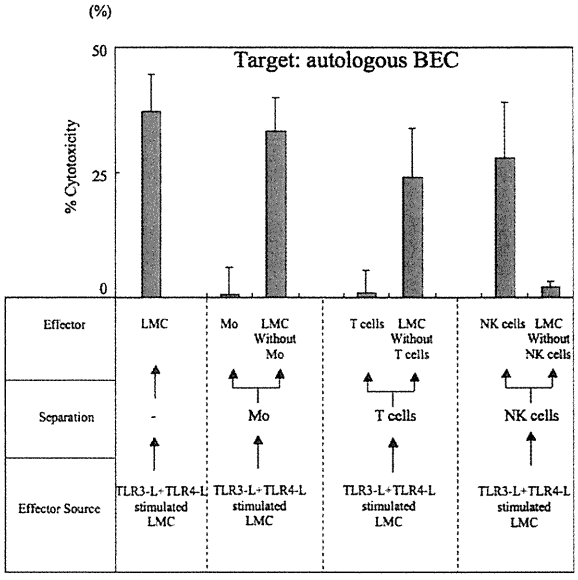

Fig. 2. Identification of the cell lineage within LMC that mediate cytotoxicity against autologous BEC. Cultures of LMC were activated *in vitro* with TLR3-L and TLR4-L and then aliquots assayed for cytotoxicity against autologous BEC (control) or used to isolate or deplete specific cell lineages. Thus, LMC were either enriched for Mo or depleted of Mo, enriched for T cells, or depleted of T cells and enriched for NK cells or depleted of NK cells and each of these tested for cytotoxicity against autologous BEC. Results of mean cytotoxicity (mean ± SD) of data obtained on one PBC patient are displayed.

from other control liver diseases (PBC versus HBV-related cirrhosis: $P = 0.03$, PBC versus HCV-related cirrhosis: $P = 0.02$, PBC versus alcohol-related cirrhosis: $P = 0.02$). Subsequently, in efforts to confirm that the activation by TLR4-L (LPS) and TLR3-L (poly I:C) was indeed induced by way of the respective TLR pathways, use was made of pretreatment of the activation agents with previously defined optimum concentrations of polymyxin B for LPS and chloroquine for poly I:C. As shown in Fig. 1C, polymyxin B inhibited CTL activity in a dose-dependent manner and chloroquine inhibited CTL activity even at the lowest concentration used.

NK Cells Are Cytotoxic for Autologous BEC in the Presence of TLR3-L+TLR4-L-Stimulated LMC. The ability of cells to induce cytotoxic activity against autologous BEC following the ligation of TLR3-L+TLR4-L was next examined. Cultures of LMC, stimulated with TLR3-L+TLR4-L, were used to either isolate enriched populations of Mo, T cells, NK cells, or isolate cultures depleted of each of these cell lineages. These enriched and depleted cell cultures were assessed for their cytotoxicity against autologous BEC. Unfractionated TLR3-L+TLR-4-activated LMC were used for purposes of a positive control. As shown in Fig. 2, whereas Mo did not demonstrate any significant cytotoxicity against autologous BEC (CTL activity; 0.6 ± 5.4%), LMC depleted of Mo demonstrated significant cytotoxicity against autologous BEC (CTL activity; 33.2 ± 6.8%). Similarly, whereas T cells did not demonstrate significant cytotoxicity against autologous BEC (CTL activity; 0.8 ± 4.5%), LMC depleted of T cells had significant cytotoxicity against autologous BEC (CTL activity; 24.0 ± 10.0%). On the other hand, whereas NK cells demonstrated significant cytotoxicity against BEC (CTL activity; 28.0 ± 11.0%), LMC depleted of NK cells did not show significant cytotoxicity against autologous BEC (CTL activity; 2.0 ± 1.1%). These data indicate that it is the NK cell lineage following TLR3-L and TLR4-L stimulation that is responsible for significant cytotoxic activity against autologous BEC. Representative data from one PBC patient is shown in Fig. 2.

TLR4-L-Stimulated NK Cells with Supernatants from TLR3-L-Stimulated LMC Are Cytotoxic for Autologous BEC. In efforts to identify the potential mechanisms by which activation of TLR3-L+TLR4-L in cultures of LMC generate cytotoxic activity of NK cells against autologous BEC, data obtained in preliminary studies showed that the activation of enriched population of NK cells with TLR3-L+TLR4-L did not lead to significant cytotoxicity against autologous BEC (Fig. 3A). These data indicate that the generation of cytotoxic activity against autologous BEC was likely due to the presence of a second population of cells. Experiments were thus carried out to clarify the relationship of NK cells, LMC, TLR3-L, and TLR4-L. We prepared supernatant fluids from LMC cultured in the presence of the appropriate ligands for either TLR3, TLR4, or TLR3+TLR4. As shown in Fig. 3A, NK cells only demonstrated cytotoxicity against autologous BEC when cultured in the presence of TLR4-L and supernatant fluids prepared from TLR3-L-activated LMC (CTL activity; 26.3 ± 11.0%), but not when cultured in the presence of TLR3-L and supernatant fluids prepared from LMC with TLR4-L (CTL activity; 0.2 ± 2.1%). The NK cells, in addition, did not kill autologous BEC in the presence of supernatant from TLR3-L and TLR4-L-stimulated LMC (CTL activity; 0.8 ± 2.8%) as shown in Fig. 3A. These data indicate that NK cells cytotoxicity against autologous BEC requires not only the activation of TLR4-L but also cytokines that are synthesized by LMC upon TLR3-L activation.

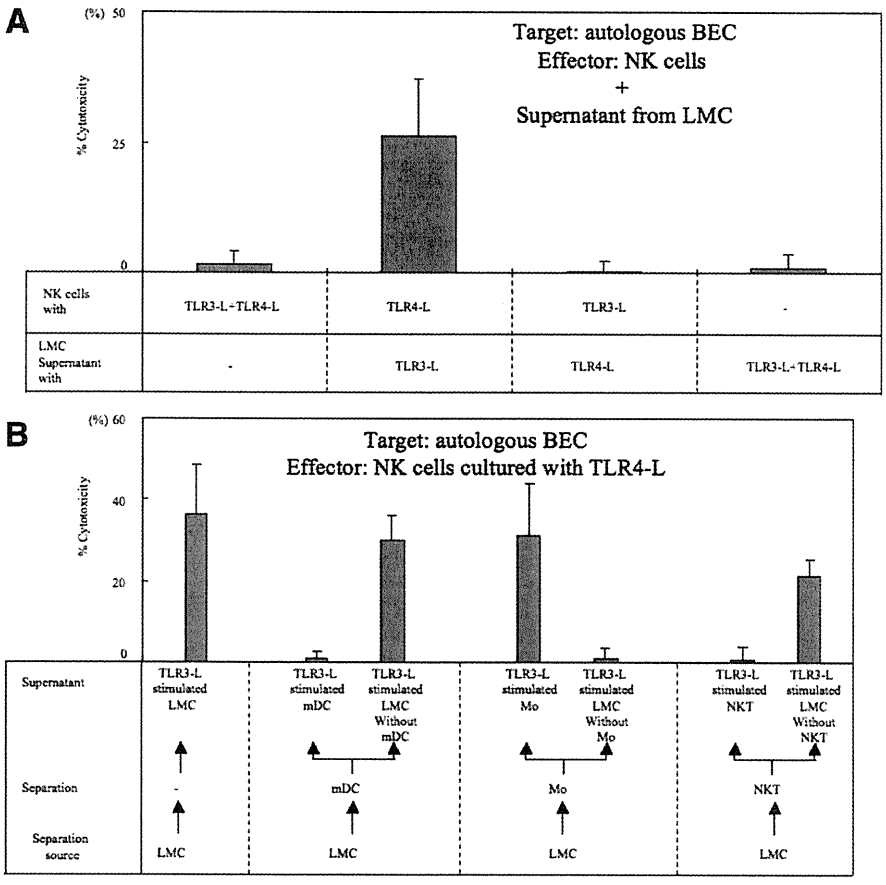

Fig. 3. (A) The activation requirements of NK cells in mediating cytotoxicity against autologous BEC. Highly enriched population of NK cells were cultured *in vitro* in the presence of (1) TLR3-L+TLR4-L; (2) TLR4-L and supernatant fluid from LMC cultured in the presence of TLR3-L; (3) TLR3-L and supernatant fluid from LMC cultured in the presence of TLR4-L; and (4) supernatant fluids from LMC cultured in the presence of TLR3-L+TLR4-L. Cultures were performed in triplicate and the mean ± SD of the net percent cytotoxicity calculated. The data shown are from one PBC patient and are representative. (B) Identification of the cell lineage that is the source of the factor required to mediate cytotoxicity of autologous BEC by TLR4-L-activated NK cells. A pool of a highly enriched population of NK cells was cultured with TLR4-L in the presence of supernatant fluids from (1) unfractionated LMC cultured with TLR3-L (control); (2) highly enriched populations of mDC or LMC depleted of mDC-stimulated with TLR3-L; (3) highly enriched population of Mo or LMC depleted of Mo-stimulated with TLR3-L; and (4) highly enriched population of NKT cells to LMC depleted of NKT cells stimulated with TLR3-L. These cultures were tested for cytotoxicity against autologous BEC. Each culture was performed in triplicate and the data shown reflect mean ± SD of net percent cytotoxicity of the triplicate cultures. The data shown are from one PBC patient and are representative.

Supernatant from TLR3-L-Stimulated Mo Induces NK Cell Cytotoxicity. We next carried out studies in efforts to identify the cell lineage that was the source of the cytokine(s) in the supernatant fluids from TLR3-L-activated unfractionated LMC that induced TLR4-L-stimulated NK cell cytotoxicity against autologous BEC. Highly enriched populations of mDC, Mo, NKT cells, and the corresponding population of LMCs depleted of mDC, Mo, and NKT cells were stimulated with TLR3-L and the supernatant harvested; insufficient quantities were available to study the pDC fraction. NK cells were cultured with TLR4-L in the presence or absence of each of these supernatant fluids and analyzed for cytotoxicity against autologous BEC as described in Materials and Methods. As noted in Fig. 3B, whereas TLR4-L-stimulated NK cells cultured in the presence of supernatant fluids from TLR3-L unfractionated LMC demonstrated significant cytotoxicity; similarly TLR4-L-stimulated NK cells, when cultured with supernatant fluids of TLR3-L, stimulated mDC, and NKT cells did not demonstrate detectable cytotoxicity against autologous BEC. However, the TLR4-L-activated NK cells, cultured in the presence of TLR3-L-activated Mo, readily demonstrated cytotoxicity. The identification of Mo as the source of the cytokine required for TLR4-L-activated NK cells to induce cytotoxicity against autologous BEC was confirmed by results obtained with

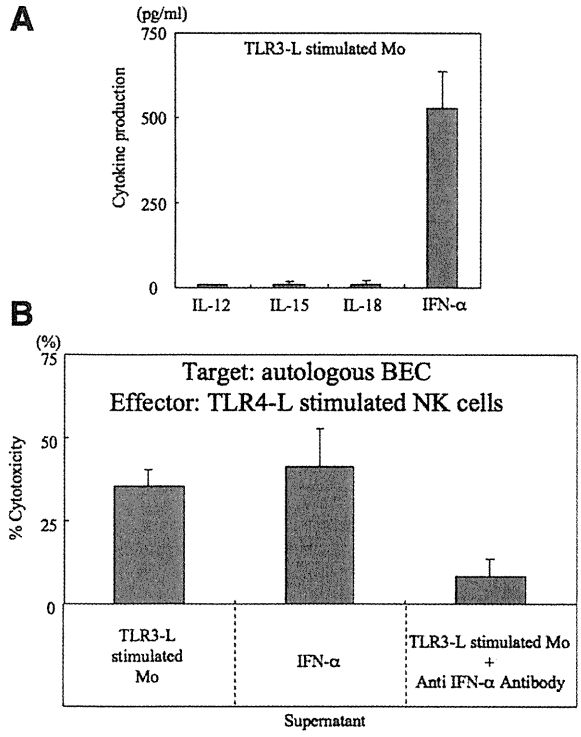

Fig. 4. (A) Analysis of cytokines synthesized by *in vitro* TLR3-L-activated hepatic Mo. Mo from the liver of the 22 patients included in the present study were isolated and cultured *in vitro* and the supernatant fluids analyzed for levels of IL-12, IL-15, IL-18, and IFN-α. Cultures were performed in triplicate and the data displayed represents mean ± SD of values obtained from cultures from one representative patient. Statistical differences between PBC patients and disease controls are described in the text. (B) IFN-α is required by TLR4-L-stimulated NK cells to mediate cytotoxicity against autologous BEC. Aliquots of TLR4-L-stimulated NK cells were cultured in the presence of either supernatant fluids from TLR3-L-stimulated hepatic Mo (control), IFN-α, or supernatant fluids from TLR3-L-stimulated Mo incubated with previously determined optimum concentration of anti-IFN-α monoclonal antibody. Cultures were performed in triplicate and assayed for cytotoxicity against autologous BEC. Data displayed are net percent cytotoxicity and the data shown are from one representative PBC patient.

supernatant fluids from TLR3-L-stimulated LMC depleted of mDC, and NKT cells, respectively.

TLR3-L-Stimulated Mo Produce IFN-α. The nature of the cytokine synthesized by TLR3-L-activated Mo that promoted cytotoxicity in TLR4-L-activated NK cells was studied next. We reasoned that the cytokine responsible for this activity was most likely IL-12, IL-15, IL-18, or IFN-α, which have previously been shown to generally activate NK cells. As seen in Fig. 4A, whereas TLR3-L-stimulated Mo produced low but detectable levels of IL-12 (7.9 ± 3.4 pg/mL), IL-15 (9.8 ± 8.0 pg/mL), and IL-18 (10.0 ± 9.6 pg/mL), the major cytokine synthesized was shown to be IFN-α (530.1 ± 106.2 pg/mL). In efforts to confirm that it was indeed IFN-α that was responsible for inducing TLR4-L-activated NK cell cytotoxicity, aliquots of TLR4-L-activated NK cells were cultured in the presence or absence of various concentrations of either IL-12, IL-18, IL-15, or IFN-α (Fig. 4A). Data derived from such studies demonstrated that whereas TLR4-L-activated NK cells cultured in the presence of IL-12, IL-18, or IL-15 (10-20 pg/mL) had no detectable cytotoxicity (data not shown), TLR4-L-activated NK cells cultured in the presence of recombinant IFN-α (500 pg/mL) readily induced cytotoxicity against autologous BEC (cytotoxicity; 41.2 ± 11.4%) (Fig. 4B). The identity of IFN-α as the cytokine responsible for inducing cytotoxicity in cultures of TLR4-L-activated NK cells was confirmed with the use of anti-IFN-α antibody. Thus, pretreatment of supernatant fluids from TLR3-L-activated Mo with anti-IFN-α reduced the cytotoxicity of TLR-4-stimulated NK cells against autologous BEC (cytotoxicity; 8.5 ± 5.2%). We also examined the relative levels of IFN-α synthesized by TLR3-L-activated Mo from patients with other diseases as compared with Mo from PBC patients in efforts to determine whether there was a qualitative and/or quantitative difference in the synthesis of this cytokine. IFN-α production from TLR3-L-activated Mo from PBC patients (n = 8; 355 ± 132 pg/mL) was significantly higher than similarly activated Mo from HBV-related cirrhosis (n = 3; 175 ± 74 pg/mL: $P < 0.03$), HCV related cirrhosis (n = 8; 175 ± 57 pg/mL: $P < 0.01$), or those from alcohol-related cirrhosis (n = 3; 180 ± 54 pg/mL: $P < 0.03$).

Contribution of Other Molecules to Liver NK Cell Cytotoxicity Against Autologous BEC. Although the above studies identified IFN-α as the cytokine synthesized by TLR3-L-activated Mo, we next attempted to identify the nature of the molecules synthesized by NK cells that were potentially involved in mediating cytotoxicity against autologous BEC. First, we evaluated the expression of activating receptors, inhibitory receptors, and effectors using reverse transcriptase (RT)-PCR methods on mRNA isolated from unstimulated NK cells, TLR4-L-stimulated NK cells, IFN-α-stimulated NK cells, and the combination of TLR4-L and IFN-α-stimulated NK cells. As shown in Fig. 5A, based on the activation signals the cultured cells expressed effector molecules such as FasL, TRAIL, and/or Granzyme B. Among these effector molecules, TRAIL appeared to be the molecule involved in promoting the cytotoxicity of TLR4-L-activated NK cells. Thus, as shown in Fig. 5B, the addition of monoclonal anti-TRAIL antibody but not anti-FasL antibody or anti-Granzyme B significantly reduced the cytotoxicity

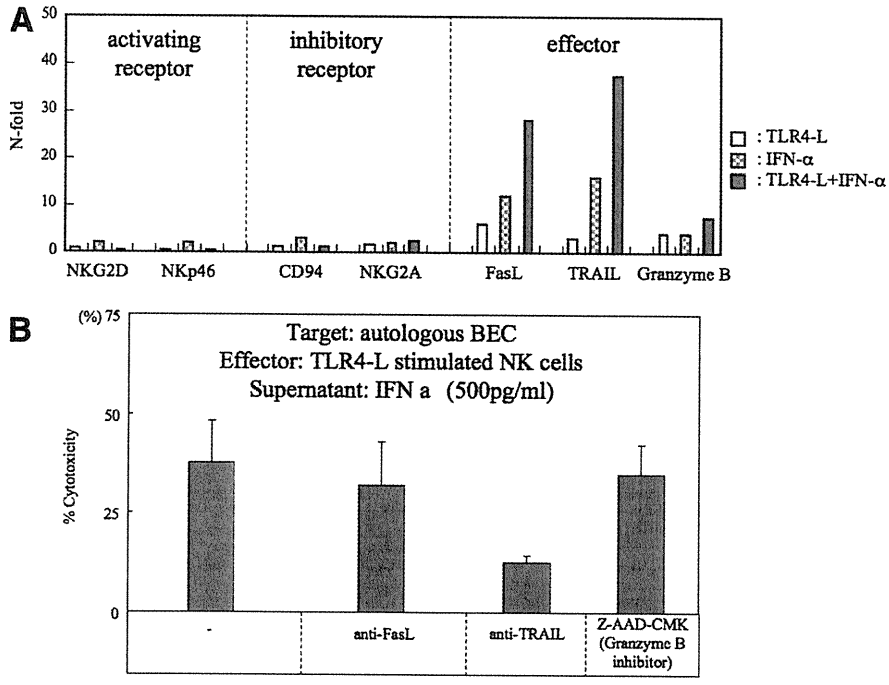

Fig. 5. (A) Expression of activating receptor, inhibitory receptor, and effectors based on TLR4-L, IFN-α, or a combination of TLR4-L and IFN-α stimulation. Activating receptors and inhibitory receptors were not expressed following stimulation from TLR4-L, IFN-α, or their combination. Effectors such as FasL, TRAIL, and Granzyme B were synergistically expressed dependent on the stimulation. Data displayed are from one representative PBC patient. (B) Contribution of TRAIL to liver NK cell cytotoxicity against autologous BEC. Aliquots of NK cells were cultured in the presence of TLR4-L and 500 pg/mL of IFN-α alone (control) or with the addition of predetermined optimum concentrations of anti-FAS-L, anti-TRAIL, or Z-AAD-CMK (inhibitor of Granzyme B) and then assayed for cytotoxicity against autologous BEC. Cultures were performed in triplicate and the data displayed reflect net percent cytotoxicity expressed as mean ± SD of the triplicate cultures. The data shown are from one PBC patient and are representative.

of TLR4-L-activated NK cells. These data indicate that IFN-α from Mo and TLR4-L-activated NK cells induce TRAIL to mediate cytotoxicity against liver BEC.

NK Cells Around BEC in the Liver. Finally, we investigated the relative levels of NK cells around bile ducts in sections of liver by immunohistochemistry. Comparative analyses of sections of liver from PBC patients and patients with liver diseases other than PBC demonstrated that $CD56^+$ NK cells predominantly invaded the portal area only in sections from PBC patients. Thus, whereas the number of $CD56^+$ NK cells invading portal areas was determined to be 8 ± 4.4 cells per small bile duct from PBC patients, those for sections of liver from patients with hepatitis C gave values of 2.7 ± 2.1 CD56+NK cells per small bile duct ($P < 0.01$), those from PSC gave values of 1.1 ± 1.2 CD56+NK cells ($P < 0.01$), and those from normal liver gave a value of 0.8 ± 1.0 CD56+NK cells ($P < 0.01$). Representative histochemical images are displayed in Fig. 6.

Discussion

Studies of the mechanisms of a variety of autoimmune diseases, including PBC, have predominantly focused on the contributory role of adaptive T and B cell responses in the pathogenesis of disease.[14-16] It is thus generally assumed that the major effector mechanisms that induce tissue pathology are those mediated by autoantigen-specific $CD8^+$ T cells and autoantigen-specific antibodies that directly and/or indirectly contribute to tissue pathology. Interestingly, the institution of immunosuppressive agents that predominantly target pathways involved in the activation and effector mechanisms employed by cells of the adaptive immune system have so far failed to result in clear therapeutic benefit in patients with PBC. This therapeutic failure of inhibiting adaptive immunity in patients with chronic autoimmune diseases such as PBC has prompted a need for the reevaluation of this line of thinking. Thus, it is reasonable to consider that alternate immune effector mechanisms are functioning and contributing to the pathogenesis of human PBC.

We submit that the involvement of innate immune effector mechanisms in any chronic disease including

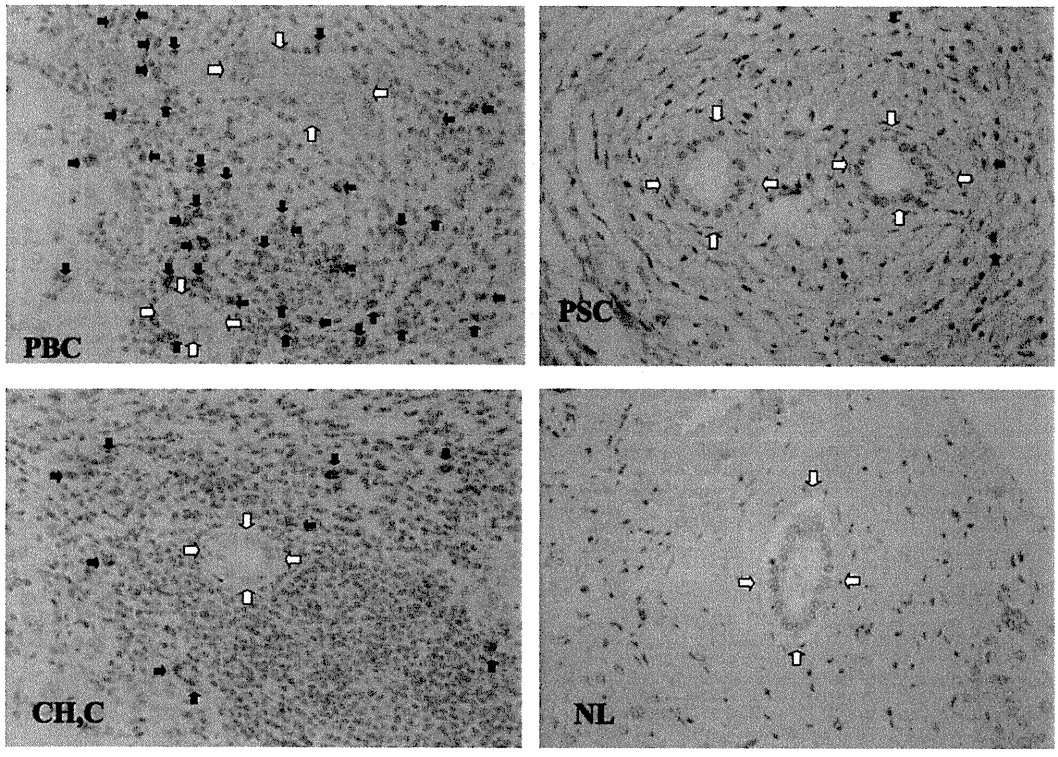

Fig. 6. Immunohistochemistry of CD56-positive cells in liver. Mononuclear cells expressing CD56 are seen in the biliary epithelial layer and periductal tissue. In PBC, $CD56^+$ cells are seen within the biliary epithelium (white arrow) and also at high density around the bile ducts (black arrow). In PSC and hepatitis C, $CD56^+$ cells are scattered, and in normal liver $CD56^+$ cells are rare around the bile ducts. Statistical differences between PBC patients and controls are described in the text.

autoimmune diseases such as PBC needs to be considered and evaluated. Thus, whereas it is easy to visualize a role for innate immune involvement in the initial stages of the disease process followed by the emergence of adaptive immune responses, it is clear that destruction of tissues during the chronic stages must require removal of dying cells and products of lytic cells. The removal of such unwanted tissues in addition to autophagy must involve the function of innate immune mechanisms. It naturally follows that the activated state of the innate immune system must result in proinflammatory cascades contributing to the pathology of the autoimmune disease. It should also be noted that the adaptive immune system has been shown to affect the character and magnitude of innate inflammatory responses.[17]

One of the major cell lineages of the innate immune system that is known to mediate target cell destruction are cells of the NK cell lineage.[18] Our previous findings of a high frequency of NK cells within cellular infiltrates around small bile duct cells of the liver in PBC patients[12] prompted us to examine the potential role this cell lineage plays in the pathogenesis of human PBC. Data presented herein demonstrate that NK cells from TLR3-L and TLR4-L-stimulated LMC kill autologous BEC, especially in PBC patients when compared to other control liver diseases; there have been descriptions of crosstalk between NK cells and other innate immune populations by way of TLRs.[13,19] One explanation for this observation is the finding of high levels of IFN-α in the sera of patients with PBC as compared with sera from patients with other liver diseases and otherwise control individuals. Thus, IFN-α is known to activate NK cell and contributes to enhance NK cell mediated cytotoxicity (Supporting Fig. 2 highlights these pathways).

The data herein also demonstrate that CD56-expressing NK cells upon ligation of TLR4 in the presence of IFN-α activates NK cells[20] and induces TRAIL.[21] The function of NK cells appears to vary depending on the disease process.[22] For example, the phenotypes of NK cells in patients with inflammatory bowel disease are different from those from normal intestinal mucosa.[23] NK cell activation receptor NKp46-positive NK cells have been shown to recognize and destroy beta cells in type I diabetes.[24] However, as previously shown, NKp46 is not induced on NK cells by

TLR4-L in the presence of IFN-α. Hence, it is our working hypothesis that the function of local resident NK cells in CNSDC is distinct from that noted in patients with PBC as exemplified by the unique expression of TRAIL in the latter but not the former.

There was no detectable cytotoxic effect when BECs were cultured with either TLR3-L or TLR4-L alone in our assay. Up-regulation of NK cell activating ligands has been reported in several liver subpopulations, including BEC, and has been implicated in liver injury.[25] It is not clear whether NK cell-activating ligands are also up-regulated on BEC in PBC and involved in the increased sensitivity to NK cell killing. Studies are in progress to define the relative sensitivity of BEC to NK cell cytotoxicity.

NK cells are cytotoxic for autologous BEC in the presence of TRAIL. The fact that human cholangiocytes constitutively express death receptor 5, which is the natural receptor for TRAIL, coupled with the finding of elevated levels of TRAIL expression and apoptosis in cholangiocytes of PBC patients,[26] suggests that TRAIL/Death receptor 5-mediated apoptosis may be the major pathway involved in the pathogenesis of chronic cholestatic disease.

Our data indicate that there are two requirements for NK cell-mediated cytotoxicity. One requirement depends on the source of TLR4 ligation and the other is the source of IFN-α, reportedly elevated in CNSDC.[27] Indeed, IFN-α appears to be derived from a monocytoid lineage, potentially plasmacytoid dendritic cells (pDC)[28]; in our study we were not able to address this issue because of insufficient quantities of pDC in this experimental protocol. This is an issue that should be examined in the future. An additional remaining question is the identification of the source of the ligands for TLR3 and TLR4 that activate Mo and NK cells, respectively. Thus, whereas exogenous sources of ligands for TLR3 and 4 were used herein, it will be important to identify the natural ligands that are functional in patients with PBC because such data will be of major clinical importance. In this regard, it is of interest to note that there is a correlation of urinary tract infections and PBC[29,30]; this could be the candidate source for TLR-L.

Sera from patients with autoimmune diseases often reflect the presence of elevated levels of inflammatory cytokines, including type 1 and 2 interferons (IFN), TNF-α, and IL-12.[31-33] IFN is induced by both a TLR-dependent and independent pathway in systemic autoimmunity.[34] Additionally, activation and proliferation of both autoantigen specific and non-specific CD8 T cell responses are characterized by the expression of CD38 and Ki-67 expression.[35] Previous work has demonstrated that pDC is a major source of type 1 IFN in response to ligation of TLR7.[36] In this regard, the characteristics of pDC that contribute to their pathogenic role include the observation that TRAIL-expressing pDC induces death of CD4 T cells that express TRAIL-associated death receptors.[37] In addition, pDC inhibit T cell proliferation through an indoleamine oxidase (IDO)-dependent pathway[38] and, finally, pDC rapidly migrate to the site of autoimmune mediated injury and/or infection and attract CD4+ T cells to the site.[39] We should note that in this study we did not evaluate IFN production from pDC in the presence of TLR7/8-L (CL097), but we did note the absence of cytolytic activity of LMC incubated with TLR4-L and TLR7/8-L (CL097).

Finally, it has also been demonstrated that CX3CL1 is expressed by BEC from patients with PBC and appears involved in the recruitment of intrahepatic lymphocytes into bile ducts.[8,40] This interaction promotes NK cell activation.[41] In conclusion, therefore, there is a complex but nonetheless well-defined relationship between liver mononuclear cell subpopulations and the biliary cell pathology of PBC. These interactions provide several steps that can potentially be modulated to reduce inflammation and will be the focus of further studies.

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CIN85 is required for Cbl-mediated regulation of antigen receptor signaling in human B cells

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The aberrant regulation of B-cell receptor (BCR) signaling allows unwanted B cells to persist, thereby potentially leading to autoimmunity and B-cell malignancies. Casitas B-lineage lymphoma (Cbl) proteins suppress BCR signaling; however, the molecular mechanisms that control Cbl function in human B cells remain unclear. Here, we demonstrate that CIN85 (c-Cbl interacting protein of 85 kDa) is constitutively associated with c-Cbl, Cbl-b, and B-cell linker in B cells. Experiments using CIN85-overexpressing and CIN85-knockdown B-cell lines revealed that CIN85 increased c-Cbl phosphorylation and inhibited BCR-induced calcium flux and phosphorylation of Syk and PLCγ2, whereas it did not affect BCR internalization. The Syk phosphorylation in CIN85-overexpressing and CIN85-knockdown cells was inversely correlated with the ubiquitination and degradation of Syk. Moreover, CIN85 knockdown in primary B cells enhanced BCR-induced survival and growth, and increased the expression of BcLxL, A1, cyclin D2, and myc. Following the stimulation of BCR and Toll-like receptor 9, B-cell differentiation-associated molecules were up-regulated in CIN85-knockdown cells. Together, these results suggest that CIN85 is required for Cbl-mediated regulation of BCR signaling and for downstream events such as survival, growth, and differentiation of human B cells. (*Blood.* 2012;119(10):2263-2273)

Introduction

B-cell receptor (BCR) signaling guides critical cell fate decisions in B cells during ontogeny.[1,2] BCRs can generate tolerogenic signals to purge or silence B cells that bind to self-antigens, and immunogenic signals to expand B cells that are specific for foreign antigens. Thus, BCR signaling must be properly regulated at the various stages of B-cell development, as aberrant regulation of BCR signaling potentially leads to autoimmunity and B-cell malignancies.

On BCR ligation by antigens, the Src-family protein tyrosine kinase (PTK) Lyn and Syk are initially activated. Syk propagates the signal by phosphorylating downstream signaling molecules, causing the activation of critical signaling intermediates phosphoinositol 3-kinase (PI3K) and phospholipase C (PLC)γ2. PI3K activates Akt kinase, which is important for B-cell survival.[3] PLCγ2 activation induces the release of intracellular Ca^{2+} and the activation of protein kinase C (PKC), which cause the activation of mitogen-activated protein kinases (MAPKs; ERK, JNK, and p38 MAPK) and of transcription factors, including NF-κB and NF-AT. These molecules regulate further downstream molecules that are responsible for determining B-cell fates such as survival, growth, and differentiation.[1,2]

Casitas B-lineage lymphoma (Cbl) proteins are E3 ubiquitin ligases that regulate signals of various receptors by promoting the ubiquitination of signaling components.[4,5] Tyrosine phosphorylation of Cbl proteins is critical for their function.[6] Mammalian Cbl proteins consist of 3 members, c-Cbl, Cbl-b, and Cbl-3, among which c-Cbl and Cbl-b are expressed in hematopoietic cells.[7] In B cells, Cbl proteins associate with Syk and B-cell linker (BLNK), and negatively regulate BCR signaling.[8,9] B cell-specific ablation of c-Cbl/Cbl-b proteins in mice causes aberrant BCR signaling as well as impaired B-cell anergy, culminating in the development of systemic lupus erythematosus (SLE)–like disease.[10] In addition, c-Cbl is hypophosphorylated on tyrosine in advanced stages of chronic lymphocytic leukemia (CLL).[11] These findings suggest that Cbl-mediated regulation of BCR signaling is critical for the fate decisions of self-reactive and malignant B cells.

Adaptors are noncatalytic molecules that integrate the spatial and temporal assembly of multiprotein complexes involved in the survival, growth, and differentiation of B cells. We previously showed that the B lymphocyte adaptor molecule of 32 kDa (Bam32)/DAPP1 regulates BCR signaling/internalization and B-cell survival.[12,13] The *SH3KBP1* (SH3-domain kinase-binding protein 1) gene, which is also known as CIN85 (c-Cbl interacting protein of 85 kDa), encodes an adaptor that is independently identified by several groups and contains 3 SH3 domains, a proline-rich region, and a coiled-coil domain.[14-17] Early studies showed that in nonimmune cells, CIN85 regulates the clathrin-dependent internalization of receptor tyrosine kinases (RTKs) such as epidermal growth factor receptors (EGFRs).[18,19] The formation of the ternary complex of CIN85, c-Cbl, and endophillin is critical for this process. In immune cells, however, little is known approximately the function of CIN85. CIN85 facilitates ligand-induced FcεRI internalization in RBL-2H3 mast cells.[20] In addition, it regulates FcεRI signaling via Cbl-mediated regulation of

Submitted April 29, 2011; accepted January 13, 2012. Prepublished online as *Blood* First Edition paper, January 18, 2012; DOI 10.1182/blood-2011-04-351965.

The online version of this article contains a data supplement.

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Syk expression in RBL-2H3 cells.[21] A recent study showed that CIN85 modulates c-Cbl–mediated down-regulation of FcγRIIa in human neutrophils.[22] It is thus of interest to determine whether CIN85 regulates the signaling pathways of other multimeric immune receptors, such as the T- and B-cell receptors.

Here, we demonstrate that CIN85 is constitutively associated with c-Cbl, Cbl-b, and BLNK in human B cells. Gain-of-function and loss-of-function experiments revealed that CIN85 up-regulated c-Cbl phosphorylation and inhibited BCR-induced calcium flux and phosphorylation of Syk and PLCγ2, without affecting BCR internalization. CIN85 also promoted c-Cbl–dependent ubiquitination and degradation of Syk. Moreover, CIN85 knockdown in primary B cells caused enhanced BCR-induced survival and growth, and augmented BCR/TLR9-induced expression of B-cell differentiation-associated molecules. Collectively, these results suggest that CIN85 is required for Cbl-mediated regulation of BCR signaling and for downstream events such as the survival, growth, and differentiation of human B cells.

Methods

Reagents

Goat anti–human IgM and IgG/IgA/IgM F(ab′)$_2$ fragments were purchased from Jackson ImmunoResearch Laboratories. Rabbit anti–human phospho-Zap-70 (Y319)/Syk (Y352), anti–human phospho-PLCγ2 (Y1217), anti–human phospho-Akt, anti–mouse Akt, anti–human phospho-JNK, anti–human phospho-ERK, and anti–human PLCγ2 pAbs as well as anti–human BclxL and Blimp-1 mAbs were purchased from Cell Signaling Technology. Mouse anti–human phospho-Btk, anti–human phospho-BLNK, anti–human c-Cbl, and anti–human Rac1 mAbs were from BD Immunocytometry. Mouse anti–human Cbl-b, anti–human Syk (4D10), anti–human BLNK, and anti-ubiquitin mAbs as well as rabbit anti–human c-Cbl and anti–mouse cyclin D2 pAbs were from Santa Cruz Biotechnology. Mouse anti-V5 mAb was from Invitrogen. Mouse anti-phosphotyrosine and anti–human CIN85 mAbs were from Upstate Biotechnology. Rabbit anti–human Vav2 mAb was from Epitomics. Sheep anti–human CD2AP pAb was from R&D Systems. Mouse anti–β-actin mAb was from Sigma-Aldrich.

B cell lines and primary B cells

The B lymphoma cell line BJAB was cultured in RPMI 1640 medium supplemented with 10% FCS. Human peripheral blood mononuclear cells, kindly provided by healthy volunteers, were separated from their buffy coats. Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. The Institutional Review Board of Kyushu University Hospital approved all research on human subjects. B cells were isolated with Dynabeads M450 CD19 and DETACHaBEAD CD19 (Dynal Biotech), according to the manufacturer's instructions. The isolated B cells exhibited greater than 99.5% viability on trypan blue exclusion and more than 95% purity on flow cytometry. Trace levels of phosphorylation of BCR-signaling molecules were observed in the B cells immediately after purification, probably because of mechanical stress.[23] The cells were thus rested for a couple of hours before further analysis. The cells were cultured at a density of 1×10^6 cells/mL in a 96 flat-bottom microtiter plate in complete RPMI 1640 medium supplemented with 10% FCS.

Expression constructs and transfection

Constructs encoding V5-tagged wild-type (WT) and 3 SH3 domain-deleted mutants of human CIN85 (pEF1/V5-CIN85 and –CIN85-dSH3ABC) were previously described.[24] The BJAB cells were transfected with the aforementioned constructs using a Gene Pulser apparatus (Bio-Rad Laboratories). The control cells were transfected with an empty vector. Stably transfected BJAB clones were selected in the presence of G418 (2 mg/mL) and screened with anti-V5 mAb.

RNA interference

The pSUPER-based strategy was adopted to knockdown hCIN85 expression. To generate CIN85 small-hairpin RNA (shRNA), a 19-nucleotide sequence (CAGCAATGACATTGACTTA) selected from human CIN85 cDNA was annealed and ligated into the pSUPER or GFP-pSUPER vector. A scrambled sequence (GTTACTAACGCGAATTAAC) was used as negative control. hCIN85 or the control shRNA vector was transfected into BJAB cells using a Gene Pulser apparatus, and stable hCIN85-knockdown BJAB clones were selected in the presence of puromycin (0.5 μg/mL). Transient transfections of primary B cells with the pSUPER-hCIN85 vector were performed using the Nucleofection kit from AMAXA Biosystems as previously described.[23]

Measurement of intracellular free calcium

B cells were washed with RPMI 1640 medium containing 10% FCS and adjusted to a concentration of 1×10^6 cells/mL. After incubation at 37°C for 15 minutes, 1 μg/mL of Fluo 4/AM (Dojindo) was added, and the cells were incubated for an additional 30 to 45 minutes with resuspension every 15 minutes. The cells were centrifuged and resuspended in RPMI 1640 at a density of 2×10^6 cells/mL and stimulated with anti-IgM (20 μg/mL). The fluorescence intensity of intracellular Fluo 4 was monitored and analyzed using flow cytometry.

Immunoprecipitation

Cells were lysed as described.[13] Subsequently, protein G-Sepharose (Amersham Pharmacia Biotech) precleared lysates were incubated with anti-V5, -BLNK, -Syk, -Cbl, -Vav2 mAb, or -CD2AP pAb for 1 hour at 4°C and then immunoprecipitated with protein G-Sepharose overnight at 4°C. The precipitated proteins were resolved by 10% SDS-PAGE; transferred onto a Millipore Immobilon polyvinylidene difluoride membrane; and blotted with anti-phosphotyrosine (4G10), -V5, -c-Cbl, -Cbl-b, -BLNK, -Vav2, -Syk, or -ubiquitin mAbs, followed by incubation with secondary HRP-conjugated IgG (Jackson ImmunoResearch Laboratories) specific for the primary Ab. The blots were developed with an ECL Plus kit (Amersham Biosciences).

Western blot analysis

Nonstimulated or stimulated cells (1×10^6) were lysed as described.[12] The lysates were then denatured in an equal volume of 2 × SDS sample buffer, resolved on a 10% SDS-PAGE gel, and electro-transferred to nitrocellulose membranes in non-SDS–containing transfer buffer (25mM Tris, 0.2M glycine, and 20% methanol; pH 8.5). Western blotting was performed with anti–phospho-Syk (1:2000), anti–phospho-PLCγ2 (1:2000), anti–phospho-BLNK (1:2000), anti–phospho-JNK (1:2000), anti–phospho-ERK (1:2000), anti–phospho-Akt (1:2000), anti-Akt (1:2000), anti-CIN85 (1:2000), anti–β-actin (1:2000), or anti-Vav2 (1:2000), followed by a 1:15 000 dilution of anti–rabbit or anti–mouse HRP-conjugated IgG. The blots were developed with the ECL plus kit (Amersham Biosciences). The chemiluminescence intensity was monitored using a Laser3000 (FujiFilm) instrument. We quantitated the band intensity of the proteins using ImageGauge Version 4.22 software (FujiFilm). The resulting values were expressed as fold changes in protein expression relative to the protein expression in unstimulated control cells.

Luciferase assays

Cells (1×10^7) were transfected by electroporation with the NF-AT-reporter construct, which was generously provided by Dr Shoichiro Miyatake (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). After 18 to 20 hours, cells were harvested and plated on 96-well plates at a density of 2×10^5/well. Triplicate cultures were incubated in the media alone with graded doses of anti-IgM or with 50nM PMA and 2.5μM ionomycin. After 6 hours, the cells were lysed in 50 μL reporter lysis buffer (Promega) for 15 minutes at room temperature. The luciferase activity was assayed by adding 20 μL luciferase substrate (Promega) to 50 μL lysate

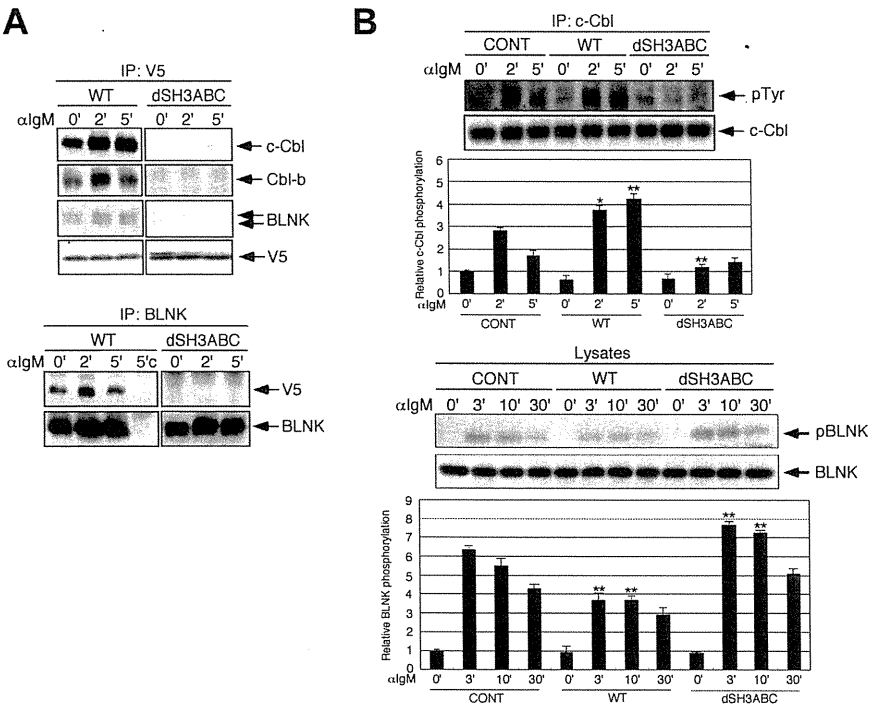

Figure 1. CIN85 associates with Cbl and BLNK and regulates their phosphorylation in B cells. (A) BJAB cells stably expressing either WT or SH3-deleted CIN85 were stimulated with 20 μg/mL of F(ab′)$_2$ goat anti–human IgM for the indicated time periods. Immunoprecipitates with anti-V5 or anti-BLNK mAb were separated on a 10% SDS-PAGE gel and analyzed by Western blotting with anti–c-Cbl, anti–Cbl-b, anti-BLNK, or anti-V5 mAb. 5′c, immunoprecipitation of the cell lysates at 5 minutes with isotype control. (B) Control BJAB cells and stable transformants expressing either WT or SH3-deleted CIN85 were stimulated with 20 μg/mL of F(ab′)$_2$ goat anti–human IgM for the indicated time periods. Immunoprecipitates with anti–c-Cbl mAb were separated on a 10% SDS-PAGE gel and analyzed by Western blotting with anti-phosphotyrosine or anti–c-Cbl mAb. The resulting values are expressed as fold changes in protein expression compared with unstimulated control cells. The values are the mean ± SD of 3 independent experiments (*$P < .05$, **$P < .01$ vs controls). (C) Control BJAB cells and stable transformants expressing either WT or SH3-deleted CIN85 were stimulated with 20 μg/mL of F(ab′)$_2$ goat anti–human IgM for the indicated time periods. The cell lysates were subsequently separated on a 10% SDS-PAGE gel and analyzed by Western blotting with anti–phospho-BLNK or anti-BLNK mAb. The resulting values are expressed as fold changes in protein expression compared with unstimulated control cells. The values are the mean ± SD of 3 independent experiments (**$P < .01$ vs controls).

and immediately measuring the luminescence on a Lumat LB9507 luminometer (EG & G Berthold). To serve as a control for the transfection efficiency, the relative luciferase activity of the medium and cells stimulated with BCR was calculated relative to stimulation with PMA/ionomycin.

Flow cytometric analysis

BJAB cells were incubated on ice for 15 minutes with 20 μg/mL goat-unlabeled anti-IgM before they were washed with ice-cold medium and warmed at 37°C for the indicated time intervals. The cells were washed with ice-cold PBS containing 2% FBS and 0.2% sodium azide (Fisher Scientific) to stop internalization at the assigned time points and to remove the unbound Ab. The remaining surface BCRs were stained with FITC-labeled rabbit anti–goat Ig and quantified by flow cytometry. The data are presented as the percentage of surface BCR remaining.

Fluorescence microscopic analysis

BJAB cells were incubated with 10 μg/mL of unlabeled goat anti–human IgM sera (20 μg/mL) at 4°C for 30 minutes and warmed to 37°C for the indicated time periods. The cells were then fixed with 3.7% paraformaldehyde and permeabilized with PBS containing 1% BSA and 0.05% saponin (wash buffer). The cells were then incubated for 30 minutes with FITC-conjugated anti–goat IgG pAb (Jackson ImmunoResearch Laboratories) at 4°C. The stained cells were centrifuged onto slides and analyzed with inverted fluorescent microscopy (BZ-9000; Keyence).

Quantitative real-time PCR

The total RNA was extracted from the primary B cells using Isogen reagent (Nippon gene) and was treated with DNase I (Invitrogen) to remove contaminating genomic DNA. First-strand cDNA was synthesized using a QuantiTect reverse transcription kit (QIAGEN). Quantitative real-time PCR was performed in the ABI Prism 7700 Sequence Detector (Applied Biosystems). The reactions were performed in triplicate wells in 96-well plates. TaqMan target mixes for Cyclin D2, Myc, *BCL2L1*/BclxL, *BCL2A1*/A1, *PRDM1*/Blimp-1, and *XBP1* were purchased from Applied Biosystems. 18S ribosomal RNA (rRNA) was separately amplified in the same plate as an internal control for variation in the amount of cDNA in PCR. The collected data were analyzed using the Sequence Detector software (Applied Biosystems). The data were expressed as the fold change in gene expression relative to the expression in the control cells.

Annexin V staining

After culture, cells (1-2 × 10^5) were washed twice with PBS and suspended in 85 μL binding buffer (MBL) containing Ca^{2+}. The cell suspension supplemented with 10 μL annexin-V–FITC or annexin-V–PE (MBL) and 5 μg of propidium iodide (PI) or 1 μg of 7-ADD was incubated at room temperature for 15 minutes in the dark. Subsequently, binding buffer was added, and the fraction of early apoptotic cells was measured using flow cytometry.

BrdU assay

DNA synthesis was monitored by pulse-labeling cells for 2 hours with the thymidine analog 5-bromo-2′-deoxyuridine (BrdU). The cells were washed 3 times with PBS and fixed for 20 minutes at −20°C in an ethanol fixative (0.15mM glycine in 70% EtOH, pH 2.0). After rehydration in PBS, BrdU incorporation was detected by incubation with an anti-BrdU mAb for 1 hour at 37°C, followed by a rhodamine-conjugated anti–mouse antibody (1:500; Jackson ImmunoResearch Laboratories) and staining of the nucleus with 4′-6-diamidino-2-phenylindole for 1 hour. The proportion of BrdU-positive nuclei (BrdU labeling index) was assessed, based on a sample size of 500 cells per data point.

Statistical analysis

Statistical analysis was performed using the Student t test. $P < .05$ was considered statistically significant.

Results

CIN85 associates with Cbl and BLNK and regulates their phosphorylation

The tyrosine phosphorylation of signaling molecules is a critical event in BCR signaling.[1,2] Because SH3 domains play an important role in the function of CIN85,[25] we focused on

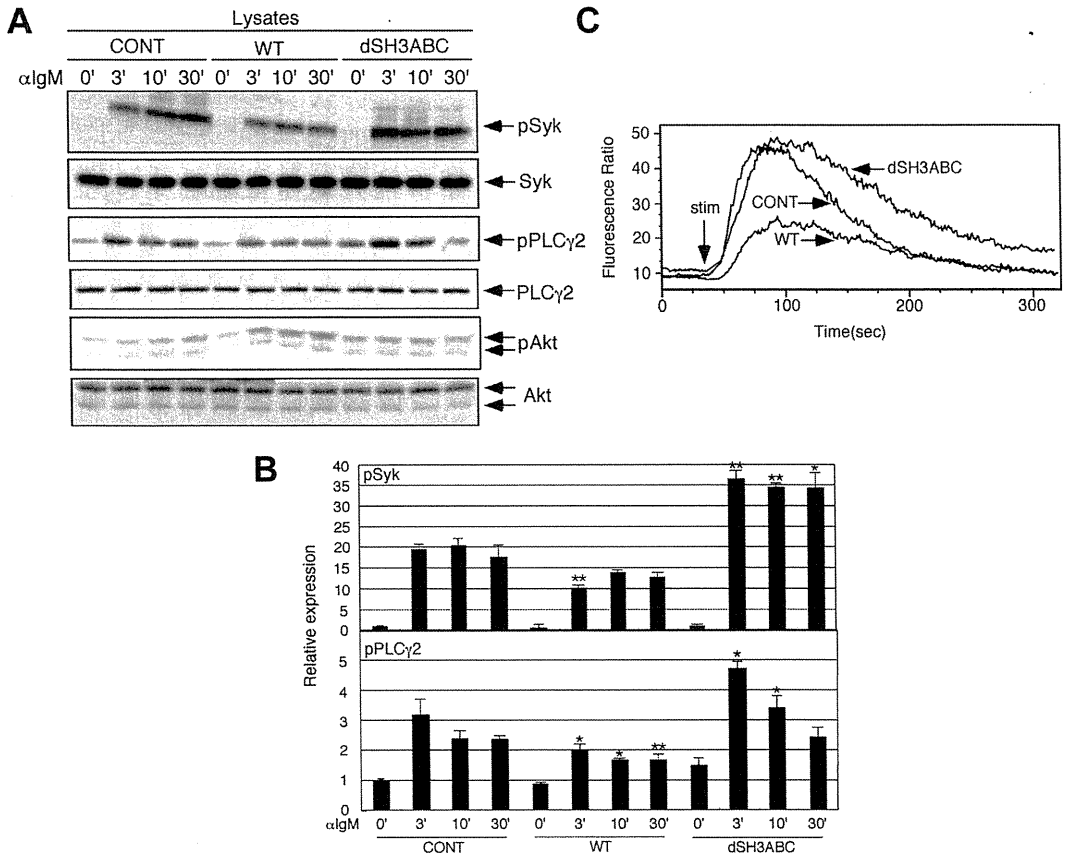

Figure 2. Forced CIN85 expression inhibits BCR-induced calcium flux and phosphorylation of Syk and PLCγ2. (A-B) Control BJAB cells and stable transformants expressing either WT or SH3-deleted CIN85 were stimulated with 20 μg/mL of $F(ab')_2$ goat anti–human IgM for the indicated time periods. The cell lysates were subsequently separated on a 10% SDS-PAGE gel and analyzed by Western blotting with anti–phospho-Syk pAb, anti-Syk mAb, anti–phospho-PLCγ2 pAb, anti-PLCγ2 pAb, anti–phospho-Akt pAb, or anti-Akt pAb. The resulting values are expressed as fold changes in protein expression compared with unstimulated control cells. The values are the mean ± SD of 3 independent experiments (*$P < .05$, **$P < .01$ vs controls). (C) Ca^{2+} influx in control BJAB cells and stable transformants expressing either WT or SH3-deleted CIN85. The intracellular free calcium levels in Fluo 4/AM-loaded cells were analyzed using flow cytometry after the cells were stimulated with 20 μg/mL $F(ab')_2$ goat anti–human IgM. The results shown are representative of 4 independent experiments.

tyrosine-phosphorylated molecules downstream of the BCR that could associate with the SH3 domains of CIN85. Specifically, we focused on the 2 molecules, BLNK and c-Cbl, that function as key positive and negative regulators of BCR signaling,[1,10] respectively; both proteins can associate with the SH3 domains of CIN85.[14,26]

We first determined the association of Cbl and BLNK with CIN85 using WT and SH3-deleted CIN85-expressing B cell lines. Consistent with previous reports,[14,26] WT CIN85 was constitutively associated with c-Cbl and BLNK, and these associations were increased after BCR stimulation (Figure 1A). Cbl-b was similarly associated with WT CIN85, albeit to a lesser extent. Although the association of WT CIN85 and BLNK appeared modest, the inverse immunoprecipitation of BLNK confirmed the association (Figure 1A). As expected, the association of Cbl and BLNK with CIN85 was abrogated in SH3-deleted CIN85-expressing B cells, suggesting that the SH3 domains of CIN85 are required for its association with Cbl and BLNK. Because the tyrosine phosphorylation of c-Cbl and BLNK is critical for their function,[6,27] we next determined whether the overexpression of WT and SH3-deleted CIN85 affects BCR-induced phosphorylation of c-Cbl and BLNK. Compared with control cells, WT and SH3-deleted CIN85 sustained and inhibited c-Cbl phosphorylation, respectively (Figure 1B). In addition, WT and SH3-deleted CIN85 inhibited and enhanced BLNK phosphorylation, respectively (Figure 1B). These findings suggest that CIN85 associates with Cbl and BLNK and regulates their phosphorylation in an opposite manner.

Forced CIN85 expression inhibits BCR-induced calcium flux and the phosphorylation of Syk and PLCγ2

We tested whether the overexpression of WT and SH3-deleted CIN85 affects early BCR signaling. Syk phosphorylation, which is one of the earliest events in BCR signaling, was inhibited in WT CIN85-expressing cells, whereas it was sustained in SH3-deleted CIN85-expressing cells (Figure 2A-B). Two enzymes, PLCγ2 and PI3K, function as critical mediators downstream of BCR signaling.[1,2,28] WT and SH3-deleted CIN85 partially inhibited and enhanced BCR-induced phosphorylation of PLCγ2, respectively (Figure 2A-B). In contrast, the phosphorylation of Akt, which is a downstream molecule of PI3K, was not affected in WT or SH3-deleted CIN85-expressing cells (Figure 2A). Activated PLCγ2 converts PIP2 into IP3 and diacyl glycerol, of which PIP2 is critical for calcium flux in B cells.[1,2,12] Consistent with the levels of PLCγ2 phosphorylation, the BCR-induced calcium flux was significantly inhibited in WT CIN85-expressing cells, whereas it was slightly sustained in SH3-deleted CIN85-expressing cells (Figure 2C). These results suggest that CIN85 inhibits BCR-induced calcium flux and the

phosphorylation of Syk and PLCγ2, and that the SH3 domains of CIN85 are required for its inhibitory function.

CIN85 knockdown enhances BCR-induced calcium flux and the phosphorylation of Syk, Vav2, and PLCγ2, leading to augmented NF-AT activation and CD69 expression

To elucidate the role of endogenously expressed CIN85 in BCR signaling, we generated CIN85-knockdown B cell lines. In contrast to the CIN85-overexpressing cells (Figures 1 and 2), CIN85-knockdown cells exhibited enhanced phosphorylation of Syk, BLNK, and PLCγ2 (Figure 3A-B). Akt phosphorylation was comparable between control and CIN85-knockdown cells (Figure 3A). Consistent with the levels of PLCγ2 phosphorylation, BCR-induced calcium flux was accentuated in CIN85-knockdown cells (Figure 3C). Vav2 positively regulates PLCγ2 activation in B cells.[29] Vav2 phosphorylation was enhanced in CIN85-knockdown cells (Figure 3D). These BCR signaling profiles in CIN85-knockdown cells are reminiscent of those in c-Cbl/Cbl-b double-knockout B cells.[10] The phosphorylation of c-Cbl was significantly inhibited in CIN85-knockdown cells (Figure 3E). BCR-induced calcium flux plays a crucial role in the activation of the transcription factor NF-AT, the disruption of which results in significant defects in B-cell function.[30] BCR-induced NF-AT activation was enhanced in CIN85-knockdown cells (Figure 3F). In addition, BCR-induced up-regulation of the activation marker CD69 was pronounced in CIN85-knockdown cells (Figure 3G). These phenotypes in CIN85-knockdown cells were again similar to those observed in Cbl-deficient B cells.[10] Given that CIN85 strongly associates with Cbl proteins (Figure 1A), these results suggest that CIN85 plays a vital role in Cbl-mediated regulation of BCR signaling.

CIN85 promotes the ubiquitination and degradation of Syk in B cells

Cbl proteins function as E3 ubiquitin ligases and target PTK substrates, including Syk, for degradation.[31,32] We thus tested whether CIN85 affects Syk ubiquitination in B cells. Syk ubiquitination was induced on BCR stimulation. Compared with control cells, Syk ubiquitination was increased in WT CIN85-expressing cells (Figure 4A). In contrast, an impairment in Syk ubiquitination was noted in CIN85-knockdown cells (Figure 4A). These results suggest that CIN85 positively regulates Cbl-mediated ubiquitination of BCR-signaling molecules including Syk. Despite the altered levels of Syk ubiquitination, the level of total Syk protein was not altered in the WT CIN85-expressing or CIN85-knockdown cells throughout the stimulation (Figures 2A and 3A). Because only a small pool of Syk is phosphorylated on stimulation and targeted for degradation in B cells,[31] we tested the degree of Syk phosphorylation among the total Syk immunoprecipitate. The levels of phosphorylated Syk were reduced in WT CIN85-expressing cells but enhanced in CIN85-knockdown cells (Figure 4B), suggesting that CIN85 promotes Cbl-dependent loss of the phosphorylated pool of Syk in B cells.

CIN85 does not affect BCR internalization

CIN85 regulates Cbl-mediated internalization of the EGFR in several cell types other than B cells.[18,19] To test whether CIN85 affects BCR internalization, we first monitored the levels of surface BCR expression after stimulation. Without stimuli, the levels of surface BCR were similar on CIN85 overexpression and CIN85 knockdown. In parental cells, BCR crosslinking caused a rapid decrease in surface BCR levels, suggesting that BCR was efficiently internalized after stimulation (Figure 5A; supplemental Figure 1A, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). BCR internalization was not affected in the WT or SH3-deleted CIN85-expressing cells. Moreover, the absence of endogenous CIN85 did not affect BCR internalization (Figure 5B, supplemental Figure 1A). Next, we directly visualized BCRs in B cell lines using fluorescence microscopy. In control cells, the BCR complexes exhibited a slightly patchy distribution before stimulation, and within 3 minutes after stimulation, the BCRs formed polarized tight caps on the cell surface. After 10 minutes of BCR stimulation, a punctate pattern of internalized BCRs was clearly visualized (Figure 5C). Consistent with the findings obtained with flow cytometry (Figure 5A-B), the spatial and temporal distribution of BCR complexes in CIN85-overexpressing and CIN85-knockdown cells appeared similar to that in control cells (Figure 5C, supplemental Figure 1B-C). These findings suggest that CIN85 does not affect BCR internalization.

CIN85 knockdown enhances the survival, growth, and differentiation of primary B cells

BCR signaling plays a critical role in determining the survival, growth, and differentiation of B cells.[1] It was thus of interest to test whether CIN85 affects B cell fate. A major obstacle, however, is that the survival, growth, and differentiation of B cell cannot be properly assessed in transformed B cells. We therefore sought to knock down CIN85 expression in human primary B cells. After introduction of the GFP-CIN85 knockdown vector, GFP-positive B cells were sorted and used for further experimentation. Under these conditions, we were able to knock down the CIN85 mRNA expression in B cells by 60%-80% (Figure 6A).

We first tested whether CIN85 knockdown affects the expression of the B-cell survival-associated genes BcLxL and A1. Consistent with previous studies,[33] BCR stimulation induced BcLxL and A1 mRNA expression in control cells. This induction was far more drastic in CIN85-knockdown B cells (Figure 6A). The costimulation of TLR9 with its ligand CpG enhances BCR-induced expression of B-cell survival genes.[34] This enhancement was less evident in CIN85-knockdown cells than in control cells (Figure 6A), suggesting that CIN85 knockdown requires less costimulation for the full induction of B-cell survival genes. Consistent the findings for the transcript levels, the BCR-induced expression of BcLxL protein was more pronounced in CIN85-knockdown cells (Figure 6B). We also tested whether CIN85 knockdown affects BCR-induced death of B cells using the annexin-binding assay. The CIN85-knockdown cells exhibited less BCR-induced cell death (Figure 6C). We next tested whether CIN85 knockdown affects the expression of the B-cell growth-associated genes cyclin D2 and myc. Again, BCR-induced expression of these genes was more pronounced in CIN85-knockdown cells (Figure 6A), and costimulation with TLR9 did not enhance induction compared with the control cells. Consistent with the findings for the transcript levels, BCR-induced expression of cyclin D2 protein was more pronounced in CIN85-knockdown cells (Figure 6B). We also tested whether CIN85 knockdown affects B-cell growth using the BrdU uptake assay. Consistent with the expression levels of cyclin D2 and myc, CIN85 knockdown enhanced BCR-induced cell growth (Figure 6D). On activation, B cells undergo plasma cell differentiation along with the expression of critical differentiation-associated genes such as Blimp-1 and Xbp-1. Consistent with previous studies,[35] BCR stimulation alone was not sufficient to induce the expression of Blimp-1 and Xbp-1 in human B cells (data not shown). However, the combined stimulation of BCR and TLR9 clearly induced the expression of these genes in control cells,

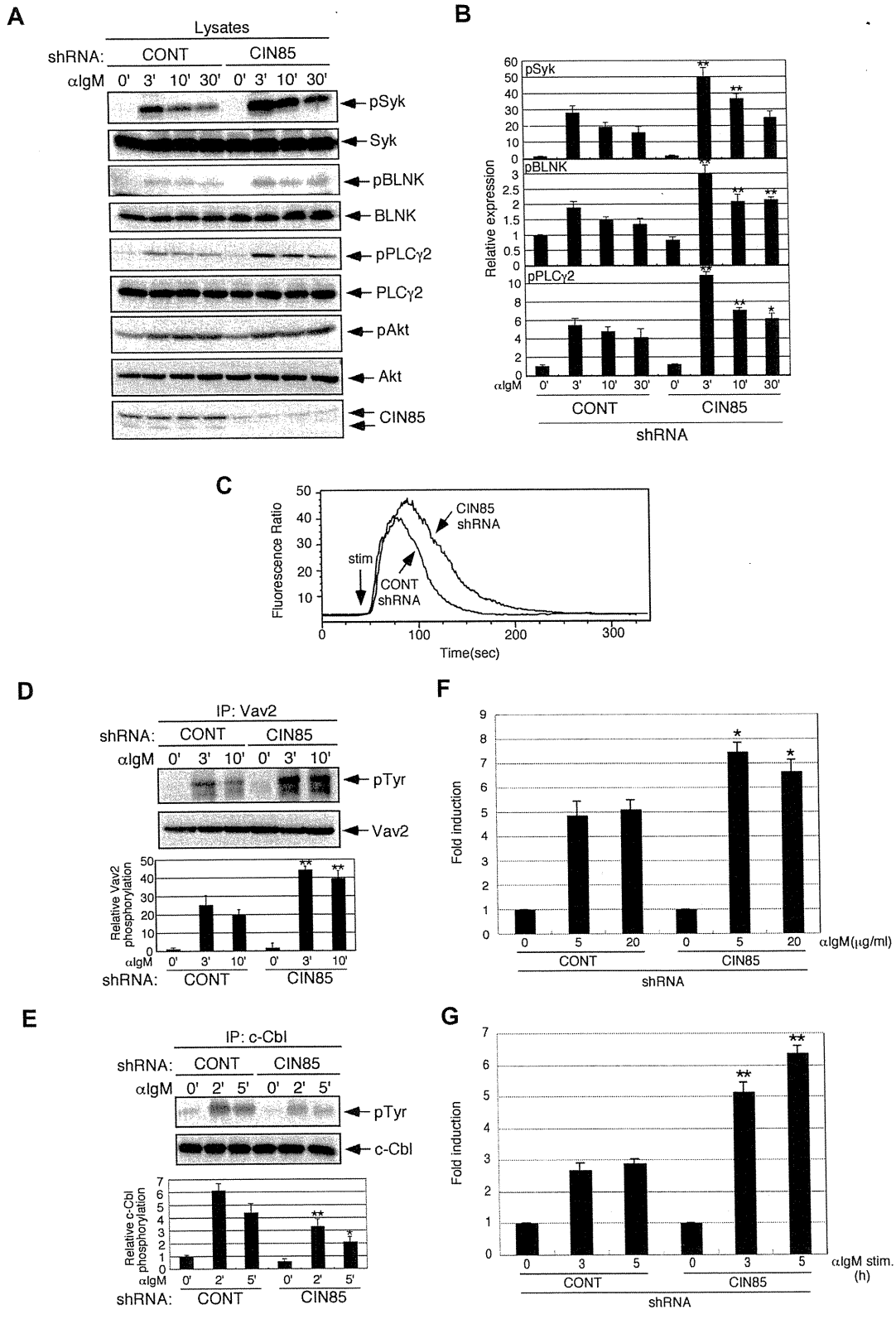

Figure 3. CIN85 knockdown enhances BCR-induced calcium flux and phosphorylation of Syk, Vav2, and PLCγ2, leading to augmented NF-AT activation and CD69 expression. (A-B) Stable control and CIN85-knockdown BJAB cells were stimulated with 20 μg/mL F(ab') $_2$ goat anti–human IgM for the indicated time periods. The cell lysates were subsequently separated on a 10% SDS-PAGE gel and analyzed by Western blotting with anti–phospho-Syk pAb, anti-Syk mAb, anti–phospho-BLNK mAb, anti-BLNK mAb, anti–phospho-PLCγ2 pAb, anti-PLCγ2 pAb, anti–phospho-Akt pAb, anti-Akt pAb, or anti-CIN85 mAb. The resulting values are expressed as fold changes in protein expression compared with unstimulated control cells. The values are the mean ± SD of 3 independent experiments (*$P < .05$, **$P < .01$ vs controls). (C) Ca^{2+} influx in stable control and CIN85-knockdown BJAB cells. Intracellular free calcium levels in Fluo 4/AM-loaded cells were analyzed using flow cytometry after the cells were stimulated with 20 μg/mL F(ab')$_2$ goat anti–human IgM. The results shown are representative of 4 independent experiments. (D-E) Stable control and CIN85-knockdown BJAB cells were stimulated with 20 μg/mL F(ab')$_2$ goat anti–human IgM for the indicated time periods. Immunoprecipitates with anti-Vav2 or anti–c-Cbl mAb were separated on a 10% SDS-PAGE gel and analyzed by Western blotting with anti-phosphotyrosine mAb, anti-Vav2 mAb, or anti–c-Cbl mAb. The resulting values are expressed as fold changes in protein expression compared with unstimulated control cells. The values are the mean ± SD of 3 independent experiments (*$P < .05$, **$P < .01$ vs controls). (F) Stable control

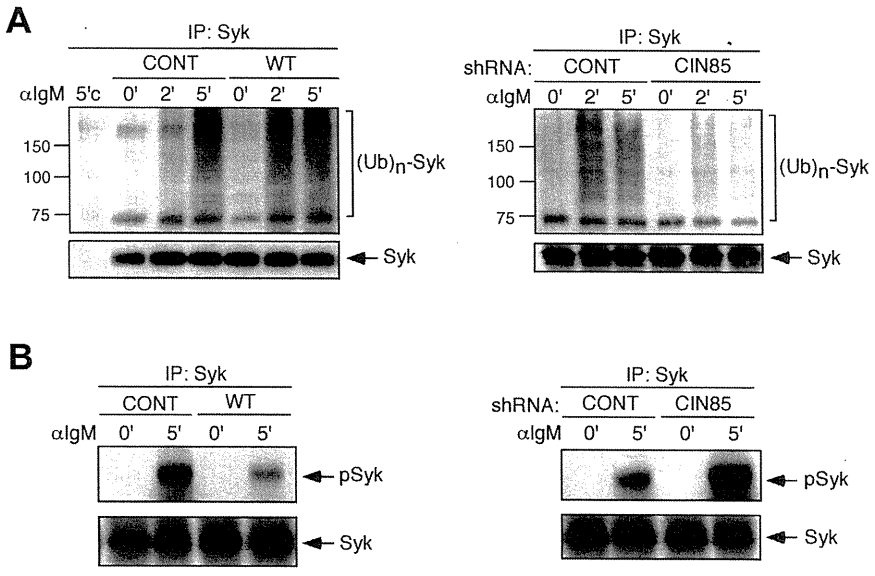

Figure 4. CIN85 promotes Syk ubiquitination and degradation in B cells. (A) Control BJAB cells, stable transformants expressing WT CIN85, and CIN85-knockdown BJAB cells were stimulated with 20 μg/mL $F(ab')_2$ goat anti–human IgM for the indicated time periods. Immunoprecipitates with anti-Syk mAb were separated on a 10% SDS-PAGE gel and analyzed by Western blotting with anti-ubiquitin or anti-Syk mAb. 5′′c, immunoprecipitation of the cell lysates at 5 minutes with isotype control. The molecular weight is indicated on the left side of the blots. (B) Control BJAB cells, stable transformants expressing WT CIN85, and CIN85-knockdown BJAB cells were stimulated with 20 μg/mL $F(ab')_2$ goat anti–human IgM for 5 minutes. Immunoprecipitates with anti-Syk mAb were separated on a 10% SDS-PAGE gel and analyzed by Western blotting with anti–phospho-Syk pAb or anti-Syk mAb.

whereas this induction was more pronounced in CIN85-knockdown B cells (Figure 6E). Consistent with the findings for the transcript levels, BCR stimulation alone did not induce detectable levels of Blimp-1 protein. However, the combined stimulation of BCR and TLR9 clearly induced the expression of the Blimp-1 protein in control cells, although this was more pronounced in CIN85-knockdown cells (Figure 6F). These results suggest that CIN85 is required for Cbl-mediated regulation of BCR signaling and downstream events such as the survival, growth, and differentiation of human B cells.

Discussion

We demonstrated here that CIN85 functions as a novel adaptor to regulate proximal BCR signaling. Gain-of-function and loss-of-function experiments revealed that CIN85 not only enhances BCR-induced c-Cbl phosphorylation but also inhibits BCR-induced calcium flux and the phosphorylation of Syk and PLCγ2. CIN85 promotes c-Cbl–dependent ubiquitination and degradation of Syk, which is a key upstream kinase that propagates BCR signaling by phosphorylating downstream molecules including PLCγ2. Because Cbl proteins directly associate with Syk and inhibit its function,[6] it is probable that CIN85 acts as a critical scaffolding adaptor for Cbl proteins and is indispensable for Cbl-mediated regulation of Syk activation in B cells.

Consistent with our findings, a critical role of CIN85 in Cbl-mediated regulation of Syk activation was recently shown in FcεRI signaling in mast cells.[21] In mast cells, CIN85 enhances c-Cbl-mediated ubiquitination and the degradation of Syk protein.[21] In B cells, however, CIN85 overexpression significantly increased Syk ubiquitination (Figure 4), but CIN85 knockdown did not alter the total levels of Syk protein throughout stimulation (Figure 3A), as previously shown in c-Cbl/Cbl-b double-knockout B cells.[10] This apparent discrepancy in Syk degradation between mast cells and B cells could be explained by the findings of Rao et al,[31] who showed that on BCR stimulation, only a small portion of Syk is phosphorylated and then degraded by c-Cbl. Rao et al also showed that c-Cbl does not directly affect the catalytic activity of Syk.[31] Consistent with these findings, our study showed that CIN85 promotes c-Cbl–mediated ubiquitination and degradation of the phosphorylated pool of Syk (Figure 4A-B).

What, then, are the possible mechanisms by which CIN85 enhances BCR-induced c-Cbl phosphorylation in B cells? Src-family PTKs and Syk are proposed to phosphorylate c-Cbl on tyrosines.[6] We previously showed that CIN85 directly interacts with the SH3 domain of Src-family PTKs including Lyn.[17] In addition, CIN85 directly associates with BLNK, PLCγ and Vav, all of which are direct Syk interactors,[17,36] and thus, CIN85 is indirectly associated with Syk via binding to BLNK, PLCγ, and Vav. In view of these findings, it seems probable that CIN85 acts as a key scaffolding adaptor that permits the spatial proximity of Src-family PTKs, Syk, and Cbl proteins and thus facilitates their phosphorylation of Cbl proteins.

Although CIN85 appears to function in concert with Cbl proteins to regulate BCR signaling, an additional mechanism is possible. Previous in vitro binding experiments showed that CIN85 directly binds to Src-family tyrosine kinases, PLCγ, p85 PI3K,Vav, Btk, and SHIP, all of which are involved in BCR signaling, through its SH3 domains and proline-rich region.[15,24,25] In addition, a recent study showed that the SH3 domains of CIN85 could uniquely bind to ubiquitin.[37] Thus, after various BCR-signaling molecules are ubiquitinated by Cbl proteins on stimulation, the competition between canonical SH3 ligands and ubiquitin binding to CIN85 may affect BCR signaling in a temporal and spatial manner. Therefore, it is probable that CIN85 also directly regulates BCR signaling by a Cbl-independent mechanism.

A recent study using liquid chromatography-coupled tandem mass spectrometry showed that 3 SH3 domains of CIN85 could recruit protein molecules required for the proper formation and

Figure 3 (continued) and CIN85-knockdown BJAB cells transfected with the NF-AT luciferase reporter construct were stimulated with graded doses of $F(ab')_2$ goat anti–human IgM for 8 hours and lysed, and the luciferase activity was assayed using a luminometer. The relative luciferase activity of the medium and BCR-stimulated cells was expressed with respect to that of the PMA/ionomycin stimulation. The results were presented as the mean and SEM of triplicate cultures. One experiment representative of 4 independent experiments is shown (*$P < .05$ vs controls). (G) Stable control and CIN85-knockdown BJAB cells before and after stimulation with 20 μg/mL $F(ab')_2$ goat anti–human IgM (3 and 5 hours) were analyzed for surface expression of CD69. One experiment representative of 3 independent experiments is shown (**$P < .01$ vs controls).

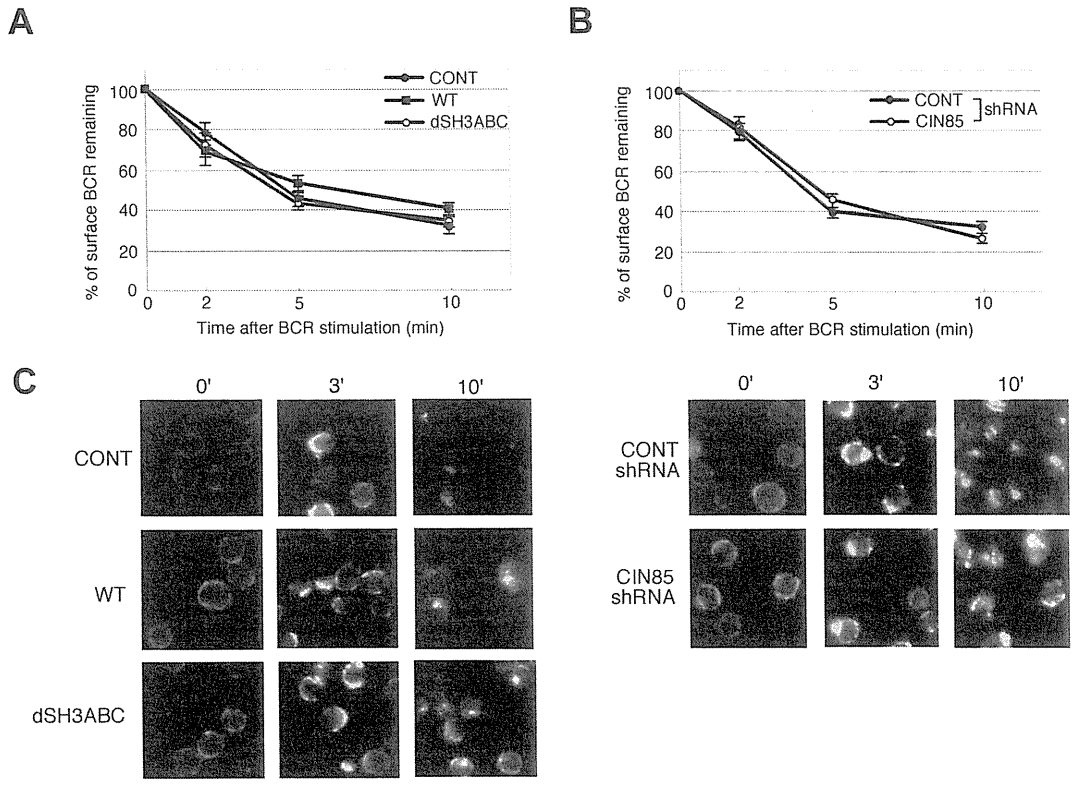

Figure 5. CIN85 does not affect BCR internalization. (A) BJAB cells (control and stable transformants expressing either WT or SH3-deleted CIN85) and (B) BJAB cells (control and CIN85-knockdown) were incubated at 4°C with F(ab')$_2$ goat anti–human IgM for 30 minutes. The cells were washed, warmed to 37°C for the indicated time intervals, stained at 4°C for 30 minutes with a FITC-labeled anti–goat IgG pAb, and analyzed by flow cytometry. The results are expressed as the percentage of surface BCRs remaining. The data are presented as the average and SEM of 3 independent experiments. (C) Control BJAB cells, stable transformants expressing either WT or SH3-deleted CIN85, and CIN85-knockdown BJAB cells were incubated at 4°C with 20 μg/mL F(ab')$_2$ goat anti–human IgM for 30 minutes. The cells were washed and warmed to 37°C for the indicated time periods. The cells were fixed, permeabilized, stained with a FITC-labeled anti–goat IgG pAb, and analyzed by fluorescence microscopy. The images shown are representative of 3 independent experiments.

function of coated vesicles.[25] Similarly, early studies showed a characteristic feature of CIN85 in the formation of clathrin-coated vesicles during the internalization of RTKs such as EGFRs in nonimmune cells.[18,19] Brain-specific CIN85-deficient mice manifest impaired internalization of D2 dopamine receptors, which belong to the 7-transmembrane G protein-coupled receptor superfamily.[38] In addition, CIN85 facilitates ligand-induced FcεRI internalization in RBL-2H3 mast cell lines.[20] Because BCR internalization is regulated via a clathrin-dependent pathway,[39] it was of interest to determine whether CIN85 regulates BCR internalization. Our study, however, shows that CIN85 does not affect BCR internalization (Figure 5, supplemental Figure 1). These data are somewhat surprising, given that Cbl proteins control BCR internalization by a ubiquitin-dependent mechanism.[10,40] However, the role of Cbl proteins in BCR ubiquitination and internalization is still rather controversial. The HECT family member Itch, but not c-Cbl, is an E3 ubiquitin ligase that is involved in BCR ubiquitination.[41] In addition, the ubiquitination of Igβ, which is a component of BCR, does not facilitate BCR internalization but is required for the sorting of early endosomes and for trafficking into late endosomes,[41] which suggests that BCR ubiquitination is more critical at the later stage of its trafficking. Because our imaging analysis (Figure 5C) cannot clearly distinguish the spatial distribution of early and late endosomes, it is of great interest to test whether CIN85 affects postendocytotic BCR trafficking. A recent study showed that in human neutrophils, CIN85 modulates c-Cbl–mediated down-regulation of FcγRIIa in the later stages of receptor trafficking without affecting the internalization of this receptor.[22]

During the submission of this paper, 2 studies were published that, in contrast to our findings, showed that CIN85 positively regulates BCR signaling in mouse and chicken B cells.[42,43] These studies found that CIN85 associates with BLNK and regulates BCR-induced NF-κB activation. However, the detailed profiles of BCR signaling differ between the 2 studies: the BCR-induced phosphorylation of BLNK and PLCγ2 and the calcium flux are significantly decreased on the loss of CIN85 in chicken B cells, whereas they are apparently normal in CIN85-deficient mouse B cells.[42,43] It should be noted that the former study did not actually use CIN85-deficient cells; rather, it used cells expressing a mutant BLNK that failed to bind to CIN85 or its homolog CD2AP.[42] Although these findings are intriguing, it is rather surprising that Cbl-mediated function of CIN85 in B cells was barely investigated in these studies. As previously mentioned, it is becoming evident that Cbl proteins play a critical role in the function of CIN85 in immune cells.[20-22] In addition, BCR-induced association of CIN85 with c-Cbl was recently shown even in mouse B cells.[44] We thus find that in human B cells, CIN85 negatively regulates BCR signaling via a Cbl-dependent mechanism. Our data obtained using CIN85-knockdown primary B cells also support this hypothesis. The molecular reason underlying the apparent discrepancy between our study and the aforementioned ones[42,43] remains unclear. One possibility, however, is that the relative contribution of CIN85-binding partners varies depending on the source of B cells used. In human B cells, Cbl proteins seem to preferentially associate with CIN85 over BLNK (Figure 1A). Notably, we found that CD2AP seems to preferentially associate with BLNK over c-Cbl in human B cells (supplemental Figure 2). It is therefore of potential interest