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CD137 Promotes Proliferation and Survival of Human B Cells

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CD137 (4-1BB)-mediated costimulation plays an important role in directing the fate of Ag-stimulated T cells and NK cells, yet the role of CD137 in mediating B cell function is unknown. We found that CD137 is expressed in vitro on anti-Ig–stimulated peripheral blood B cells and in vivo on tonsillar B cells with an activated phenotype. In vitro CD137 expression is enhanced by CD40 stimulation and IFN-γ and is inhibited by IL-4, -10, and -21. The expression of CD137 on activated human B cells is functionally relevant because engagement with its ligand at the time of activation stimulates B cell proliferation, enhances B cell survival, and induces secretion of TNF-α and -β. Our study suggests that CD137 costimulation may play a role in defining the fate of Ag-stimulated human B cells. ***The Journal of Immunology*****, 2010, 184: 787–795.**

A member of the TNFR superfamily, CD137, is predominantly found on activated T and NK cells (1–3). CD137L is present on APCs, including dendritic cells, macrophages, monocytes, and B cells (4, 5). Stimulation of CD137, through its natural ligand or agonistic Ab, induces potent antitumor immunity (6–8); it also effectively ameliorates disease severity in several mouse models of autoimmunity, including systemic lupus erythematosus (SLE) (9), chronic graft-versus-host disease (10), collagen-induced arthritis (11, 12), inflammatory bowel disease (13), and experimental autoimmune encephalitis (14). Thus, immunotherapeutics targeting CD137 represent promising new approaches to a wide array of distinct immune disorders.

The explanation for the apparent disparity between the ability to promote tumor rejection and treat autoimmune disease seems to be predicated on CD137-mediated manipulation of T cell function. Specifically, in conceptually overlapping experimental models of autoimmunity, CD137 ligation induces T cell deletion or hyporesponsiveness (10, 13), stimulation of $CD4^+CD25^+$ Ag-specific regulatory T cell subsets (15), or proliferation of Ag-specific $CD8^+$ $CD11c^+$ T cells, which suppresses $CD4^+$ T cell responses (11). This CD137-mediated immune modulation of T cells is postulated to be mechanistically responsible for observed changes in B cell function, including diminished isotype-specific Ab responses and changes in B cell survival. For example, in murine and primate models, administration of agonistic Abs against CD137 reduces T cell-dependent Ab production, and CD137-deficient mice demonstrate reduced IgG2a and IgG3 responses to keyhole limpet hemocyanin (16–18). Additionally, the importance of CD137–CD137L costimulation in B cell survival is evidenced in CD137L transgenic mice, whereby B cells are noted to decline in absolute number with advancing age (19). Importantly, murine B cells do not express CD137, whereas human B cells were reported to upregulate CD137 in response to anti-IgM stimulation (1, 20, 21). Therefore, it is uncertain whether murine-based animal models will accurately predict the clinical response to CD137 manipulation. Considering the importance of B cells in antitumor immune regulation and autoimmunity (22, 23), it is striking that the function of CD137 on human B cells has not been elucidated.

The goals of our study were to evaluate which external signals regulate CD137 expression on human B cells and to define the biological effect of CD137-mediated costimulation on human B cells. We demonstrate that the expression of CD137 on human B cells in vitro is initiated by BCR stimulation, whereas CD40 ligation and cytokines provide second-tier regulation. Furthermore, we show that CD137 is naturally expressed on B cells in tonsillar tissue in vivo and that this expression is temporally distinct from CD137L in vitro. The presence of CD137 on human B cells is functionally relevant, because stimulation with human CD137L-transfected cell lines at the time of activation induces proliferation, protects B cells from activation-induced cell death, and promotes the secretion of TNF-α and -β. Thus, our findings demonstrate that CD137 costimulation may play a role in defining the fate of Ag-stimulated human B cells.

Materials and Methods

Cells

Buffy coats from healthy donors were purchased (Biological Specialty Corporation, Colmar, PA), and PBMCs were prepared by density centrifugation (Ficoll-Paque, GE Healthcare, Piscataway, NJ). B lymphocytes were purified from PBMCs by negative selection using B cell isolation kit II (Miltenyi Biotec, Auburn, CA), and T lymphocytes were purified by positive selection using CD3 microbeads (Miltenyi Biotec), according to the manufacturer's instructions. The purity of cell separations was typically >98% for B and T lymphocytes, with <0.2% contamination of $CD3^+$ T cells in purified B cell populations (as assessed by flow cytometry). For the isolation of naive and memory B cell subsets, $CD19^+$ cells were

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Abbreviations used in this paper: 7-AAD, 7-aminoactinomycin D; BAFF-R, B cell activating factor receptor; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TdR, thymidine deoxyribose.

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positively selected using a CD19 multisort kit (Miltenyi Biotec), followed by separation of $CD19^+CD27^+$ (purity >80%) and $CD19^+CD27^-$ (purity >90%) cell subsets using CD27 microbeads (Miltenyi Biotec), according to the manufacturer's instructions.

Tonsils were obtained with informed consent from patients undergoing routine tonsillectomies at the University of Maryland Medical Center. Cells were mechanically homogenized in complete medium and mashed through a 40-μm sieve to clear the cell solution from tissue fragments and cell clusters. The mononuclear cells were isolated by Ficoll-Paque density centrifugation.

B cell-activation experiments

All in vitro cell cultures were performed in RPMI 1640 (Mediatech, Manassas, VA) supplemented with 10% FCS (Atlanta Biologicals, Lawrenceville, GA), 1% penicillin/streptomycin, 1% HEPES, and 1% Glutamax (all from Life Technologies, Carlsbad, CA).

Stimulation of PBMCs. PBMCs (2×10^6) were stimulated with 2 μg/ml PWM (Sigma-Aldrich, St. Louis, MO), 5 μg/ml PHA (Calbiochem, San Diego, CA), 25 ng/ml PMA/1 μg/ml ionomycin (Sigma-Aldrich), 2.5 μg/ml CpG (InvivoGen, San Diego, CA), or 5 μg/ml LPS (Sigma-Aldrich) in 24-well plates. After 72 h, PBMCs were harvested, washed, and assessed for CD137 expression by flow cytometry.

B/T cell coculture experiments. Purified B cells (1×10^6) and T cells (2×10^6) were cocultured and stimulated with PWM under the conditions described above. Direct B–T cell interactions were inhibited by the addition of transwell membranes. In brief, 2×10^6 T cells were added to the upper chamber of transwell plates (polyester membranes 6.5 mm, 0.4 μm; Corning Costar, Lowell, MA), and 1×10^6 B cells were added to the lower chamber.

Anti-CD40L–blocking experiments. To block CD40–CD40L interactions, various concentrations (1–20 μg/ml) of purified mouse anti-human CD40L (BD Biosciences, San Jose, CA) mAb and the isotype control were added to PBMC and B/T cell cocultures.

BCR-mediated stimulation experiments. Purified B cells or B cell subsets were cultured at a concentration of 1×10^6/ml in 48- or 96-well flat-bottom plates. B cells were activated with 10 μg/ml anti-Igs $F(ab')_2$ fragments (goat anti-human IgA+IgG+IgM [H+L], Jackson ImmunoResearch Laboratories, West Grove, PA), with or without the addition of 1 μg/ml purified goat anti-human CD40 Ab (R&D Systems, Minneapolis, MN). The following human recombinant cytokines were used to evaluate their impact on B cell stimulation: 100 U/ml IL-2 (Proleukine, Chiron, Emeryville, CA), 20 ng/ml IL-4 (R&D Systems), 1 ng/ml IL-6 (BD Biosciences), 50 ng/ml IL-10 (eBioscience, San Diego, CA), 50 ng/ml IL-15 (R&D Systems), 100 ng/ml IL-21 (BioSource International, Camarillo, CA), 500 U/ml IFN-γ (eBioscience), and 50 ng/ml TNF-α (BD Biosciences).

Real-time RT-PCR analysis

Total RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI), and cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostic, Indianapolis, IN). Real-time

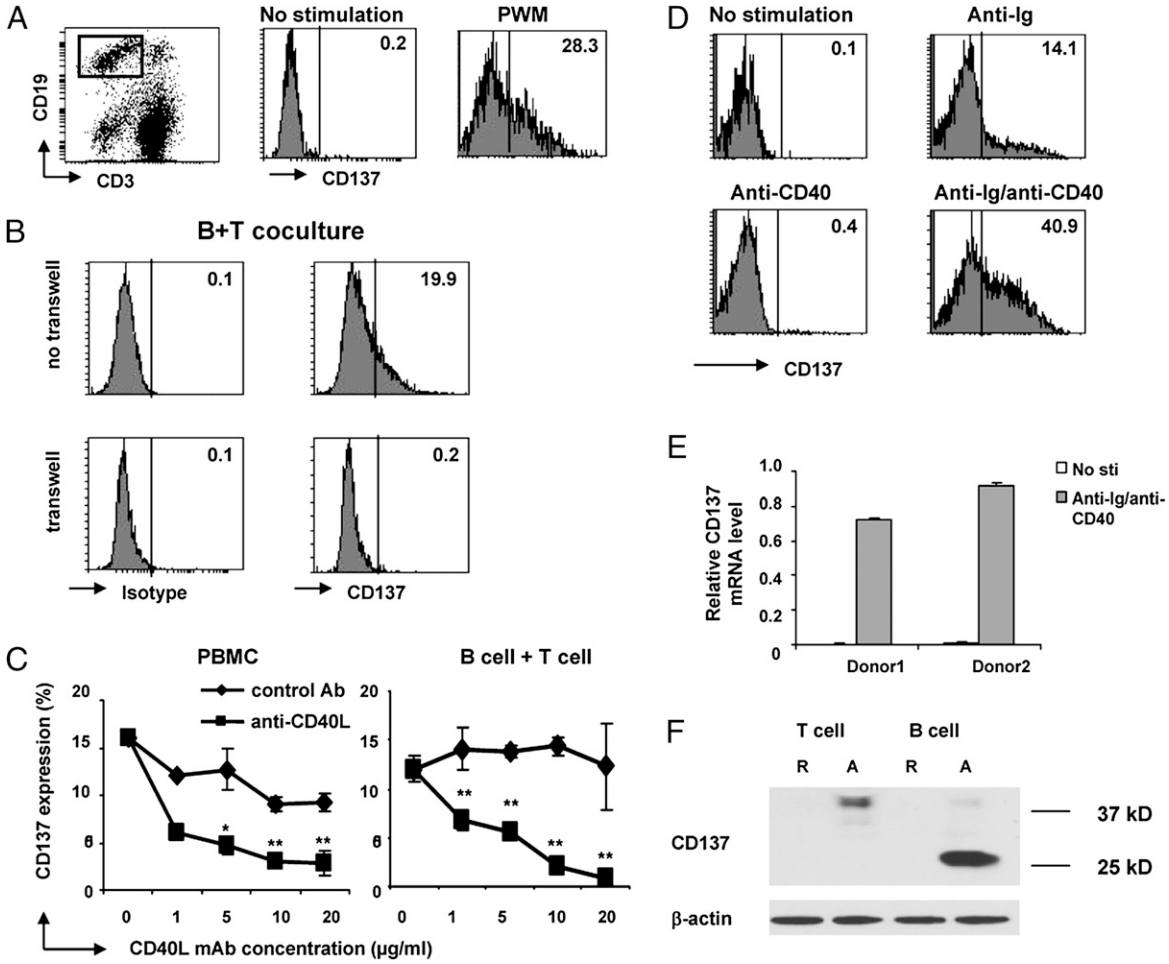

FIGURE 1. CD137 is induced on human B cells following activation and the expression level is enhanced by interaction between CD40 and CD40L. *A*, CD137 is upregulated on B cells following PWM stimulation of PBMCs from healthy individuals ($n = 18$). A representative FACS analysis is shown as the percentage of CD137 expression on gated B ($CD19^+CD3^-$) cells in whole PBMCs. *B*, B cells require T cell contact to upregulate CD137 during PWM stimulation. Isolated B cells and T cells from healthy individuals ($n = 10$) were cocultured in the presence of PWM with or without transmembrane for 3 d. Graphs indicate the percentage CD137 expression on gated B ($CD19^+CD3^-$) cells from one donor. *C*, Anti-CD40L mAb blocks CD137 expression on PWM-activated B cells in PBMCs and B/T cell cocultures in a dose-dependent fashion. Data are mean ± SD and are representative of five individual experiments. $*p < 0.05$; $**p < 0.0001$. *D*, CD137 is induced on human B cells upon BCR stimulation with enhancement of cell surface expression level following CD40 ligation with agonistic anti-CD40 Ab. Graphs indicate the percentage of CD137 expression on B cells. The isotype control was used to define the gate in all FACS analyses. The expression of CD137 mRNA (*E*) and protein (*F*) in anti-Ig/anti-CD40–activated B cells from two different donors was assessed by quantitative RT-PCR and Western blot. R, resting; A, activated.

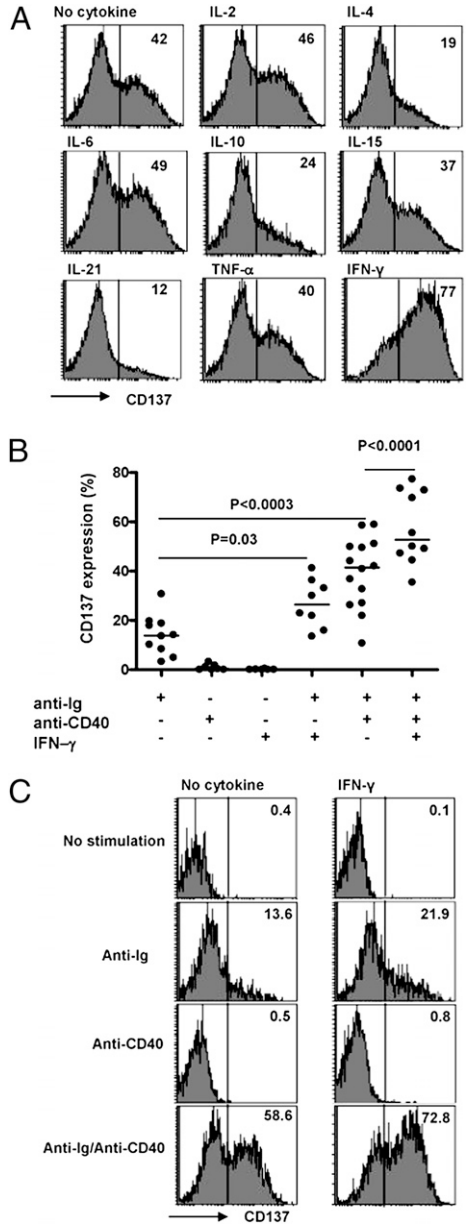

FIGURE 2. Cytokines provide a second level of regulatory control for the expression of B cell-associated CD137. *A*, Purified B ($CD19^+$) cells were stimulated with anti-Ig/anti-CD40 in the presence of no cytokine, IL-2, -4, -6, -10, -15, or -21, TNF-α, or IFN-γ. After 3 d of culture, B cells were harvested and assessed for CD137 expression by flow cytometry. *B*, CD40 ligation and/or IFN-γ enhance CD137 expression levels on human B cells, but primary BCR stimulation is required. *C*, Representative FACS analysis from one donor is shown as the percentage of CD137 expression on B cells. Data are representative of ≥10 individual experiments. The isotype control was used to define the gate in all FACS analyses.

quantitative PCR was performed using 7500-Fast Real-Time PCR Systems (Applied Biosystems, Foster City, CA) with specific primers, probes, and software (Applied Biosystems). The level of CD137 mRNA was quantified based on a titrated standard curve co-run in the same experiment and calibrated with the expression level of GAPDH. All samples were done in triplicate.

Western blot analysis

Proteins were separated by 4–20% SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham Biosciences). Membranes were blocked and incubated overnight at 4°C with goat anti-human CD137 Ab (R&D Systems). Murine mAb against β-actin was used as an internal control. Membranes were then washed and incubated with HRP-conjugated secondary Ab. The protein bands were detected by chemiluminescence (General Electric, Fairfield, CT).

Flow cytometry

Peripheral blood and tonsillar B cells were phenotyped by staining with directly conjugated mouse anti-human mAbs against CD3, CD19, CD20, CD32, CD69, CD86, CD95, CD137, CD137L, IgD, IgM (all from BD Biosciences), CD5, CD23, CD25, CD27, CD38, and CD71 (all from eBioscience). Directly conjugated mouse IgGs were used as isotype controls. Labeled cells were acquired on an LSRII flow cytometer and analyzed with FACS Diva (BD Biosciences) and Winlist (Verity Software House, Topsham, ME) software.

To evaluate cell proliferation by flow cytometry, B cells and separated B cell subsets were labeled with CFSE (Enzo Life Sciences, Farmingdale, NY), according to the manufacturer's instructions. CFSE-labeled cells were cultured as described above. At day 3 or 4 of culture, cells were harvested, stained with cell surface markers, and analyzed by flow cytometry. The total number of mitoses per 100 gated cells was calculated using the formula: number of mitoses = $\sum(Xn*100 - Xn*100/2n)$, where X is the percentage of cells that underwent n divisions (24). Cell cycle analysis was performed using a BrdU-FITC flow kit (BD Biosciences). In brief, activated purified B cells were pulsed with 10 μM BrdU. After 4 h, cells were harvested, and cell surfaces were stained with mAbs, followed by intracellular staining with anti-BrdU mAb. 7-aminoactinomycin D (7-AAD) was used to evaluate DNA content. B cell apoptosis was determined by staining with Annexin V/7-AAD (Annexin V-PE apoptosis detection kit I, BD Biosciences), according to the manufacturer's recommendations. Accucount particles (Spherotech, Lake Forest, IL) were added before analyzing samples to obtain accurate absolute cell numbers, which were calculated using the manufacturer's instructions.

Immunohistological analysis

OCT-embedded sections (5 μm) of tonsil were fixed for 15 min in 4% paraformaldehyde and washed with PBS for 15 min. After incubating with 10% normal human serum, the sections were double-stained for 45 min with the following primary Abs: goat anti-human CD137 (2 μg/ml, R&D systems)/mouse anti-human CD20 (1:500; eBioscience), and goat anti-human CD137/FITC-mouse anti-human IgD (1:20; BD Biosciences). Tissue sections were washed with PBS and blocked with 5% rabbit serum. Then each section was incubated with secondary Abs: Alexa Fluor 594 conjugated rabbit anti-goat IgG, $F(ab')_2$ fragment (1:1000, Invitrogen, Carlsbad, CA)/Alexa Fluor 488 conjugated rabbit anti-mouse IgG, $F(ab')_2$ fragment (1:1000, Invitrogen) for 30 min. Slides were washed and counterstained with DAPI (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 10 min. To control for nonspecific binding, control stains with isotype-matched primary Abs were included. Tissue sections were viewed with a Nikon Eclipse E6000 fluorescent microscope and were photographed with a Retiga 4000 camera (Q-Imaging, Austin, TX).

Functional B cell studies

Generation of CD137L transfectants. Human CD137L cDNA was obtained by RT-PCR from total RNA extracted from human PBMCs and subcloned into a mammalian expression vector (pcDNA3.1; Invitrogen). P815 cells were transfected with human CD137L using Lipofectamine (Invitrogen). After selection with G418 (800 μg/ml) for 1–2 wk, drug-resistant cells were FACS sorted for CD137L expression. CD137L positive cells were further cloned by limiting dilution. A clone with high levels of CD137L expression (hereafter called P815-CD137L) was selected and used in subsequent functional B cell experiments. P815 cells transfected with vector alone were used as a negative control (hereafter called P815-mock).

CD137–CD137L interaction experiments. γ-Irradiated (100 Gy) P815-CD137L cells or P815-mock cells were cultured with purified human B cells for 3–7 d. All cytokines and stimuli were added at the initiation of culture.

^{3}H-thymidine deoxyribose incorporation assays. Purified B cells were seeded at 2×10^5/well in triplicate wells in a 96-well flat-bottom plate and stimulated, as described under *B cell-activation experiments*, in the presence of irradiated P815-mock or P815-CD137L cells. To block the interaction of CD137 with CD137L, 10 μg/ml soluble CD137 protein (Prospec, Rehovot, Israel) was added at the initiation of culture. ^{3}H-thymidine deoxyribose (TdR) (37 kBq/well) was added 16 h before completion of the experiment, and thymidine incorporation was measured using a liquid scintillation counter (PerkinElmer, Waltham, MA).

Induction of apoptosis. Activation-induced cell apoptosis was performed by culturing B cells with anti-Ig alone or in combination with IFN-γ or anti-CD40, as described under *B cell-activation experiments*.

Cytokine ELISAs. Supernatants were collected 24–48 h after cultures were initiated. The concentrations of IL-4 and TNF-β (eBioscience), IL-6 and -10 (BD Bioscience), and IFN-γ and TNF-α (Cell Science, Canton, MA) were measured by a standard ELISA, following the manufacturer's protocol.

Statistical analysis

A repeated-measures model was used to compare CD137 expression under different conditions (reagents) to account for potential intradonor correlation.

The calculation for the repeated-measures model was implemented in SAS PROC MIXED. Residual diagnosis, such as Q-Q plot, was used to check the normality and model goodness of fit. The model reduces to *t* test and paired *t* test when there is only one sample per donor in each group.

Results

CD137 is expressed on human B cells following activation and expression is regulated by interactions between CD40–CD40L and proinflammatory cytokines

We first characterized activating signals required to induce the expression of CD137 on human B cells by stimulating whole PBMCs with various mitogenic stimuli. Human B cells upregulated CD137 in the presence of PWM (12.05% ± 9.76%, $n = 18$; Fig. 1*A*), whereas stimulation with PHA, LPS, PMA/ionomycin, or CpG was not effective (data not shown). Because PWM is recognized to activate T and B cells, we next sought to determine whether CD137 expression on human B cells is T cell dependent. We observed that purified B cells did not upregulate CD137 in the presence of PWM (data not shown). However, PWM stimulation of cultures containing isolated T and B cells induced B cell-associated CD137 expression (19.78% ± 10.52%; $n = 10$). This expression of CD137 is dependent on cell-to-cell contact, because B cells separated from T cells, by a transwell membrane, did not upregulate CD137 (Fig. 1*B*). Because the CD40–CD40L costimulatory pathway is integral to T cell-dependent B cell function, we defined the relative import of CD40–CD40L interactions in the upregulation of CD137 on B cells. The addition of escalating concentrations of CD40L mAb to PWM- stimulated PBMCs and purified T/B cell cocultures significantly reduced the percentage of CD137 expression ($p < 0.0001$) in a dose-dependent fashion (Fig. 1*C*). To confirm the significance of CD40–CD40L interactions in the induction of CD137, purified B cells were stimulated with anti-Ig in the presence or absence of anti-CD40–stimulating Ab. Resting (nonstimulated) B cells and B cells stimulated with anti-CD40 Ab alone did not upregulate CD137. However, B cells stimulated with anti-Ig upregulated CD137, whereas CD137 expression was dramatically enhanced at both mRNA and protein levels with concurrent CD40 ligation (Fig. 1*D–F*). These data demonstrate that CD137 is induced on human B cells following BCR stimulation and that expression levels are enhanced by interactions between CD40 and CD40L. Importantly, CD137 expression levels on B cells varied among different healthy individuals, ranging from 3.2–30.7% ($n = 10$; median = 13.9%) and from 10.7–62.2% ($n = 14$; median = 43.1%) upon stimulation with anti-Ig or anti-Ig/anti-CD40, respectively.

It is notable that the predominant CD137 band differed between anti-CD3–stimulated T cells and anti-Ig/anti-CD40–stimulated B cells (Fig. 1*F*). Specifically, activated B cells displayed a dominant band at ~28 kDa, whereas activated T cells had a dominant band at ~40 kDa. Importantly, activated B cells also have an identical 40-kDa subdominant band. These distinct protein sizes might reflect cell-specific posttranslational modifications of CD137 or the presence of isoforms in different cell types.

Because cytokines are recognized to influence human B cell function, we evaluated the impact of cytokine stimulation on CD137 expression. Purified B cells were stimulated with anti-Ig/anti-CD40 Abs in combination with defined cytokines recognized to mediate B cell function. IL-2, -6, and -15 and TNF-α did not directly affect CD137 expression on human B cells. However, coculture of anti-Ig/anti-CD40–stimulated B cells with IFN-γ dramatically enhanced ($p < 0.0001$) the percentage of CD137-expressing cells, whereas IL-4 ($p < 0.0001$), IL-10 ($p = 0.0188$), and IL-21 ($p = 0.0037$) induced the opposite effect (Fig. 2*A*). Similar to the effects of CD40 ligation, the ability of IFN-γ to enhance CD137 expression is dependent upon BCR signaling (Fig. 2*B*, 2*C*), because B cells stimulated with IFN-γ alone or in combination with anti-CD40 did not upregulate CD137. These data suggest that exposure to Ag is required for CD137 expression and that CD40 signaling and select cytokines provide a second level of regulatory control for CD137 expression on human B cells.

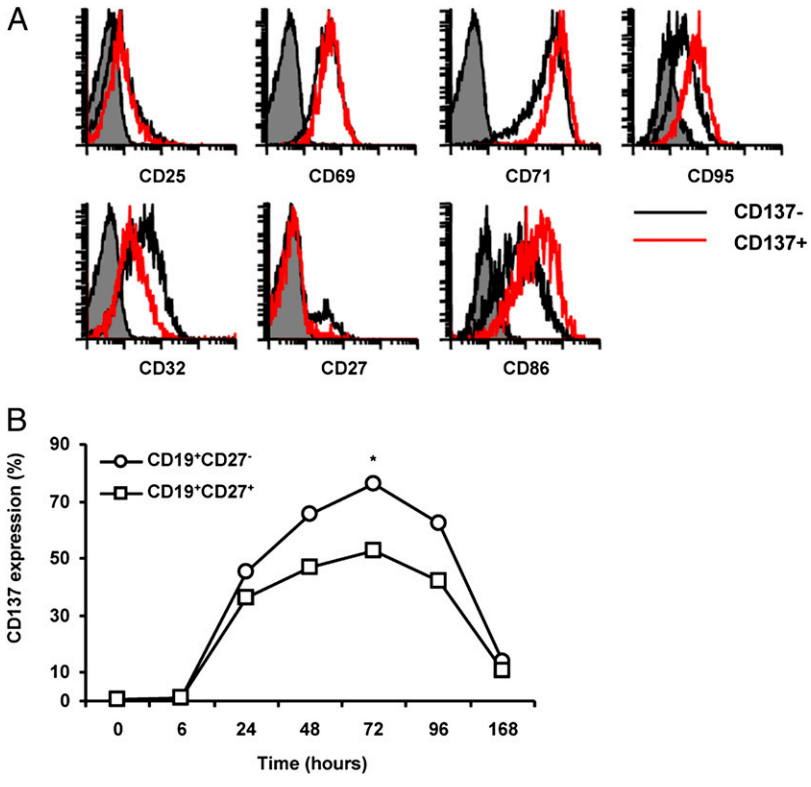

FIGURE 3. CD137 is preferentially expressed on activated B cells of naive origin. *A*, Purified human B cells were activated with anti-Ig/anti-CD40. After 3 d, B cells were harvested, and CD137-expressing B cells and non-CD137–expressing B cells were assessed for cell surface phenotype by flow cytometry. Graphs show surface expression of indicated markers on $CD137^+$ B cells and $CD137^-$ B cells. Filled peaks represent isotype controls. Data are representative of five individual experiments. *B*, Purified human B cells were separated into naive ($CD19^+CD27^-$) and memory ($CD19^+CD27^+$) B cells and subsequently stimulated with anti-Ig/anti-CD40. CD137 surface expression was determined at indicated time points. Data shown are representative of five individual experiments. $*p < 0.05$.

CD137 is preferentially expressed on activated B cells of naive origin

As a first step in characterizing the function of CD137 on human B cells, we compared the cell surface phenotype of $CD137^+$ and $CD137^-$ B cells. Anti-Ig/anti-CD40–stimulated $CD137^+$ B cells demonstrated elevated levels of CD71, CD86, and CD95 but diminished expression of CD32 (Fig. 3*A*). Interestingly, although $CD137^-$ B cells expressed small amounts of CD27, this marker was virtually absent on $CD137^+$ B cells. Because CD27 distinguishes naive B cells ($CD19^+CD27^-$) from memory B cells ($CD19^+CD27^+$), we evaluated whether CD137 was differentially upregulated on these distinct populations. Naive and memory B cells were purified based on their levels of CD27 expression, cultured in the presence of anti-Ig/anti-CD40, and harvested at defined time intervals. In both cell populations, CD137 expression was present on day 1 and reached a peak at day 3. By day 7, the expression of CD137 had returned to baseline. Overall, the percentage of $CD137^+$ B cells was higher in cultures originating from $CD27^-$ B cells versus $CD27^+$ B cells at every time point tested (Fig. 3*B*), with $p = 0.0014$ on day 4. These data demonstrate that following anti-Ig/CD40 ligation, CD137 is preferentially, but not exclusively, found on activated naive B cells.

Because CD137 was not found on unstimulated peripheral blood B cells from healthy donors, we next sought to determine the expression of CD137 on B cells in secondary lymphoid organs. Ex vivo analysis of human tonsillar B cells revealed that 0.8% ± 0.3% ($n = 6$) of total B cells expressed CD137 (Fig. 4*A*). Consistent with the in vitro phenotype in peripheral blood B cells, these CD137-expressing tonsillar B cells exhibit a phenotype associated with cell activation, e.g., elevated expression of CD23, CD5, CD86, CD71, and CD95. Additionally, $CD137^+$ tonsillar B cells are surface IgM^{hi}, $CD38^{+/-}$, and $IgD^{+/-}$ (Fig. 4*B*). The majority of $CD137^+$ B cells are found in the germinal center; fewer cells are found in the follicular mantle zone (Fig. 4*C*, 4*D*).

The differentiation of human B cells into effector Ig-secreting cells requires cell division (25, 26). Therefore, we sought to determine whether CD137 expression on human B cells is associated with B cell division. Based on CFSE dilution, we observed that higher levels of CD137 are expressed on divided B cells (Fig. 5*A*) compared with nondivided B cells (Fig. 5*B*). Specifically, CD137 is predominantly expressed on divided memory B cells. In contrast, divided and nondivided naive B cells express high levels of CD137, although higher CD137 expression levels are found on naive B cells with low CFSE intensity. Importantly, the expression of CD137 does not solely rely on B cell division, because CD137 is present on the B cell surface prior to cell division, and blocking of B cell division with mitomycin C does not abrogate the ability of anti-Ig/anti-CD40–activated B cells to upregulate CD137 (data not shown). Furthermore, cell cycle analysis confirmed that more $CD137^+$ B cells (30%) are present in the S phase than $CD137^-$ B cells (15%; Fig. 5*C*).

CD137 ligation enhances B cell proliferation

To expand upon the observation that CD137 expression on B cells is associated with B cell activation and cell division, we evaluated whether CD137 costimulation of activated B cells induces

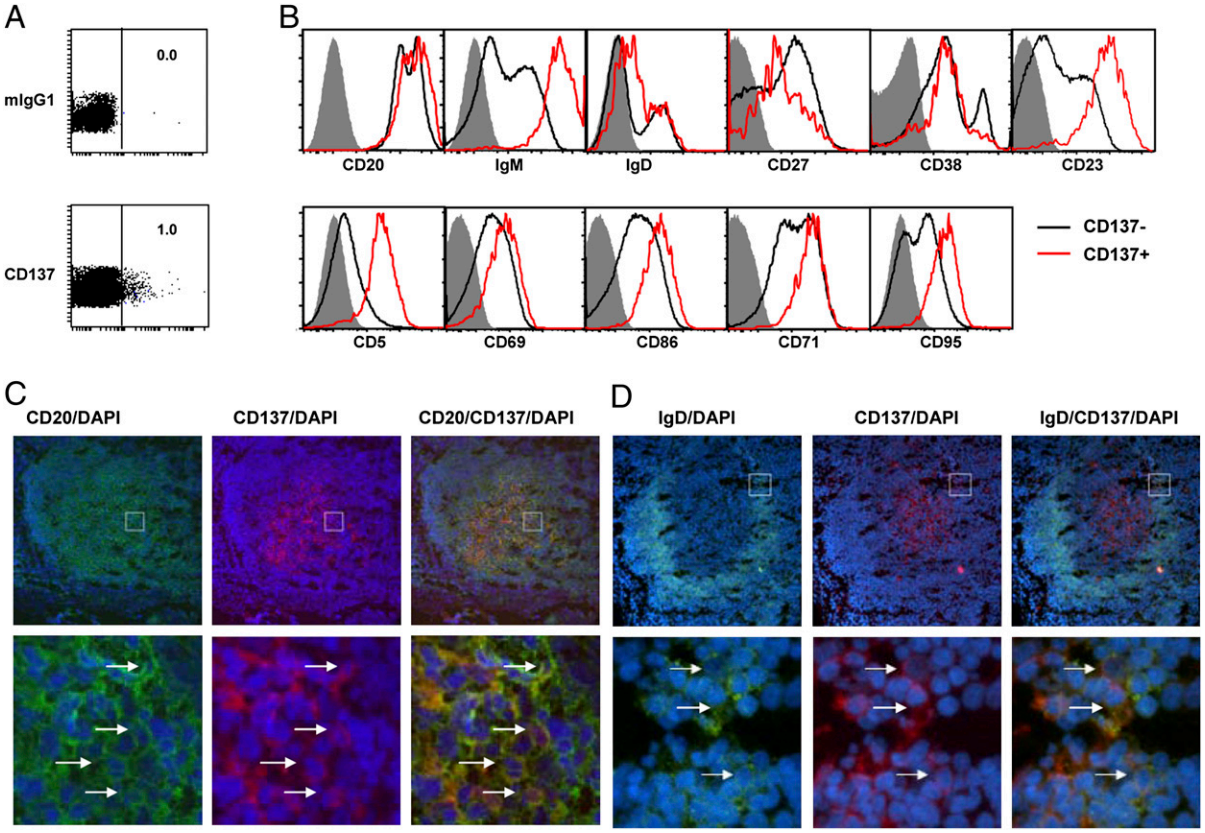

FIGURE 4. Ex vivo expression of CD137 in tonsillar B cells. *A*, Tonsillar mononuclear cells were analyzed for CD137 expression on B cells by FACS. B cells ($CD19^+CD3^-$) were gated, and the percentage of CD137 expression on B cells is indicated in dot plot. Data are representative of six individual experiments from different donors. *B*, The phenotype of $CD137^+$ B cells was analyzed in comparison with $CD137^-$ B cells. Filled peaks indicate isotype controls. Data are representative of five individual experiments. *C* and *D*, Three-color immunohistochemistry of human tonsil sections was used to identify the localization of CD137-expressing B cells: CD20 (green)/CD137 (red)/DAPI (blue) (*C*) and IgD (green)/CD137 (red)/DAPI (blue) (*D*). The upper row of panels shows a tonsillar germinal center (original magnification ×20). IgD staining defines mantle zone B cells. Indicated area from germinal center (*C*) and follicular mantle zone (*D*) was enlarged (original magnification ×200) to identify the CD20/CD137 and IgD/CD137 double-positive cells (arrows, *lower panels*).

proliferation. To study the effect of CD137 costimulation on human B cells, P815 cell clones expressing human CD137L were generated. The P815-CD137L cells were recognized by mouse anti-human CD137L mAb and human CD137 fusion protein, confirming cell surface expression of CD137L and the ability to interact with human CD137 receptor, respectively (data not shown). Purified B cells were stimulated with anti-Ig, anti-Ig/IFN-γ, or anti-Ig/anti-CD40 in the presence of P815-CD137L or P815-mock cells. In comparison with B cell cultures with P815-mock cells, the presence of P815-CD137L significantly enhanced ^{3}H-TdR incorporation in all three culture conditions (Fig. 6*A*). Because IFN-γ and anti-CD40 activate B cells, the baseline of ^{3}H-TdR incorporation without P815 transfectants was significantly higher in the group with anti-Ig/anti-CD40, followed by anti-Ig/IFN-γ, compared with stimulation with anti-Ig only. The specificity of the observed effects was confirmed in CD137–CD137L blocking experiments, in which the addition of soluble human CD137 protein to anti-Ig–stimulated B cell cultures completely abrogated the observed differences (Fig. 6*B*). These data suggest that the observed changes in thymidine incorporation are specifically mediated through CD137–CD137L interactions.

Subsequent studies using CFSE dilution analysis confirmed that CD137 enhanced anti-Ig–activated B cell proliferation in all five donors tested ($p = 0.0076$) (Fig. 6*C*). Specifically, anti-Ig–activated B cells only completed one cell division in the presence of P815-mock cells, whereas in the presence of CD137 costimulation (P815-CD137L), cells completed two or more divisions. Additionally, CD137L-stimulated B cells exhibited a greater percentage of cells in the S phase of the cell cycle compared with those cultured with P815-mock cells (Fig. 6*D*). When B cells were activated with anti-Ig/anti-CD40, a significantly higher percentage of cells (66.1%) underwent cell division compared with anti-Ig stimulation (23.9%). However, in this setting, CD137 stimulation did not enhance the cell division and cell cycle progression (data not shown). These data suggest that the initial B cell activation signal determines the net effect of CD137-mediated B cell proliferation.

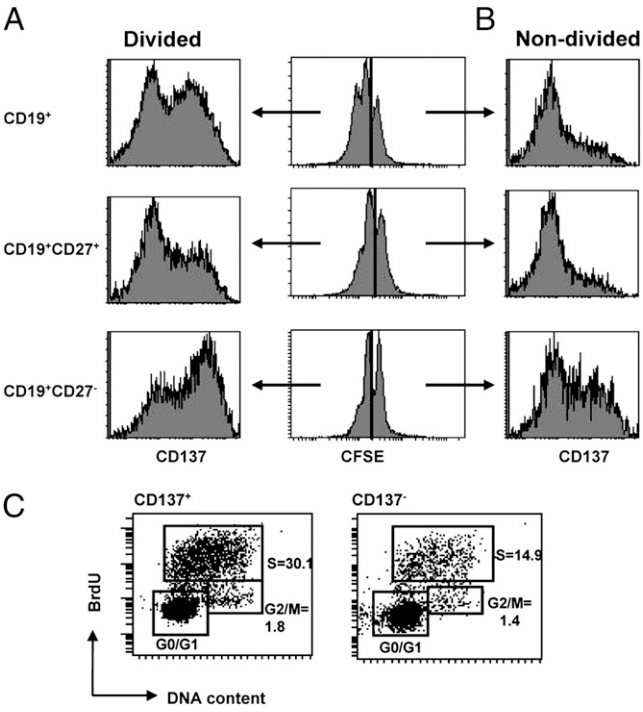

FIGURE 5. CD137 expression is associated with enhanced B cell proliferation. High levels of CD137 are expressed on divided human B cells. Unseparated B cells (bulk; CD19^{+}), memory B cells (CD19^{+}CD27^{+}), and naive B cells (CD19^{+}CD27^{-}) were labeled with CFSE and stimulated with anti-Ig/anti-CD40. After 4 d, cells were harvested and analyzed for CFSE dilution and CD137 expression by flow cytometry. Based on CFSE dilution, divided and nondivided B cell subsets were gated and analyzed for CD137 surface expression. Graphs indicate the percentage of CD137 expression on subsets of divided (*A*) and nondivided (*B*) B cells. Data are representative of at least five individual experiments. *C*, CD137 expression is associated with cell cycle progression in anti-Ig/anti-CD40–stimulated human B cells. Anti-Ig/anti-CD40–stimulated B cells were cultured for 3 d and pulsed with BrdU for 4 h. Cell cycle status of CD137-expressing (CD19^{+}CD137^{+}) and non-CD137–expressing (CD19^{+}CD137^{-}) B cells was determined by flow cytometry using anti-BrdU and 7-AAD. Data are representative of three individual experiments.

CD137 ligation promotes human B cell survival

Based on our observation that CD137 ligation in B cell cultures exposed to anti-Ig/anti-CD40 enhanced thymidine incorporation, yet failed to induce clear cell division and cell cycle progression, we postulated that CD137 may provide a survival advantage for B cells. To test this hypothesis, purified B cells were stimulated with anti-Ig, anti-Ig/IFN-γ, or anti-Ig/anti-CD40 in the presence of mock or CD137L-transfected P815 cells. Cell survival was assessed at various time points by flow cytometric staining with Annexin V and 7-AAD. In the presence of P815-CD137L, the percentage and absolute number of surviving B cells (as determined by Annexin V^{-}/7-AAD^{-}) improved in all three culture conditions (Fig. 7) at days 4 and 6. However, despite the significantly higher CD137 expression level induced by anti-Ig/anti-CD40 stimulation, the difference in the CD137-mediated enhancement of survival in anti-Ig/anti-CD40 activated B cells seemed to be less than that in the B cells activated by anti-Ig alone or anti-Ig/IFN-γ. In fact, anti-Ig/anti-CD40–activated B cells exhibited a higher survival rate compared with anti-Ig alone or anti-Ig/IFN-γ in the absence of CD137 ligation, which is consistent with previous reports (27, 28) that CD40 provides a survival signal for B cells.

CD137 ligation induces secretion of TNF-α and -β

It is known that B cells produce effector cytokines upon activation (29, 30). To further define the function of CD137 stimulation on activated B cells, we stimulated B cells with anti-Ig/anti-CD40 in the presence of irradiated CD137L or mock-transfected P815 cells and measured cytokine release by ELISA. No significant differences in the production of IL-4, -6, and -10 or IFN-γ were detected between mock- and CD137L-stimulated B cells. However, B cells stimulated with CD137L demonstrated profound increases in TNF-α and -β secretion in each of the three donors tested (Fig. 8). These data are particularly interesting given the recognized role of TNF antagonists in the treatment of autoimmune diseases, such as rheumatoid arthritis (RA) and SLE, in which activated B cells are postulated to play an important role in disease pathogenesis.

Discussion

In this study, we demonstrated that, despite the reported absence of CD137 on murine B cells, CD137 is expressed on human B cells. Our studies revealed that CD137 is expressed on activated B cells following BCR stimulation. Cognate help from T cells through CD40–CD40L interaction and/or cytokines are important for the regulation of CD137 expression on human B cells. Among the cytokines tested, only the Th1 cytokine IFN-γ enhanced CD137 expression, whereas IL-4, -10, and -21 inhibited CD137 expression. Importantly, neither anti-CD40 stimulation nor cytokine alone were capable of inducing B cell expression of CD137 in the absence of BCR stimulation. In addition, polyclonal stimulation of human B cells with CpG, which stimulates TLR9-mediated B cell proliferation and differentiation in the absence of Ag, failed to upregulate CD137 (data not shown). These data suggest that CD137 expression on human B cells is

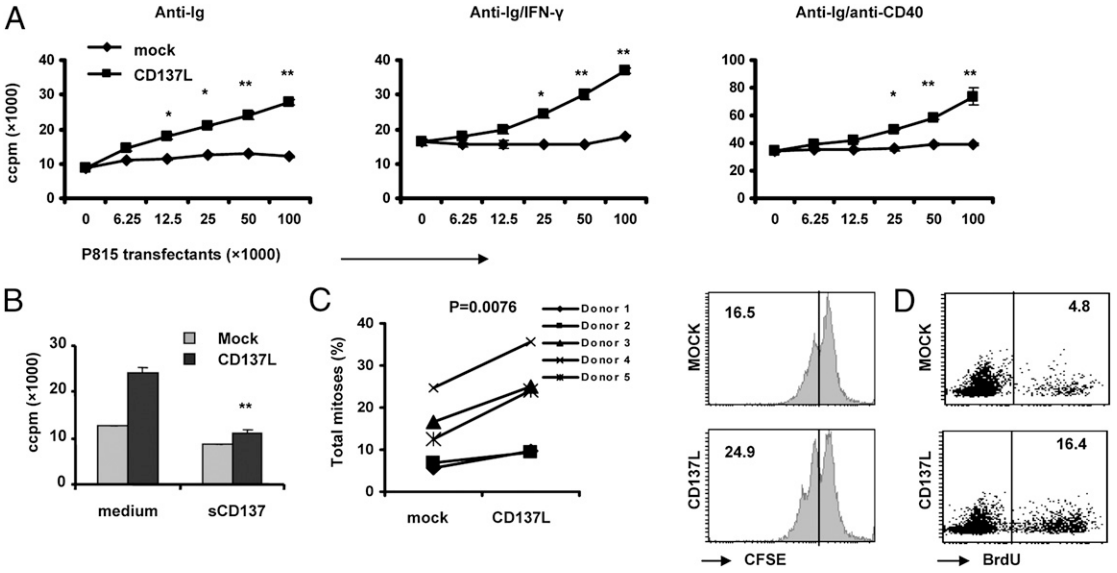

FIGURE 6. CD137 ligation stimulates B cell proliferation. *A*, Purified B cells were stimulated with anti-Ig, anti-Ig/IFN-γ, or anti-Ig/anti-CD40 in the presence of various numbers of irradiated P815-mock cells or P815-CD137L cells. Thymidine incorporation was measured after 3 d (anti-Ig, anti-Ig/IFN-γ) or 4 d (anti-Ig/anti-CD40) of culture. Data are mean ± SD of triplicate wells and are representative of five experiments. Without activation, the baseline thymidine incorporation of B cells is 100–200 Ci/min. *B*, The addition of soluble CD137 (sCD137) protein to cultures with anti-Ig–activated B cells abrogates the enhanced thymidine incorporation by P815-CD137L–activated B cells. Histograms show thymidine incorporation in anti-Ig–stimulated B cell cultures with P815-mock cells or P815-CD137L cells after the addition of sCD137. Data are mean ± SD of triplicate wells and are representative of four individual experiments. *C*, Anti-Ig–stimulated B cells exhibit a greater rate of cell proliferation in the presence of P815-CD137L cells at day 4 by CFSE dilution analysis. A representative FACS analysis of CFSE dilution assay (donor 3) is shown. The values indicate the percentage of mitoses of gated B lymphocytes. *D*, Anti-Ig–stimulated B cells have more cells in the S phase of the cell cycle in the presence of irradiated P815-CD137L cells compared with P815-mock controls. Data are representative of four individual experiments. $*p < 0.05$; $**p < 0.001$.

tightly controlled and strictly dependent upon Ag encounter and the BCR serves as the initial "switch" that enables upregulation of CD137 on the B cell surface.

Similar to T cell-associated CD137, B cells transiently upregulate CD137 with detectable cell surface levels after 24 h of activation, maximal expression levels by day 3, and a return to baseline levels by day 7. This study demonstrated that $CD137^+$ B cells are phenotypically associated with enhanced expression of CD5, CD23, CD71, CD86, and CD95, whereas CD32 expression is decreased, which implies that $CD137^+$ B cells are highly activated. Furthermore, divided B cells express higher levels of CD137 compared with nondivided B cells, although CD137 expression is not dependent on B cell division. Interestingly, upon anti-Ig/anti-CD40 stimulation, naive B cells are more prone to enhanced CD137 expression than memory B cells. We postulate that CD137 costimulation may be especially important for naive B cell regulation, because naive and memory B cells require different signals for cellular activation and differentiation (31, 32).

The functional effects of CD137-mediated costimulation on T cell proliferation and survival are well documented (33–35), and this study demonstrated that CD137 on human B cells mediates analogous functional changes. ^{3}H-thymidine incorporation by anti-Ig–, anti-Ig/IFN-γ–, or anti-Ig/anti-CD40–stimulated B cells is significantly enhanced upon ligation with CD137. Interestingly, B cell division and cell cycle progression are affected differently, depending on the initial B cell activation signal. For example, B cell division is enhanced through CD137 ligation on B cells that have been stimulated with anti-Ig alone. In contrast, B cell division and cell cycle are not affected by CD137 ligation among B cells that are stimulated with anti-Ig and anti-CD40, despite significantly higher CD137 expression levels. Because CD40 signaling is known to mediate B cell proliferation and survival (28), delicate CD137–CD137L-mediated enhancements in cell division and cell cycle progression may be masked by the potent proliferative effects of the CD40 signal itself. Despite the fact that various B cell stimuli (e.g., anti-Ig, anti-Ig/IFN-γ, and anti-Ig/anti-CD40) impact differently on CD137-mediated B cell proliferation, B cell survival was improved by CD137 ligation in all culture conditions. Specifically, greater improvement in B cell survival was observed among B cells activated by anti-Ig alone or in combination with IFN-γ compared with those activated by anti-Ig/

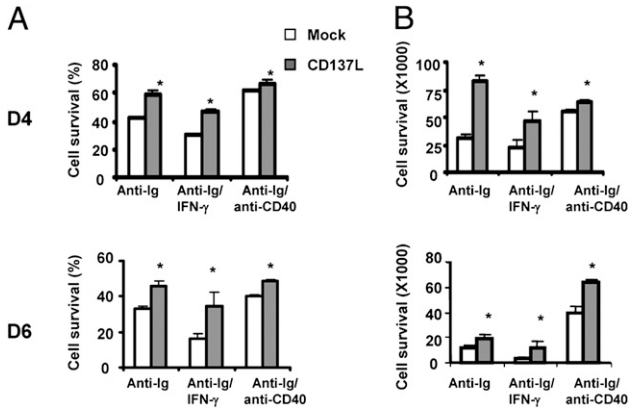

FIGURE 7. CD137 ligation enhances B cell survival. Purified B cells were stimulated with anti-Ig, anti-Ig/IFN-γ, or anti-Ig/anti-CD40 in the presence of P815-mock or P815-CD137L cells. The percentage (*A*) and absolute number (*B*) of surviving B cells were assessed by flow cytometry at culture day 4 and 6. B cells were identified from P815 cells according to forward light scatter versus side scatter, as well as CD19 staining. Annexin V and 7-AAD staining was used to distinguish the surviving cells (Annexin $V^-/7\text{-}AAD^-$) from apoptotic and dead cells. Data are mean ± SD of pooled data from five donors for anti-Ig and anti-Ig/IFN-γ stimulation and from six donors for anti-Ig/anti-CD40 stimulation. Histograms represent B cell survival in cultures with P815-mock or P815-CD137L cells. $*p < 0.05$.

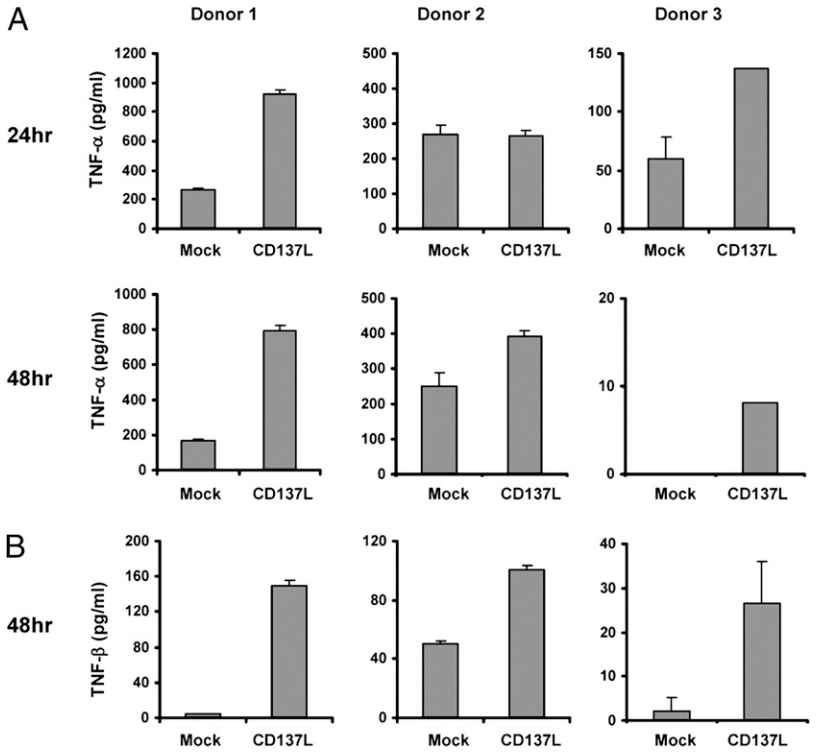

FIGURE 8. CD137 ligation enhances TNF production by anti-Ig/anti-CD40–activated B cells. Purified B cells from three donors were activated with anti-Ig/anti-CD40 in the presence of irradiated CD137L or mock-transfected P815 cells. Cell supernatants were collected at 24 and 48 h, and the level of cytokines was detected by ELISA.

anti-CD40. Overall, our data indicate that CD137-mediated changes in B cell function depend on initial B cell activation and are the net effect of CD137-mediated B cell proliferation and survival.

Ex vivo histological analysis of human tonsil showed that the $CD137^+$ B cells mainly localize to the germinal center. Taken together with the observation that CD137 ligation in vitro promotes B cell proliferation and survival, we postulate that the interaction of CD137 with CD137L expressed on APCs plays an important role during early B cell activation and expansion in the germinal center reaction.

Because B cells are reported to express CD137L (36), we examined the relationship between CD137L and CD137 expression in vitro and ex vivo (Supplemental Fig. 1). CD137L is expressed during the early stage of B cell activation (<16 h after anti-Ig/anti-CD40 stimulation), followed by the upregulation of CD137. In our in vitro culture conditions, the majority of cells did not coexpress CD137 and CD137L. However, there were activated B cells at different stages in vivo. We were able to detect $CD137^+$ and $CD137L^+$ B cells ex vivo from human tonsil, although the receptor and ligand are primarily expressed on distinct populations (Supplemental Fig. 2). In this scenario, the interaction of $CD137L^+$ B cells with $CD137^+$ B cells might bidirectionally costimulate B cells and mediate cell functional changes, because CD137L can also transduce signals in certain cell types (37–39).

In addition to Ab production and Ag presentation, activated B cells produce cytokines and, thus, regulate immune responses (29, 30). Moreover, B cells play a key role in the pathogenesis of diseases such as RA, demonstrated by the clinical efficacy of targeted B cell depletion (40). In response to CD137 stimulation, the production of TNF-α and -β was greatly enhanced by anti-Ig/anti-CD40–activated B cells. Both cytokines independently or complementarily enhanced the immune responses and augmented inflammation by targeting various immune cells. Particularly, TNF-α has been established as a central player in the pathogenesis of RA (41), TNF-β is required for the formation of germinal center-like structures within the inflamed synovium (42), and our unpublished data suggest that the CD137 expression on peripheral B cells is elevated in autoimmunity (e.g., RA). Therefore, our data suggest that CD137-mediated B cell stimulation might be involved in the pathogenesis of RA. Further investigation of the function of B cell-associated CD137 in autoimmunity is underway.

To the best of our knowledge, the current study is the first to demonstrate that CD137 promotes human B cell proliferation and survival and cytokine production. CD137 is distinct from other TNFR family members that regulate B cell proliferation and differentiation, such as CD40 and B cell activating factor-receptor (BAFF-R) (43–45). CD40 and BAFF-R are constitutively expressed on B cells, whereas the upregulation of CD137 is strictly dependent on the presence of anti-BCR stimulation. Similar to BAFF-R, CD137 costimulates the proliferation of B cells in the presence of anti-BCR. In contrast, CD40 stimulates B cell proliferation and differentiation in the absence of anti-Ig. Therefore, it is likely that CD40, CD137, and BAFF-R fulfill distinct functions in vivo. However, comparison of the signaling cascade initiated through these receptors requires further investigation.

Finally, our findings have important implications for the clinical translation of CD137-based immunotherapeutic strategies. The disparity of CD137 expression and function between human and murine B cells challenges the use of murine-based disease models for the evaluation of CD137-mediated immune regulation, and targeting the CD137 pathway with therapeutic intent may have unanticipated consequences on human B cell function. In addition, the fact that the expression of B cell-associated CD137 is regulated by many of the factors involved in the pathogenesis of RA and SLE (e.g., enhanced CD40 expression and altered cytokine production) suggests that B cell-associated CD137 might be of functional import in these diseases (46, 47).

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Disclosures

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