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Expression of the Murine CD27 Ligand CD70 In Vitro and In Vivo[1]

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The interaction between TNFR family member CD27 and its ligand CD70 promotes lymphocyte expansion and effector cell formation. In humans, control of CD27 function is partly regulated by the restricted expression of CD70. We used newly developed mAbs to characterize murine (m) CD70 expression in vitro and in vivo. On resting lymphocytes and immature dendritic cells (DC), mCD70 is absent. In vitro, Ag receptor triggering induced mCD70 mRNA in T cells, but cell surface protein expression was very low. Activated B cells synthesized much higher levels of mCD70 mRNA than activated T cells and clearly expressed mCD70 at the cell surface. mCD70 cell surface expression could also be induced on the DC line D1 and on in vitro-generated murine DC upon maturation. In lymphoid organs of naive mice, virtually no mCD70-expressing cells were found, with exception of cells in the thymic medulla, which may be epithelial in origin. However, after intranasal infection with influenza virus, lung-infiltrating T cells and T and B cells in draining lymph nodes expressed mCD70 according to immunohistology. In such activated lymphocytes, mCD70 protein is largely retained intracellularly. Plasma membrane expression of mCD70 was only detectable by flow cytometry on a small proportion of lung-infiltrating T cells and peaked at the height of the primary response. Thus, expression of CD70 in the mouse is highly regulated at the transcriptional and posttranslational level. This most likely serves to limit excessive effector cell formation after antigenic stimulation. ***The Journal of Immunology*****, 2002, 169: 33–40.**

The immune system has several effector mechanisms at its disposal to free the host of pathogens. Because these effector mechanisms can also damage the host, immune responses should be tightly controlled. TNFR family members and their respective TNF-related ligands have been implicated in the regulation of survival, proliferation, differentiation, and migration during adaptive immune responses (1–4). CD27 is a lymphoid cell-specific member of the TNFR family. Human and murine T, B, and NK cells can express CD27 (5, 6). In humans, CD27 expression distinguishes between naive and effector/memory stages of peripheral B and T lymphocytes. On B cells, CD27 is only found after B cell receptor-induced activation (7, 8). CD27-expressing B cells found in human peripheral blood all have undergone somatic hypermutation and therefore CD27 is considered a marker for memory B cells in humans (9, 10). Resting T cells constitutively express CD27, while differentiation into effector T cells is accompanied by loss of CD27 expression (11, 12).

Interaction of CD27 with its transmembrane ligand, CD70, affects Ag-driven T and B cell responses in several ways. Ligation of CD27 on human activated B cells in vitro results in plasma cell differentiation and enhanced Ig production (7, 13–15). On T cells, triggering of CD27 enhances TCR-induced expansion and promotes the generation of effector T cells with cytolytic function and IFN-γ-secreting capacity (16–20). In CD27-deficient mice, reduced numbers of $CD4^+$ and $CD8^+$ effector T cells were found in the lung after primary and particularly secondary infection with influenza virus (19). In line with this, persistent triggering of CD27 by constitutive B cell-specific expression of mCD70 in CD70 transgenic (TG)[4] mice leads to increased numbers of $CD4^+$ and $CD8^+$ effector T cells (20).

One way to control function of TNFR family members is the transient and tightly regulated expression of the ligand (4, 21, 22). In humans, CD70 is expressed on Ag receptor-activated, but not on resting, T and B cells. Although human (h) CD70 expression can be increased by CD28 or CD40 cross-linking, IL-4 down-regulates hCD70 expression (12, 23). Probably due to CD70's transient expression, human tissue sections only show CD70 expression on some T cells and extrafollicular B cells. Occasionally, germinal centers containing a large number of $CD70^+$ B cells are found (24). CD70 expression is also found in the medulla of human thymus (24). In the mouse, murine (m) CD70 has been detected on lymphocytes in vitro (25, 26). In accordance with studies on human lymphocytes, mCD70 was not detected on resting T cells. However, while in humans CD70 is expressed at significant levels at the plasma membrane of in vitro-activated T cells (23), in the mouse such cell surface expression was found to be very low (26). With regards to expression of mCD70 on resting and activated B cells, studies are partially conflicting (25, 26). Although CD70 has thus far not been detected on human DC, two reports have now documented membrane expression of CD70 on murine DC, one in infected mice and the other on in vitro-stimulated DC (27, 28).

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[4] Abbreviations used in this paper: TG, transgenic; h, human; m, murine; DC, dendritic cell; HPRT, hypoxanthine phosphoribosyltransferase.

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In this study, newly developed anti-mCD70 mAb were used to document the expression pattern of mCD70 in vitro and in vivo. These findings are important to understand in which cell-cell interactions CD27-CD70 can participate. Moreover, this information is needed to establish to which extent the mouse can be used as a model to study the role of human CD27-CD70 interaction in vivo.

Materials and Methods

Mice

C57BL/6 mice were bred in the facilities of The Netherlands Cancer Institute and CLB (Amsterdam, The Netherlands) under specific pathogen-free conditions. Mice used for experiments were between 6 and 8 wk of age at the start of the experiment. All animal experiments were conducted according to institutional and national guidelines and were approved by the Experimental Animal Committees of the respective institutes. Mice were infected intranasally with influenza virus strain A/NT/60/68 as described elsewhere (19).

Generation of anti-mCD70 mAb

Armenian hamsters (Cytogen, West Roxbury, MA) were injected three times i.p. with 10^7 irradiated (50 Gray) syngeneic fibroblasts transfected with the mCD70 cDNA, termed AR-mCD70 cells (29), at weekly intervals. Two weeks after the third injection, serum was tested for mCD70 reactivity. A booster injection (i.p., 10^7 cells) was given and 3 days thereafter, hamsters were sacrificed. Splenocytes were fused with SP2/0 murine myeloma cells as described (6, 29). Fourteen days after fusion, hybridoma supernatants were tested on AR-mCD70 cells and the 771 murine B lymphoma cell line (30) for the presence of anti-mCD70 Ab by flow cytometric analysis using PE-labeled goat anti-hamster Ig as second step reagent. After limiting dilution, two stable clones (3B9, 6D8) were obtained. Both mAbs were purified from culture supernatant using protein A bound to Sepharose CL-4B beads (Sigma-Aldrich, St. Louis, MO). $F(ab')_2$ was generated and conjugated to biotin using standard procedures.

Abs and Fc proteins

mAbs to mouse B220 (RA3.6B2), CD3ϵ (145-2C11; 500A2), CD4 (GK1.5), CD8 (2.43), CD16/CD32 (2.4G2), MHC class II (M5/114.15.2), CD27 (LG.3A10; Ref. 6), CD40 (FGK-40), Thy-1.1 (50 AD 22/15), and DEC-205 (NLDC-145) were purified from hybridoma supernatant and conjugated to FITC and biotin according to standard procedures. mAb to CD40 (HM-40.3) and CD28 (37.51), as well as B7-2-FITC (GL-1), CD4-PerCP (RM4-5), CD8a-PerCP (53-6.7), CD11c-PE (HL3), CD19-FITC (1D3), and CD3-allophycocyanin (145-2C11) were purchased from BD PharMingen (San Diego, CA) and goat anti-mouse IgM $F(ab')_2$ was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). PE-labeled goat anti-hamster Ig was from Southern Biotechnology Associates (Birmingham, AL). $F(ab')_2$ of hamster-anti hCD97 mAb 1D2 (a kind gift from Dr. J. Hamann, Academic Medical Center, Amsterdam, The Netherlands) were labeled with biotin and served as control in flow cytometry and immunohistology. Recombinant receptor Fc proteins were kindly provided by Dr. R. Goodwin (Immunex, Seattle, WA).

Cells and cell culture

771 is a cell line derived from a B lymphoma induced by neonatal inoculation of a C57BL/10 mouse with MCF 1233 murine leukemia virus (30). Expression of mCD70 on this line originally permitted the cDNA cloning (29). ARHO Armenian hamster fibroblasts (6) and mCD70-transfected ARHO cells (AR-mCD70) (29) have been described previously. Single cell suspensions were obtained by grinding tissues through nylon sieves, or flushing femurs and tibias with a needle. Erythrocytes were removed by lysis with ammonium chloride solution. For T cell purification, murine lymph node single cell suspensions were depleted of MHC class II^+, $B220^+$, IgM^+ cells using anti-MHC class II, anti-B220 mAb, goat anti-rat IgG, and rat anti-mouse IgM MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and MACS (BS) deletion columns following the manufacturer's instructions. For B cell purification, $CD19^+$ cells were positively selected from splenocytes using goat anti-CD19 MACS microbeads beads and MACS (MS^+) separation columns following the manufacturer's instructions. Purity of the resulting populations was tested by immunofluorescence with anti-CD19 and anti-CD3 mAbs and exceeded 95%. DC were generated in vitro as described (31). In short, bone marrow suspensions were depleted of $CD4^+$, $CD8^+$, $B220^+$, and MHC class II^+ cells using theindicated mAbs and goat anti-rat IgG MACS microbeads and MACS (BS) depletion columns. Cells were seeded at 10^6/ml and grown in medium containing recombinant mouse GM-CSF (1 ng/ml; BD PharMingen) and IL-4 (1 ng/ml; BD PharMingen). DC were harvested at day 8. Cells were grown in IMDM containing 10% heat inactivated FCS, penicillin, streptomycin, and LPS (serotype 026:B; Sigma-Aldrich), recombinant IL-2, IL-4, IL-12, IFN-γ (BD PharMingen), or IL-10 (Endogen, Woburn, MA). The D1 dendritic cell (DC) line was cultured as described (32).

Biochemical analysis

771 cells (3×10^6/sample) were labeled with ^{125}I (Amersham International, Amersham, U.K.) by the glucose/lactoperoxidase method and lysed in 1% Nonidet P-40 buffer, containing 10 mM triethanolamine-HCl (pH 7.8), 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 20 μg/ml ovomucoid trypsin inhibitor, 1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, and 20 μg/ml leupeptin. After centrifugation, the supernatant was divided. One part was precleared with pooled human serum (5%) and protein G-Sepharose (Pharmacia, Uppsala, Sweden), the other part was precleared with normal hamster serum (10%) and protein A-Sepharose. mCD70 was subsequently precipitated with mCD27-Fc, anti-mCD70 mAb, or an irrelevant control mAb and complexes were adsorbed onto protein G- or A-Sepharose, eluted, and electrophoretically separated by 5–15% SDS-PAGE, and visualized by autoradiography.

Flow cytometry

Cells (3×10^5) were seeded in 96-well round-bottom plates and incubated with anti-CD16/CD32 mAb (5 μg/ml) in FACS buffer (PBS, 0.5% BSA, 0.02% azide). After washing, cells were stained with the indicated mAbs and a subsequent incubation with allophycocyanin-labeled streptavidin (BD PharMingen) was performed to detect biotin-conjugated mAbs. All incubation steps were for 30 min at 4°C. Cell staining was analyzed on a FACSCalibur using CellQuest software (BD Biosciences, Mountain View, CA). Viable cell populations were gated on the basis of forward and side scatter and, where indicated, on the basis of a histogram profile.

Proliferation assay

For proliferation assays, purified murine T cells (10^5/well) were stimulated with Con A (Sigma-Aldrich) at the indicated concentrations and were cultured with mCD70-transfected or mock-transfected cells (5×10^4 cells/well). [^{3}H]Thymidine incorporation was measured after 4 days of culture. [^{3}H]Thymidine (0.4 μCi; Amersham International) was present for the last 16 h of culture.

Real-time PCR

Single cell suspensions of purified T cells (1×10^6/ml) were stimulated with immobilized anti-CD3 mAb (145-2C11, 10 μg/ml). Single cell suspensions of purified B cells (1×10^6/ml) were stimulated with anti-CD40 mAb (HM-40.3, 5 μg/ml) and LPS (10 μg/ml). After the indicated time of culture, cells (2×10^6) were collected and RNA was isolated with RNazol (Cinna/Biotecx Laboratories, Friendswood, TX). Single-strand cDNA was prepared in a 20-μl reaction volume with 500 ng of oligo$(dT)_{12-18}$ and 100 U of Superscript II (Life Technologies, Rockville, MD). Murine CD70 cDNA was quantitated with the LightCycler System (Roche, Mannheim, Germany; www.lightcycler.com, technical note no. LC 10/2000). Using a FastStart DNA Master SYBR Green I kit and 1 μl of cDNA, a 20-μl real-time PCR was set-up amplifying mCD70 or hypoxanthine phosphoribosyltransferase (HPRT) from the cDNA derived from each sample. Real-time kinetic quantification allows measurement during the log-linear phase of a PCR. Analysis of this phase allows accurate determination of the starting concentration, using the equation: number of amplified molecules = initial number of molecules $\times$ efficiency constant $(E)^{\text{cycle number}}$. In our approach, a standard curve of 771 cDNA was prepared to determine the amount of CD70 cDNA ($E = 0.986$) and HPRT cDNA ($E = 0.982$) in the T and B cell samples. The samples were normalized by dividing the amount of CD70 cDNA by the amount of cDNA of the housekeeping gene HPRT. The following primers were used: mCD70 (+ strand primer, 5′-GGATGCCGGAGGAAGGTCGCCC-3′; − strand primer, 5′-CAAGGGC ATATCCACTGAACTC-3′); and HPRT (+ strand primer, 5′-TATGGA CAGGACTGAACGTCTTGC-3′; − strand primer, 5′-GACACAAACAT GATTCAAATCCCTGA-3′). Crossing points were calculated by the second derivative method using LightCycler System software. Real-time PCR was performed on a LightCycler PCR apparatus (Roche).

Immunohistology

For light microscopy, acetone-fixed cryosections of lung were incubated in methanol containing 0.02% H_2O_2, followed by incubation with 2% BSA in PBS. Next, they were incubated overnight at 4°C with biotinylated 3B9

anti-mCD70 F(ab′)$_2$ or biotinylated anti-Thy-1.1, diluted in PBS with 1% BSA. After thorough washing in cold PBS, sections were incubated with Vectastain ABC regent (Vector Laboratories, Burlingame, CA) for 30 min and developed with 0.05% 3,3′-diaminobenzidine with 0.01% H_2O_2 in 0.1 M phosphate buffer. After counterstaining with hematoxylin, sections were coverslipped for observation. For confocal microscopy, cryostat sections were fixed in acetone and incubated with anti-CD16/CD32 mAb (5 μg/ml). Sections were subsequently incubated with biotinylated 3B9 anti-mCD70 F(ab′)$_2$ at saturating concentrations for 2 h at room temperature. After thorough washing in cold PBS, sections were incubated with FITC-labeled mAb and streptavidin-conjugated Alexa Fluor 594 (Molecular Probes, Eugene, OR) overnight at 4°C. Stained sections were washed in cold PBS and coverslipped using antifading mounting medium (DAKO, Glostrup, Denmark). Omission of incubation with biotinylated anti-mCD70 mAb was used as negative control. Suspensions of cells isolated from lung-draining lymph nodes were analyzed for mCD70 expression as follows: Cells were pipetted onto cover slips, which had been coated with poly-L-lysine (50 μg/ml in PBS, 30 min, 37°C) and incubated for 30 min at 37°C. They were fixed for 5 min with methanol kept at −20°C. All subsequent incubations were at room temperature, as outlined for tissue sections. Coverslips were mounted in Vectashield (Vector Laboratories) and viewed under a Leica TCS NT confocal laser-scanning microscope (Deerfield, IL).

Results

Generation and characterization of mAbs directed against mCD70.

To generate mAbs to characterize CD70 protein expression in the mouse, Armenian hamsters were immunized with syngeneic fibroblasts transfected with the mCD70 cDNA (29). Eight percent of the supernatants of the resulting hybridomas were reactive in immunofluorescence with the mCD70-expressing B cell line 771 and the hamster fibroblast transfectant AR-mCD70. The selected mAbs, 3B9 and 6D8, could be used to isolate mCD70 from cell surface-iodinated 771 B cells. As was shown for the murine CD27-Fc protein (29), the anti-mCD70 mAbs not only precipitated monomeric mCD70, but also the SDS-resistant dimeric and trimeric forms of mCD70 (Fig. 1*A*). Flow cytometric analysis showed that 3B9 and 6D8 blocked each other's binding to 771 cells, indicating that they react with the same epitope (data not shown). The mAbs did not cross-react with human CD70 (data not shown).

The effects of the anti-mCD70 mAb on CD27-CD70 interaction were tested in two assays. Firstly, CD27 is known to be down-modulated after contact with CD70 (20, 33). Anti-mCD70 mAb 3B9 and 6D8 were found to specifically inhibit this down-modulation (data not shown). A second well-established effect of CD27-CD70 interaction is enhancement of [^{3}H]thymidine incorporation by activated T cells. As shown in Fig. 1*B*, mCD70 transfectant AR-mCD70 incremented [^{3}H]thymidine incorporation of Con A-stimulated purified murine T cells. This was inhibited by mAb 3B9 (Fig. 1*B*) as well as 6D8 (not shown). Thus, addition of anti-mCD70 mAb 3B9 and 6D8 could block early and late consequences of CD27 triggering, indicating that these anti-mCD70 mAb prevent functional interaction between mCD70 and CD27.

Analysis of mCD70 expression on in vitro-activated cells

In humans, CD70 is absent from resting lymphocytes, but can readily be induced upon in vitro activation. Concordantly, bone marrow cells, thymocytes, splenocytes, and lymph node cells, freshly isolated from naive mice, showed no cell surface expression of mCD70, according to flow cytometry (data not shown). Expression of mCD70 upon T and B cell activation in vitro was analyzed by real-time PCR at the mRNA level and by flow cytometry at the protein level. Purified T and B cells were unstimulated, or incubated for 2 days with anti-CD3 mAb, or anti-CD40 mAb with LPS, respectively. In unstimulated cells, no PCR product was obtained with mCD70 primers, indicating a total lack of mCD70 RNA expression in resting T and B cells (not shown). In stimulated T cells, CD70 mRNA was detectable, but it was present at much lower levels than in stimulated B cells (Fig. 2*A*). In accordance with the difference in mCD70 mRNA expression between in vitro activated T and B cells, cell surface mCD70 protein expression also differed. On activated T cells, only a very low level of mCD70 was found, barely detectable by flow cytometry, whereas mCD70 was easily detectable at the cell surface of activated B cells (Fig. 2*B*). Expression of mCD70 on activated T cells was not more pronounced at other time points after activation, nor when T cells were additionally stimulated with anti-CD28 mAb, IL-2, IL-4, IL-10, IL-12, or IFN-γ (data not shown).

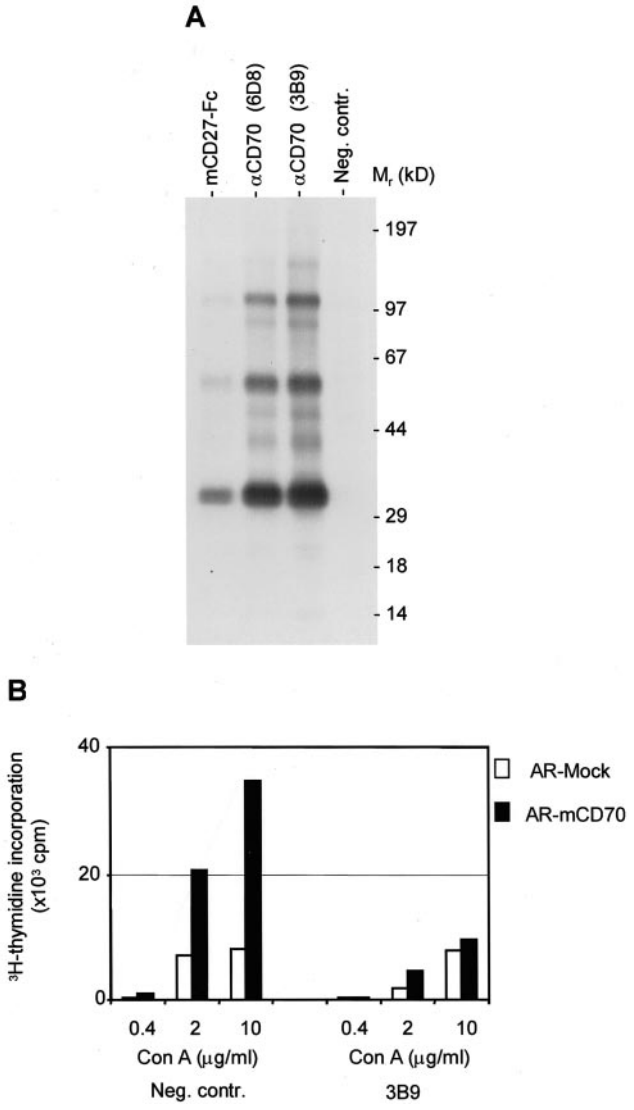

FIGURE 1. Characterization of anti-mCD70 mAbs. *A*, Immunoprecipitation of mCD70 with anti-mCD70 mAb 3B9 and 6D8, and mCD27-Fc protein. Murine CD70 was immunoprecipitated from a Nonidet P-40 lysate of cell surface-iodinated 771 B lymphoma cells. Proteins were separated by SDS-PAGE under reducing conditions and detected by autoradiography. The *outer right lane* shows the immunoprecipitate of a negative control mAb. *B*, Functional effect of anti-mCD70 mAb. [^{3}H]Thymidine incorporation by purified murine T cells, activated for 4 days with Con A in the indicated concentrations is enhanced by mCD70-transfected hamster fibroblasts (AR-mCD70), but not by mock-transfected hamster fibroblasts (AR-mock). This costimulation is blocked by anti-mCD70 mAb 3B9 (5 μg/ml), but not by a negative control mAb (5 μg/ml).

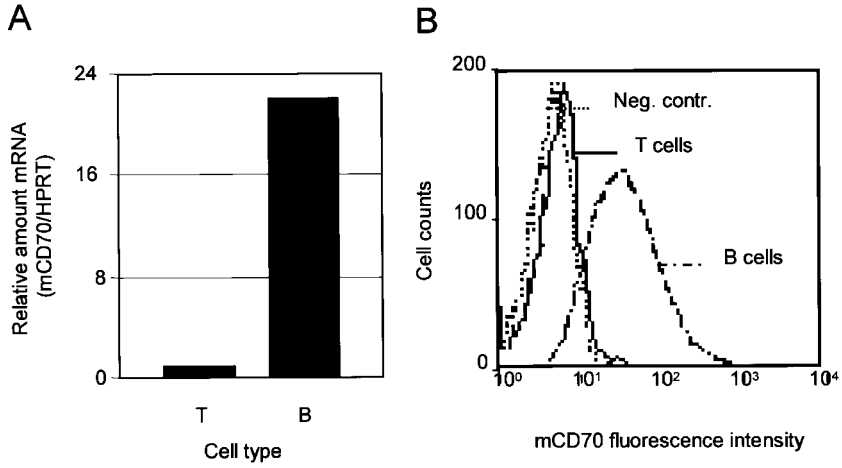

FIGURE 2. Murine CD70 mRNA expression by in vitro-stimulated cells. Purified T and B cells were stimulated with anti-CD3 mAb (immobilized, 10 μg/ml), or anti-CD40 mAb (5 μg/ml) and LPS (10 μg/ml), respectively, and collected 2 days after stimulation. Real-time PCR and flow cytometry were performed using cells from the same experiment. *A*, The result of the PCR analysis. The relative amount of CD70 mRNA, expressed in arbitrary units, was determined by real-time PCR with HPRT as an internal standard. *B*, The mean fluorescence intensity of activated T and B cells stained with anti-mCD70 mAb, relative to the control mAb (1D2, hamster anti-hCD97).

The regulation of expression of mCD70 on B cells was studied in more detail, because literature data are partially conflicting on this issue (25, 26). Purified B cells were stimulated with anti-IgM Ab, anti-CD40 mAb, and the T cell-independent stimulus LPS, either alone or in combination, and mCD70 expression was analyzed in time. Anti-IgM Ab or anti-CD40 mAb alone both induced cell surface expression of mCD70 to a similar extent, which reached maximum levels of ~50–70% positive cells at day 4 (Fig. 3*A*). These agents acted synergistically in mCD70 up-regulation, allowing for expression of mCD70 on almost all B cells, already at 2 days after activation. LPS alone was much less potent in up-regulating mCD70 cell surface expression, but acted in synergy with anti-CD40, allowing for maximal expression at day 2 after stimulation on ~70% of B cells (Fig. 3*B*).

Apart from Ag receptor signals, cytokines also control CD70 expression in humans (12, 23). We tested the influence of different cytokines on mCD70 expression on purified murine B cells activated by anti-IgM Ab or LPS with anti-CD40 mAb. Although IL-2, IL-4, or IL-12 had no effect, IFN-γ consistently inhibited the induction of mCD70 expression by anti-IgM Ab (Fig. 3*C*), but not by LPS and anti-CD40 mAb (Fig. 3*D*). Addition of IL-10 slightly (1.5-fold), but consistently, reduced induction of mCD70 expression by LPS/anti-CD40 mAb (Fig. 3*D*), or LPS alone (data not shown).

Although in the human system CD70 has only been found on cells of the lymphoid lineage, in the mouse CD70 has been found on activated $CD11c^+$ DC (27, 28). The availability of the murine DC line D1 (32) and culture procedures to obtain primary murine DC from bone marrow cells in vitro permitted us to test regulation of mCD70 expression on DC. As is shown in Fig. 4, D1 cells (Fig. 4*A*) and in vitro-generated immature DC ($CD11c^+$, MHC class II^{dull}) (Fig. 4*B*) do not express detectable mCD70 at the cell surface. However, after maturation, as induced by LPS, anti-CD40 mAb or GM-CSF (34) in particular primary DC, express high levels of mCD70.

Analysis of mCD70 expression in vivo

As mentioned above, we could not detect mCD70 by flow cytometry on cell suspensions derived from primary and secondary lymphoid organs of naive mice. To further document mCD70 expression in lymphoid organs of nonimmunized mice, immunohistology was performed. In multiple independent analyses of mice (10 animals of different strains), mCD70 expression in spleen and lymph nodes appeared to be extremely rare. Occasionally, some scattered cells in the red pulp of the spleen and dispersed cells in peripheral lymph nodes expressed mCD70 (data not shown). However,

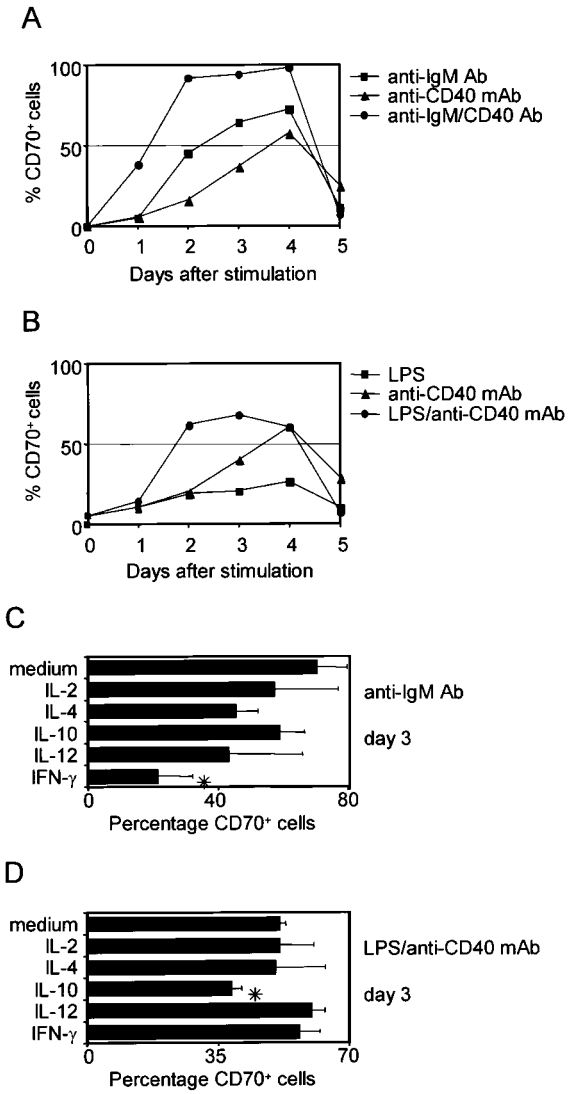

FIGURE 3. mCD70 expression on in vitro-stimulated B cells. Purified B cells were stimulated as indicated and analyzed by flow cytometry for the percentage of mCD70-expressing cells at the indicated time points after stimulation. The graphs show representative results of three or more experiments. *A* and *B*, Expression kinetics after stimulation with anti-IgM $F(ab')_2$ (5 μg/ml), anti-CD40 mAb (HM40-3, 5 μg/ml), LPS (10 μg/ml), or a combination of both stimuli. *C* and *D*, mCD70 expression on anti-IgM or LPS/anti-CD40-stimulated B cells at day 3 after addition of IL-2 (50 U/ml), IL-4 (2 ng/ml), IL-10 (2.5 ng/ml), IL-12 (2 ng/ml), or IFN-γ (7 ng/ml).

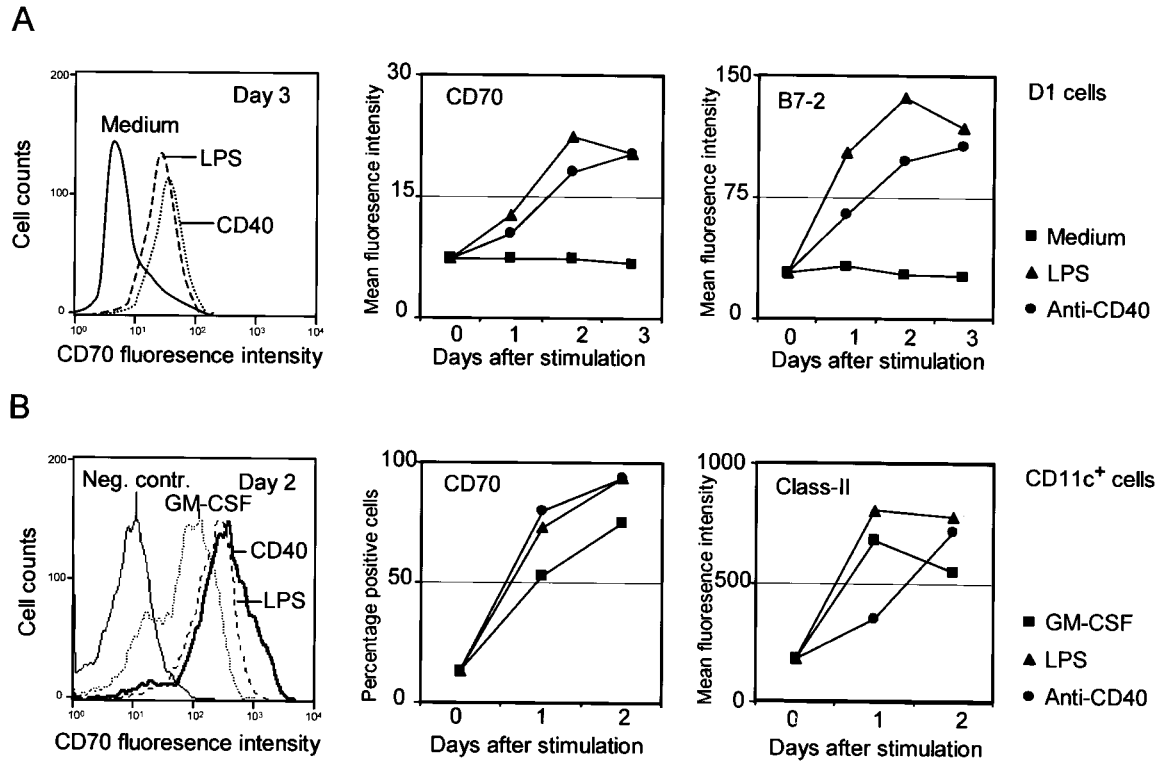

FIGURE 4. mCD70 expression on DC. *A*, D1 cells were either untreated or stimulated with LPS (10 μg/ml) or anti-CD40 mAb (FGK-40, 30 μg/ml) and analyzed by flow cytometry for mCD70 and B7-2 expression at the indicated time points after stimulation. B7-2 was used as a marker for maturation. *B*, Primary CD11c$^+$ DC were generated from bone marrow cells with GM-CSF and IL-4. After 8 days, cells were harvested and recultured in the presence of GM-CSF (2 ng/ml), LPS (5 μg/ml), or anti-CD40 mAb (HM40-3, 2.5 μg/ml). CD70 and MHC class II expression as a control were analyzed on CD11c$^+$ cells at the indicated time points after restimulation. MHC class II and mCD70 expression was analyzed within the CD11c$^+$ cell population.

consistently mCD70-expressing cells were found in the medulla of the thymus (Fig. 5). These cells also stained with an Ab to DEC-205, a carbohydrate receptor, which is expressed by DC and thymic epithelial cells (35).

Next, we examined mCD70 expression in vivo after antigenic stimulation. For this purpose, mice were infected intranasally with influenza virus. This well-characterized model is hallmarked by activation of Ag-specific T cells in lung-draining lymph nodes, followed by a transient appearance of activated T cells in the lung. In the absence of CD27, the primary T cell response in lymphoid organs and lung is significantly reduced (19). After infection, lung tissue and cell suspensions of lung-draining lymph nodes were analyzed for mCD70 expression. Lung tissue sections were prepared and stained by conventional immunohistochemistry with Abs directed at Thy-1.1 or mCD70 and examined by light microscopy (Fig. 6*A*). In these sections, conspicuous fields of mCD70-positive cells were detected in parabronchiolar regions. These were T cells, according to Thy-1.1 staining of serial sections (Fig. 6*A*). Such fields of mCD70$^+$ cells were never observed in noninfected lung tissue (not shown). Lung tissue sections were also stained with fluorescent Abs specific for T cells (CD3), B cells (B220), and DC (CD11c) and examined by confocal microscopy. In this analysis, lung-infiltrating T and B cells were detected in significant amounts. However, DC were scarce. Double staining for mCD70 revealed that a sizeable proportion of lung-infiltrating T cells expressed mCD70 (Fig. 6*B*). No mCD70-positive B cells or DC were found. Cells in lung-draining lymph nodes were examined for mCD70 expression by fluorescent staining of cytospins, followed by confocal microscopy (Fig. 6*C*). This analysis revealed significant amounts of T and B cells, identified by CD3 and B220 staining, while DC (CD11c$^+$) were very rare. Double staining for mCD70 indicated that a significant proportion of T and B cells in these lung-draining lymph nodes expressed mCD70 protein. The small number of DC present permitted no reliable conclusion on mCD70 expression in DC. Strikingly, both in T and B lymphocytes, the great majority of mCD70 staining appeared cytoplasmic, rather than at the cell membrane. This can be seen particularly well in B cells, which show peripheral staining for B220 which only rarely overlaps with the mCD70 staining (Fig. 6*C*).

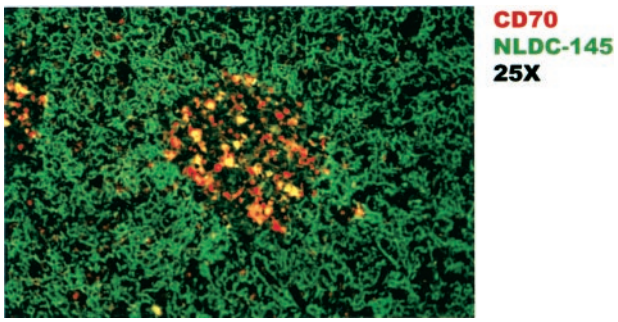

FIGURE 5. mCD70 expression in the thymus of naive mice. Thymus section of naive (nonimmunized) mouse stained with FITC-conjugated NLDC-145 mAb directed at the DEC-205 receptor (green), as well as biotinylated 3B9 anti-mCD70 F(ab′)$_2$ (red). The epithelial cells of the thymic cortex are exclusively stained by DEC-205, while the medulla contains double-positive cells.

The immunohistology data showed that mCD70 is expressed by activated T cells in lung and lung-draining lymph nodes during an ongoing immune response to influenza virus. We next questioned

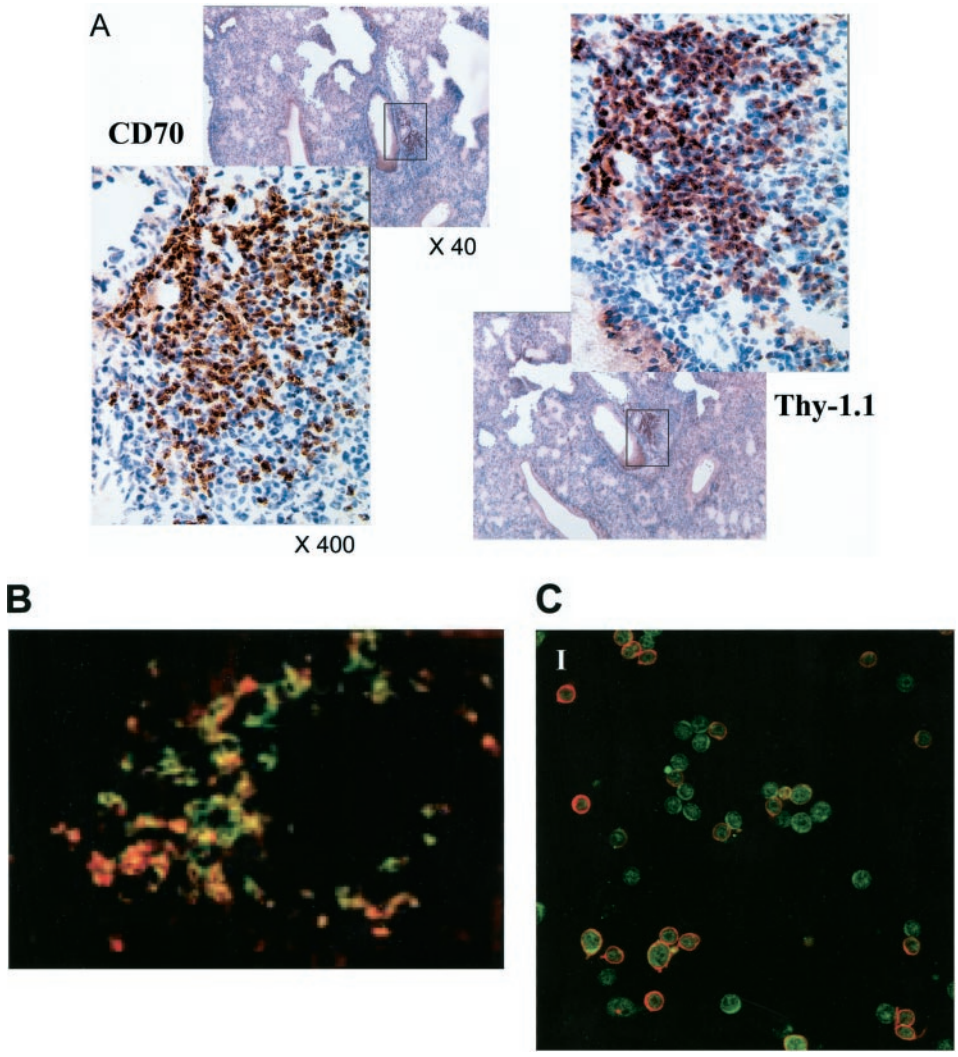

FIGURE 6. mCD70 expression in lung and lung-draining lymph nodes of infected mice. Mice were infected intranasally with influenza virus and sacrificed at day 14 (*A*), or day 8 (*B* and *C*) after infection. Lung and lung-draining lymph nodes were isolated. *A*, Light microscopic view of lung sections. Serial sections were stained with biotinylated anti-mCD70 3B9 F(ab′)$_2$) or anti-Thy1.1 mAb and were developed with streptavidin-peroxidase. Magnifications are indicated. *B*, Tissue sections of lung were stained with biotinylated anti-mCD70 (3B9 F(ab′)$_2$) (red) and FITC-conjugated anti-CD3 mAb (green). *C*, Draining lymph node cell suspensions were coated onto cover slips and stained with FITC-conjugated anti-mCD70 (3B9 F(ab′)$_2$) (green) and biotinylated anti-B220 mAb (red) to detect B cells. The mCD70-positive, B220-negative cells in this preparation are T cells according to anti-CD3 staining (not shown).

whether, in contrast to in vitro-stimulated T cells, in vivo-activated T cells express CD70 at the plasma membrane. Therefore, FACS analysis of lung-infiltrating cells during an ongoing response to influenza virus was performed. At different time points after infection, lung-infiltrating cells were isolated and double-stained with anti-CD3 and anti-CD70 mAbs (Fig. 7). This experiment showed clearly that mCD70 is detectable by flow cytometry at the plasma membrane of a small proportion of lung-infiltrating T cells. Maximal expression was found at the height of the response (day 8), as defined by the size of the T cell infiltrate and the number of Ag-specific T cells (19).

Discussion

In this report, we describe the generation of anti-mCD70 mAbs and the analysis of mCD70 expression in vivo and in vitro. The mAbs were obtained by immunizing Armenian hamsters with

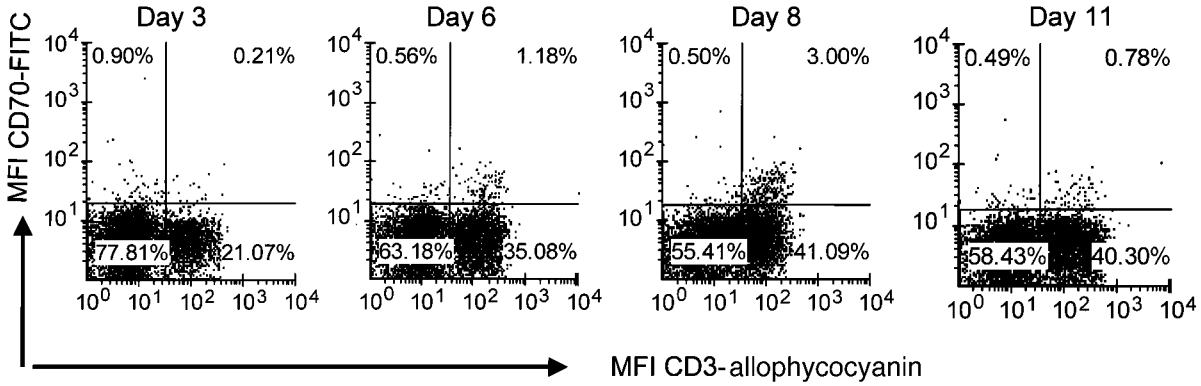

FIGURE 7. mCD70 is present at the cell surface of lung-infiltrating T cells. Mice were infected intranasally with influenza virus and sacrificed at day 3, 6, 8, or 11 after infection. Lung-infiltrating T cells were isolated as described (19) and double-stained with anti-mCD70-FITC (3B9 F(ab′)$_2$) and anti-CD3-allophycocyanin mAbs. Total cell suspensions were analyzed by flow cytometry. Mean fluorescence intensity (MFI) is indicated.

transfected syngeneic fibroblasts, a method described by Gravestein et al. (6). In this system, one epitope on mCD70 seemed to be immunodominant since the mAbs 3B9 and 6D8 could not only block each other's binding to mCD70, but both mAbs also blocked the binding of all other anti-mCD70 mAbs we generated. Accordingly, all mAbs tested also inhibited the binding of mCD70 to CD27. Still, subtle differences between the different mAbs may exist. When examining lymphoid organs of naive mice, we only consistently found expression of mCD70 on scattered cells in the thymic medulla. A similar finding was made in humans, where these cells were suspected to be of epithelial origin because CD70 was also found on epithelial cell lines derived from human thymus (24). That thymic epithelial cells may express CD70 is further supported by the recently described expression of CD70 on human thymic carcinomas (36). In the mouse, the CD70-positive cells in the thymic medulla expressed DEC-205, which is a receptor molecule found on bone marrow-derived DC as well as thymic epithelial cells (35). Therefore, it is plausible that both in humans and mice these cells are of epithelial origin. Use of anti-CD27 mAb in vivo suggested a role for CD27-CD70 interaction in murine T cell development (37). However, this proposed role, i.e., synergy of CD27 signaling with pre-TCR signaling, was not confirmed by analysis of the T cell compartment in CD27-deficient mice (19). Still, a role for CD27-CD70 interaction selection of the thymic TCR repertoire cannot be ruled out.

In line with the in vivo data, we could not detect mCD70 in vitro on resting T cells or B cells or immature DC. Hartwig et al. (25) found, in contrast to us and Oshima et al. (26) that resting murine B cells express CD70. However, this is unlikely because CD70 TG mice that constitutively express mCD70 on B cells have substantial disturbances in the composition of their immune system, notably a progressive decline in B cell numbers (20). With regard to regulation of mCD70 expression on activated B cells, our findings partly confirm data presented by Hartwig et al. (25) and Oshima et al. (26) and is most compatible with CD70 expression data for human B cells. We find induction of mCD70 expression after stimulation with anti-IgM Ab, anti-CD40 mAb, or LPS, and a synergistic effect of these B cell activators. In humans, the Th2 cytokine IL-4 negatively regulates CD70 expression on activated T and B lymphocytes (12, 23). Strikingly, we found in this study that in the mouse, the Th1 cytokine IFN-γ negatively regulates CD70 expression on IgM-activated B cells. In CD70 TG mice, persistent CD27 triggering results in an increased proportion of IFN-γ-producing T cells, which inhibit B cell maturation in the bone marrow (20). Therefore, the down-regulation of mCD70 on activated B cells by IFN-γ may represent a negative feedback mechanism.

The two signal model dictates that productive activation of naive T cells requires one signal derived from the TCR (signal 1) and one costimulatory signal (signal 2). In the current concept of an adaptive immune response, these signals are provided by mature DC in secondary lymphoid organs. The constitutive expression of CD27 on naive T cells, the in vitro consequences of CD27-CD70 interaction and the observed CD70 expression on mature murine DC suggest that CD27-CD70 can provide signal 2. In our in vivo analyses, too few DC were present in the preparation to allow for conclusions on mCD70 expression. Further studies on ex vivo-purified DC preparations are necessary to investigate this issue.

In vitro, purified T cells from CD27-deficient mice show decreased expansion in response to TCR stimulation, as compared with wild-type T cells (19), strongly suggesting that mCD70 is present at the surface of in vitro-activated murine T cells. In agreement with Oshima et al. (26), we found that mCD70 was difficult to detect by flow cytometry on such T cells. Cell surface expression was not enhanced by anti-CD28 mAb, IL-2, IL-4, IL-10, IL-12, or IFN-γ (data not shown). We addressed different posttranslational mechanisms that might explain the lack of mCD70 membrane protein expression. First, intracellular staining was done to examine whether mCD70 was retained within the cell. Second, membrane staining after culture in the presence of metalloprotease inhibitors or anti-mCD70 mAb was performed to examine whether mCD70 was shed from the membrane. Third, examined the possibility that mCD70 was shielded by soluble CD27 (17) by analyzing mCD70 expression on activated CD27-deficient T cells. In none of these experiments, we could detect substantial levels of mCD70 protein. Taken together, these data suggest that induction of CD70 mRNA in in vitro-activated T cells is too low to allow for easy detection of the protein by conventional flow cytometry.

In contrast to the in vitro experiments, analyses of mice after influenza virus infection showed a strong expression of CD70 both in the lung and in the draining lymph nodes. These data indicate that factors, other than the cytokines that were tested in the in vitro culture experiments, contribute to the induction of mCD70. In draining lymph nodes, clear expression of mCD70 was found inside the cell, and flow cytometry of cell suspensions from these nodes also revealed very low plasma membrane expression (results not shown). Recent adoptive transfer experiments with CD27-deficient cells show that CD27 contributes to activated T cell expansion in lung-draining lymph nodes, arguing that mCD70 is indeed functionally expressed at this site (J. Hendriks, Y. Xiao, and J. Borst, manuscript in preparation). In the lung, mCD70 could clearly be detected at the cell surface of activated T cells. The percentage of mCD70-positive T cells in the lung according to flow cytometry was much lower than the percentage of mCD70-positive T cells seen in sections of lung tissue. Even though mCD70 could be induced at high levels on the surface of B cells activated in vitro, B cells in lung-draining lymph nodes were barely positive according to flow cytometry. However, intracellularly they expressed mCD70. Our data argue that mCD70 is induced at the protein level in T and B cells upon in vivo activation, but is to a large extent retained intracellularly.

In summary, our combined in vitro and in vivo data indicate that mCD70 plasma membrane expression is tightly controlled by both transcriptional and posttranslational mechanisms. The consequences of constitutive high-level mCD70 expression at the cell surface of B cells in CD70 transgenic mice highlight why control of mCD70 expression levels and therewith control of CD27-CD70 interaction is crucial. In mCD70 transgenic mice that constitutively express mCD70 at a high level on B cells under the CD19 promoter, T cells are stimulated to differentiate into Th1-type effector cells, which produce IFN-γ. IFN-γ production by these T cells inhibits B cell maturation in the bone marrow, leading to progressive B cell depletion (20). In addition, the T cell compartment in these mice declines in time, most likely as a result of high turnover of the effector T cells (38).

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