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Inhibition of B-cell death does not restore T-cell-dependent immune responses in CD40-deficient mice

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SUMMARY

Signalling through CD40 is essential for the development of immunoglobulin G (IgG) antibody responses, germinal centres and B-cell memory against T-dependent antigens. In addition, engagement of CD40 in B cells promotes cell survival by inducing the expression of anti-apoptotic members of the *bcl-2* family of cell-death regulators. In the present study we analysed whether T-dependent immune responses can be developed in mice deficient in CD40 if the anti-apoptotic activity mediated by the engagement of CD40 in B cells is compensated by the constitutive over-expression of anti-apoptotic genes of the *bcl-2* family. We showed that the over-expression of either *hbcl-2* or *hbcl-x*$_L$ transgenes in B cells is not sufficient to restore IgG antibody responses and germinal centre formation in CD40-deficient mice. These results indicate that CD40 functions, other than those mediated through survival, are required for the establishment of T-dependent B-cell responses.

INTRODUCTION

Triggering of B cells begins when their membrane immunoglobulin receptor binds a specific antigen. This signal is sufficient for the activation of B cells stimulated with antigens composed of repetitive epitopes (T-independent antigens) and, in these cases, the secreted immunoglobulins are mainly of the immunoglobulin M (IgM) isotype. In contrast, for the majority of protein-derived antigens, costimulatory signals provided by CD4$^+$ T cells are required for appropriate activation of antigen-specific B cells. In this costimulatory process, the interaction between the CD40 molecule (a member of the tumour necrosis factor [TNF] receptor-1 family) and its ligand, CD40L (a TNF family molecule expressed on activated T cells), has been considered essential.[1,2] Germinal centre (GC) formation and humoral immune responses against T-dependent antigens are impaired in mice deficient in CD40 or CD40L molecules,[3,4] as occurs in patients with the hyper-IgM syndrome, owing to the lack of expression of CD40L by activated T cells.[5] In addition, the engagement of CD40 in B cells with either soluble CD40L or anti-CD40 provides survival signals that rescue both immature and mature B cells from apoptotic stimuli such as IgM cross-linking.[1,6,7] In this anti-apoptotic activity mediated through CD40, pro-survival members of the *bcl-2* gene family, such as *bcl-x*$_L$ and *A1,* which are induced through CD40 signalling,[8–11] play an important role.

Recently, it has been demonstrated that the engagement of CD40 in B cells activates several signalling pathways that could regulate the different activities mediated through CD40.[12] However, the relationship between the anti-apoptotic function of CD40 and other CD40-dependent activities in B cells, remains elusive. To explore such issues, we produced mice

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Abbreviations: *A*, absorbance; C57BL/6-SV40-Eμ-*hbcl-2* mice, B6.*bcl-2* Tg; C57BL/6-SV40-Eμ-*hbcl-x*$_L$ mice, B6.*bcl-x*$_L$; C57BL/6.129P2-Tnfrsf5tm1Imx mice, B6.CD40$^{-/-}$; GC, germinal centre; heat-aggregated human gamma globulin, AHGG; human *bcl-2*, *hbcl-2*; human *bcl-x*$_{>L}$, *hbcl-x*$_L$; pneumococcal polysaccharide, PP; tetanus toxoid, TT.

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deficient in CD40 in which the anti-apoptotic function of CD40 in B cells has been compensated for by the expression of human *bcl-2* (*hbcl-2*) or human *bcl-x$_L$* (*hbcl-x$_L$*) transgenes.[8,13] These transgenes induce a sustained over-expression of hBcl-2 or hBcl-x$_L$ at all stages of B-cell differentiation, including GC centroblasts. In these mice we evaluated the generation of GCs and the production of IgM and IgG antibodies after primary and secondary immunizations with T-dependent and T-independent antigens.

MATERIALS AND METHODS

Mice

C57BL/6.129P2-Tnfrsf5tm1Imx (B6.CD40$^{-/-}$) and C57BL/6-SV40-Eμ-*hbcl-2* transgenic (B6.*bcl-2* Tg) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). C57BL/6-SV40-Eμ-*hbcl-x$_L$* (B6.*bcl-x$_L$*) Tg mice were described recently.[8] B6.CD40$^{-/-}$ and B6.*bcl-2* Tg mice were crossed in our animal facilities and the resulting B6.CD40$^{+/-}$ hBcl-2$^{+/-}$ F$_1$ hybrids were backcrossed with B6.CD40$^{-/-}$ mice to obtain the four genetic combinations used in this study:

(1) experimental mice: B6.CD40$^{-/-}$ hBcl-2$^{+/-}$;
(2) controls for the CD40 deficiency: B6.CD40$^{-/-}$ hBcl-2$^{-/-}$;
(3) controls for the hyperexpression of hBcl-2: B6.CD40$^{+/-}$ hBcl-2$^{+/-}$; and;
(4) normal controls: B6.CD40$^{+/-}$ hBcl-2$^{-/-}$.

Similar hybrids were obtained by crossing B6.CD40$^{+/-}$ hBcl-x$_L^{+/-}$ mice with B6.CD40$^{-/-}$ mice. The expression of hBcl-2 and the deficiency in CD40 in the experimental mice was assessed in peripheral blood B cells by flow cytometry using specific monoclonal antibodies (mAbs): anti-human Bcl-2 (clone 6C8) and anti-mouse CD40 (clone HM40-3) conjugated to fluorescein isothiocyanate (FITC) and phycoerythrin (PE), respectively (Pharmingen, San Diego, CA). The identification of *hbcl-x$_L$* Tg mice was performed by polymerase chain reaction (PCR), as described previously.[8] Animals were maintained in a germ-free environment and all *in vivo* experiments with mice were performed in compliance with the Guide for the Care and Use of Laboratory Animals (ILAR, 1985).

Expression of hBcl-2 during B-cell ontogenia and cell-death assays

The expression of hBcl-2 in mature resting and GC B cells in hBcl-2 Tg mice was evaluated by flow cytometry in the spleen, as described previously,[14] using the following mAbs (Pharmingen): FITC-labelled hamster anti-hBcl-2; PE-conjugated rat anti-mouse B220 (clone RA3-6B2); biotinylated rat anti-mouse IgM (clone R6-60.2); and PE-conjugated rat anti-mouse IgD (clone 217-170). Streptavidin-RED670^{TM} was purchased from Invitrogen (Carlsbad, CA). The labelling of GC B cells was performed by combining the anti-B220 mAb with peanut agglutinin (PNA) (Vector Laboratories, Burlingame, CA). For intracellular hBcl-2 labelling, the Intrastain Fixation and Permeabilization Kit (Dako, Gloostrup, Denmark), which does not modify PNA fixation, was employed.

The effect of hBcl-2 over-expression on B-cell survival in hBcl-2 Tg mice was assessed *in vitro* using spleen cells enriched in B lymphocytes, as described previously.[8,13]

Immunization with T-independent and T-dependent antigens

Mice were immunized intraperitoneally (i.p.) with pneumococcal polysaccharide (PP) contained in the Pneumo-23 vaccine (Pasteur Merieux, Lyon, France) at a dose of 100 μg in a volume of 100 μl. Tetanus toxoid (TT; Anatoxal TE, Berna, Switzerland) and heat-aggregated human gamma globulin (AHGG) were used as T-dependent antigens for immunizations. TT was injected in the base of the tail at a dose of 1 LF/mouse in a volume of 100 μl of saline solution containing 200 μg of Al(OH)$_3$. For immunization with AHGG, lyophilized HGG (Sigma Chemical Co., St Louis, MO) was heat aggregated and emulsified in complete Freund's adjuvant (CFA) at a final concentration of 1 mg/ml for i.p. injection at a dose of 400 μg/mouse or for subcutaneous administration at a dose of 200 μg in the footpad. In some experiments, mice primed i.p. with AHGG-CFA were boosted i.p. with 400 μg of AHGG emulsified in incomplete Freund's adjuvant (IFA) 2 months after primary immunization. In all situations mice were bled weekly from the retro-orbital plexus, and the resulting sera were stored at -20° until use.

Serological studies

Serum levels of anti-PP IgM and anti-TT IgG or anti-HGG IgG were determined by enzyme-linked immunosorbent assay (ELISA), at the indicated days, in microplates (Linbro Titertek, ICN Biomedicals Inc, Aurora, OH) coated overnight with 10 μg/ml of PP or HGG, or with 5 μg/ml of TT. Sera were diluted 1 : 200 in 0·15-M phosphate-buffered saline (PBS), pH 7·4, containing 0·05% Tween-20 and 0·02% sodium azide. Goat anti-mouse IgM and IgG (Sigma) were used (as alkaline phosphatase conjugates) after dilution 1 : 1000 in the same buffer used to dilute the sera. Results are expressed as absorbance (*A*) at 405 nm.

Histological analysis

Popliteal lymph nodes were obtained from mice 15 days after immunization with AHGG-CFA in the footpads, fixed in 4% phosphate-buffered formalin, and embedded in paraffin. The generation of GCs was analysed in histological sections (4–6 μm) stained with haematoxylin and eosin (H & E).

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test. Probability values of $< 0{\cdot}05$ were considered significant.

RESULTS

In the present study we investigated whether substitution of the anti-apoptotic effect mediated through CD40 (by the over-expression of an *hbcl-2* transgene in B cells) allows the development of T-dependent antibody responses in CD40-deficient mice. For this purpose, B6.*bcl-2* Tg mice were crossed with B6.CD40$^{-/-}$ mice, and the resulting F$_1$ mice bearing the *hbcl-2* transgene were backcrossed with B6.CD40$^{-/-}$ mice. B6.*bcl-2* Tg mice were chosen as all of their mature B cells, including GC B cells, were positive for hBcl-2 (as assessed by flow cytometry Fig. 1a) and exhibited a prolonged *in vitro* survival (Fig. 1b), as previously reported.[13] The over-expression of hBcl-2 was restricted to B cells and promoted an increase in the number

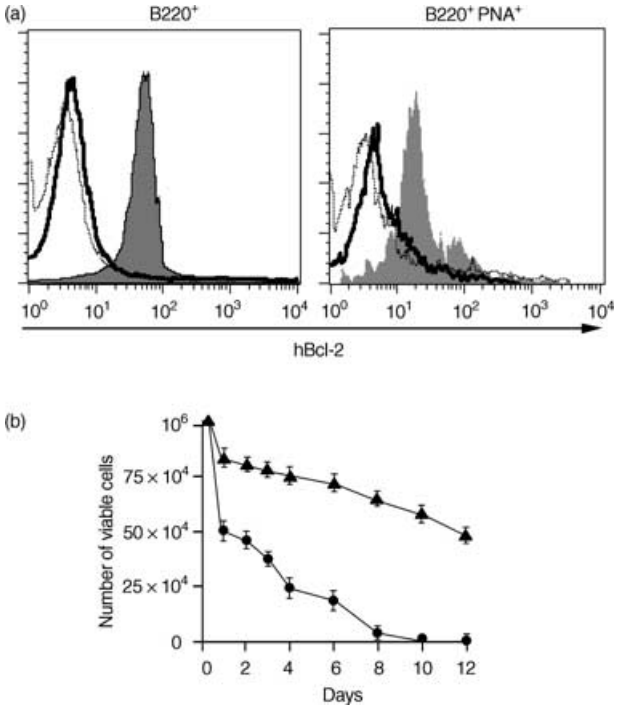

Figure 1. Expression of human Bcl-2 (hBcl-2) and *in vitro* survival of B cells from C57BL/6-SV40-Eμ-*hbcl-2* mice (B6.*bcl-2* Tg) mice. (a) The expression of hBcl-2 in popliteal lymph node $B220^+$ B cells (left panel) and $B220^+$ PNA^+ germinal centre (GC) B cells (right panel) from B6.*bcl-2* Tg (dark histograms) and non-Tg (solid lines) mice was analysed by flow cytometry 15 days after immunization with heat-aggregated human gamma globulin (AHGG) in complete Freund's adjuvant (CFA). Histogram plots were generated from the analysis of 50 000 viable cells. Dotted lines represent background fluorescence. Results are representative of three independent experiments. (b) Enriched spleen B cells (10^6 cells/well) from B6 non-Tg (●) and B6.*bcl-2* Tg (▲) mice were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and collected every 24 hr. *In vitro* B-cell survival was assessed by Trypan blue exclusion. Data represent the mean and standard deviation of surviving cells in triplicate cultures from three independent experiments.

of mature splenic B cells of both B6.CD40$^{-/-}$ and B6.CD40$^{+/-}$ mice in comparison to the non-Tg controls (data not shown). However, the phenotype of these B cells was essentially identical between B6.CD40$^{-/-}$ or B6.CD40$^{+/-}$ hBcl-2 Tg mice and their respective non-Tg controls (data not shown).

Development of T-independent, but not T-dependent, immune responses in CD40$^{-/-}$ mice over-expressing hBcl-2 in B cells

We first evaluated the effects of hBcl-2 over-expression in the development of T-independent immune responses in CD40-deficient mice. Groups of B6.CD40$^{+/-}$ hBcl-2$^{+/-}$, B6.CD40$^{+/-}$ hBcl-2$^{-/-}$, B6.CD40$^{-/-}$ hBcl-2$^{+/-}$ and B6.CD40$^{-/-}$ hBcl-2$^{-/-}$ mice were immunized i.p. with the T-independent antigen, PP. All groups of immunized mice produced high levels of anti-PP IgM 20 days after immunization (Fig. 2a). The titres of anti-PP IgM were slightly higher in both groups of hBcl-2$^+$ Tg mice than in mice lacking over-expression of hBcl-2, in correlation with the increased numbers of mature B cells present in these animals.[13]

To explore whether the over-expression of hBcl-2 in B lymphocytes restored the capacity of CD40-deficient mice to mount IgG responses against T-dependent antigens, the above-described groups of mice were immunized with either TT mixed with $Al(OH)_3$ as adjuvant, or with AHGG emulsified in CFA. In both instances, mice in which B cells expressed the CD40 molecule produced significant levels of specific IgG (Fig. 2b, 2c) 20 days after immunization. As expected,[3] B6.CD40$^{-/-}$ hBcl-2$^{-/-}$ mice were unable to produce anti-TT IgG (Fig. 2b) or anti-HGG (Fig. 2c). Interestingly, the increase in B-cell survival in B6.CD40$^{-/-}$ hBcl-2$^{+/-}$ mice, secondary to hBcl-2 over-expression, did not restore normal IgG responses against TT or HGG (Fig. 2b, 2c) 20 days after immunization. It should be noted that in B6.CD40$^{-/-}$ hBcl-2$^{+/-}$ mice, the levels of anti-TT or anti-HGG IgG were also undetectable 10 and 30 days after immunization (data not shown), indicating that the deficient T-dependent activation of B cells observed in these animals did not reflect differences in the kinetics of such antibody responses. The failure of B6.CD40$^{-/-}$ hBcl-2$^{-/-}$ and B6.CD40$^{-/-}$ hBcl-2$^{+/-}$ mice to develop IgG responses against T-dependent antigens was also observed after a secondary challenge with antigen. Thus, B6.CD40$^{-/-}$ hBcl-2$^{+/-}$ mice primed with AHHG in CFA and boosted i.p. 2 months later with AHGG in IFA did not produce significant levels of anti-HGG IgG, 10 and 15 days after secondary immunization (Fig. 3).

The generation of GCs in the four groups of mice was also investigated. To achieve this, animals were injected in the footpad with 200 μg of AHGG in CFA and, 2 weeks later, the popliteal lymph nodes were dissected and used for histological studies. In mice expressing the CD40 molecule, the immunization induced the formation of typical secondary follicles that were much more prominent in *hbcl-2* Tg mice (Fig. 4). By contrast, the formation of GCs was not observed in either B6.CD40$^{-/-}$ hBcl-2$^{+/-}$ or B6.CD40$^{-/-}$ hBcl-2$^{-/-}$ mice (Fig. 4).

T-independent and T-dependent immune responses in CD40$^{-/-}$ mice over-expressing hBcl-x$_L$ in B cells

As the engagement of CD40 with anti-CD40 mAb or soluble CD40L molecules on B cells promotes the expression of Bcl-x$_L$ and A1 instead of Bcl-2,[8–11] it can be argued that Bcl-2 is not as efficient as Bcl-x$_L$ in compensating the B-cell defects associated with the absence of CD40 signalling. To explore this possibility, B6.CD40$^{-/-}$ mice were crossed with mice Tg for *hbcl-x$_L$* in B cells.[8] Like B6.CD40$^{-/-}$ hBcl-2$^{+/-}$ mice, CD40-deficient mice over-expressing hBcl-x$_L$ in B lymphocytes developed effective antibody responses against T-independent antigens (PP) but not IgG responses against the T-dependent antigens TT and HGG (Table 1).

DISCUSSION

In the present study we explored the effects of the inhibition of B-cell apoptosis in the functionality of B lymphocytes from CD40-deficient mice. Our results clearly indicate that, *in vivo*,

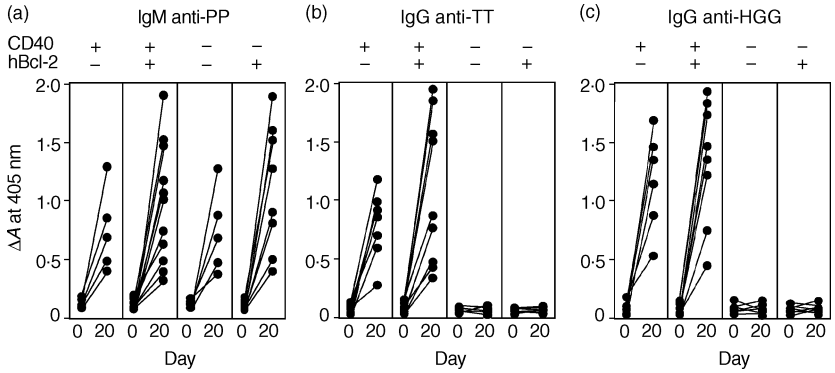

Figure 2. Immune response to T-independent and T-dependent antigens in B6.CD40$^{-/-}$ mice over-expressing human Bcl-2 (hBcl-2) in B cells. (a) Serum levels of anti-pneumococcal polysaccharide (PP) immunoglobulin M (IgM) in B6.CD40$^{+/-}$ hBcl-2$^{-/-}$ (+/–), B6.CD40$^{+/-}$ hBcl-2$^{+/-}$ (+/+), B6.CD40$^{-/-}$ hBcl-2$^{-/-}$ (–/–) and B6.CD40$^{-/-}$ hBcl-2$^{+/-}$ (–/+) mice on days 0 and 20 after intraperitoneal (i.p.) immunization with 100 μg of PP. (b) Serum levels of anti-tetanus toxoid (TT) immunoglobulin G (IgG) in B6.CD40$^{+/-}$ hBcl-2$^{-/-}$ (+/–), B6.CD40$^{+/-}$ hBcl-2$^{+/-}$ (+/+), B6.CD40$^{-/-}$ hBcl-2$^{-/-}$ (–/–) and B6.CD40$^{-/-}$ hBcl-2$^{+/-}$ (–/+) mice on days 0 and 20 after i.p. immunization with 200 μg of TT-Al(OH)$_3$. (c) Serum levels of anti-human gamma globulin (HGG) IgG in B6.CD40$^{+/-}$ hBcl-2$^{-/-}$ (+/–), B6.CD40$^{+/-}$ hBcl-2$^{+/-}$ (+/+), B6.CD40$^{-/-}$ hBcl-2$^{-/-}$ (–/–) and B6.CD40$^{-/-}$ hBcl-2$^{+/-}$ (–/+) mice at days 0 and 20 after i.p. immunization with 400 μg of heat-aggregated HGG (AHGG) in complete Freund's adjuvant (CFA). Results from individual animals are represented as absorbance (*A*) units at 405 nm.

the over-expression of hBcl-2 or hBcl-x$_L$ in B cells is not sufficient to restore IgG immune responses against T-dependent antigens in the absence of CD40 B-cell expression. These results further stress the critical dependency of the CD40L–CD40 interaction in the establishment of T-dependent immune responses.[3,4] In this regard, dendritic cells expressing ectopically CD40L can promote protective humoral immune responses against bacterial infections in the absence of T cells.[15]

The lack of IgG responses and GC formation in B6.CD40$^{-/-}$ hBcl-2$^{+/-}$ and B6.CD40$^{-/-}$ hBcl-x$_L^{+/-}$ mice suggest that the increased survival observed after CD40 engagement in B cells is not sufficient to induce other CD40-dependent activities, such as immunoglobulin class switching, affinity maturation and B-cell proliferation. In this regard, it has been shown that in B lymphocytes, B-cell survival, immunoglobulin class switching and proliferation are activities regulated by different, and partially non-overlapping, CD40-dependent signalling pathways.[12] Thus, CD40-induced B-cell proliferation, but not B-cell survival, is highly sensitive to pharmacological inhibitors of the extracellular signal-regulated protein kniase (ERK), p38 or phosphoinositide 3-kinase (PI-3 kinase) pathways,[12] and tumour necrosis factor receptor-associated factor (TRAF)2 and TRAF3, but not TRAF6, are involved in the regulation of CD40-mediated immunoglobulin class switching.[16] In support of this notion it has been recently demonstrated that LMP1, an Epstein–Barr virus-encoded protein, which signals through pathways similar to CD40, can rescue IgG immune responses, but not GC formation, in CD40-deficient mice.[17] The failure of hBcl-2 or hBcl-x$_L$ B-cell over-expression to compensate the defects observed in B lymphocytes from CD40$^{-/-}$ mice is very similar to that reported in T cells from CD28-deficient mice, in which over-expression of hBcl-x$_L$ cannot restore proliferative and effector functions.[18]

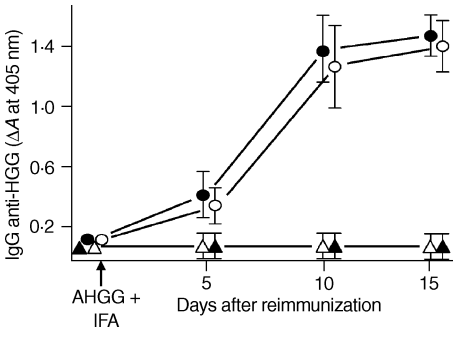

Figure 3. Serum levels of anti-human gamma globulin (anti-HGG) immunoglobulin G (IgG) after a secondary immunization with heat-aggregated HGG (AHGG) in B6.CD40$^{+/-}$ hBcl-2$^{-/-}$ (○), B6.CD40$^{+/-}$ hBcl-2$^{+/-}$ (●), B6.CD40$^{-/-}$ hBcl-2$^{-/-}$ (△) and B6.CD40$^{-/-}$ hBcl-2$^{+/-}$ (▲) mice. Mean levels [± 1 standard deviation (SD)] of anti-HGG IgG are expressed as absorbance (*A*) units at 405 nm (five to 10 mice in each group).

Another non-exclusive possibility is that the *in vivo* deficiency of CD40 not only prevents the activation and differentiation of B cells, but also the triggering and/or terminal differentiation of T lymphocytes. In fact, it has been demonstrated that in T–B-cell cognate interactions, the binding between CD40 and CD40L mediates the activation of both types of lymphocytes.[19] Accordingly, the absence of the CD40–CD40L interaction facilitates the establishment of peripheral T-cell tolerance, as demonstrated in experiments in which the blockade of CD40L interactions inhibits alloreactive T-cell responses.[20] The detection of CD40 within the thymus on cells that mediate selection of thymocytes, such as dendritic cells and macrophages, strongly suggests that signalling through the CD40L may be involved also in the selection of the T-cell repertoire.[21] In this respect, interference of the CD40–CD40L interaction with anti-CD40L mAbs impairs negative selection of thymocytes reactive to self-antigens and superantigens.[21]

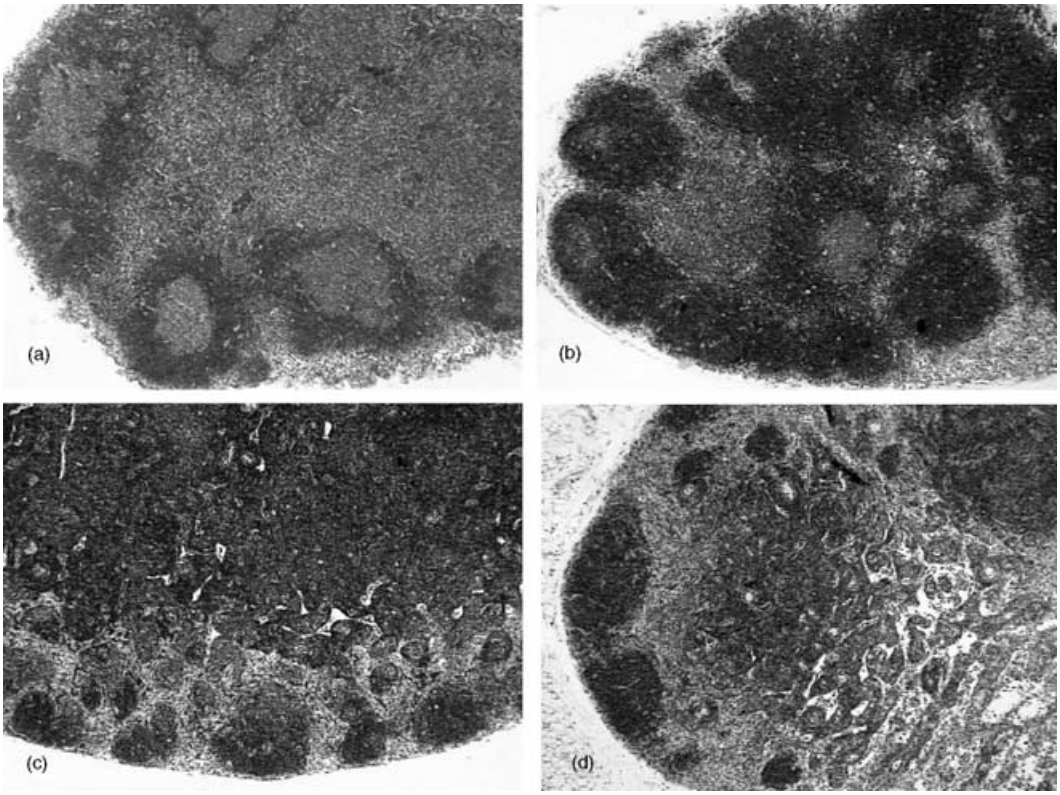

Figure 4. Over-expression of human Bcl-2 (hBcl-2) by B cells is not sufficient to restore germinal centre (GC) formation in CD40-deficient mice. The generation of GCs in popliteal lymph nodes of 2-month-old B6.CD40$^{+/-}$ hBcl-2$^{-/-}$ (a), B6.CD40$^{+/-}$ hBcl-2$^{+/-}$ (b), B6.CD40$^{-/-}$ hBcl-2$^{-/-}$ (c) and B6.CD40$^{-/-}$ hBcl-2$^{+/-}$ (d) mice, immunized with 200 μg of heat-aggregated human gamma globulin (AHGG) in complete Freund's adjuvant (CFA) in the footpads, are analysed in histological sections (4–6 μm), stained with haematoxylin and eosin (H & E) (objective 10×), 15 days after immunization.

Table 1. T-dependent and T-independent antibody responses in CD40$^{+/-}$ and CD40$^{-/-}$ mice over-expressing or not over-expressing *hBcl-x$_L$* in B cells

Mice	Anti-PP IgM*	Anti-TT IgG†	Anti-HGG IgG‡
B6.CD40$^{+/-}$ hBcl-x$_L^{-/-}$	0·878 ± 0·321	1·109 ± 0·251	1·452 ± 0·521
B6.CD40$^{+/-}$ hBcl-x$_L^{+/-}$	1·216 ± 0·512	1·625 ± 0·756	1·595 ± 0·328
B6.CD40$^{-/-}$ hBcl-x$_L^{-/-}$	0·724 ± 0·128	0·125 ± 0·063	0·097 ± 0·031
B6.CD40$^{-/-}$ hBcl-x$_L^{+/-}$	1·105 ± 0·431	0·201 ± 0·105	0·118 ± 0·043

*Mice (8–10 weeks old) were immunized intraperitoneally (i.p.) with 100 μg of pneumococcal polysaccharide (PP). Anti-PP immunoglobulin M (IgM) were determined in sera, 20 days after immunization, by enzyme-linked immunosorbent assay (ELISA). Results from three to six mice/group were expressed [mean values ± 1 standard deviation (SD)] in absorbance (*A*) units at 405 nm.

†Mice (8–10 weeks old) were immunized at the base of the tail with 1 LF/mouse of tetanus toxoid (TT) in 100 μl of saline solution containing 200 μg of $Al(OH)_3$. Serum levels of anti-TT immunoglobulin G (IgG) were determined 20 days after immunization by ELISA. Results are expressed of three to five mice/group (mean values ± 1 SD) in *A* units at 405 nm.

‡Mice (8–10 weeks old) were immunized i.p. with 400 μg of heat-aggregated human gamma globulin (AHGG) emulsified in complete Freund's adjuvant (CFA). Serum levels of anti-HGG IgG were determined 20 days after immunization by ELISA. Results from three to five mice/group were expressed (mean values ± 1 SD) in *A* units at 405 nm.

Finally, it should be stressed that the interaction between CD40 expressed on dendritic cells and CD40L on T cells seems to be essential for the migration of CD4$^+$ cells within B-cell follicles, an event crucial for the appropriate maturation of B-cell antibody responses.[22] In light of these findings, it would be of interest to perform studies, similar to those reported here, in animals over-expressing anti-apoptotic genes in both T and B lymphocytes in the absence of CD40 signalling in systems where antigen-specific B and T lymphocytes can be easily identified.

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