Effects of BCL-2 over-expression on B cells in transgenic rats and rat hybridomas

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Abstract

The rat is an important biomedical experimental model that benefited from the recent development of new transgenic and knockout techniques. With the goal to optimize rat mAb production and to analyze the impact of Bcl-2 on B-cell development, we generated bcl-2 transgenic rats. Transgenic rats showed Bcl-2 over-expression in B cells, increased B cell numbers in lymphoid organs, elevated production of immunoglobulins (Igs) and prolonged B-cell survival in vitro. Transgenic rats remained healthy, reproduced normally and did not develop autoimmunity. Fusions with bcl-2 transgenic splenocytes did not result in increased hybridoma generation. A comparison of on- and off-rates of 39 mAbs generated with bcl-2 transgenic and wild-type animals revealed no significant differences. Over-expression of Bcl-2 in hybridomas did not change cell proliferation but resulted in increased Ig production. Bcl-2 transgenic rats will be a useful tool for the generation of rat mAbs, the analysis of B cells in different pathophysiological models, such as autoimmunity, cancer or organ transplantation, and the study of rat B-cell biology.

Keywords: Apoptosis, B lymphocytes, immunoglobulins, monoclonal antibodies

Introduction

Members of the Bcl-2 pro- and anti-apoptotic family of genes (Bcl-2, Bcl-xL, Bim, Bax and A1) are differentially expressed during T- and B-cell development and play an important role in cell survival and development. The Bcl-2 gene was originally identified (1–3) as the locus linked to the immunoglobulin (Ig) heavy chain locus by the 14:18 translocation associated with lymphomas of follicular center B-cell origin (4, 5). Subsequently, Bcl-2 was shown to protect lymphocytes from certain forms of death by neglect, including growth factor withdrawal, but did not promote proliferation (6). The expression of Bcl-2 is normally tightly regulated: Bcl-2 is expressed in pre-B cells, down-regulated in resting B cells and expressed in proliferating B cells but down-regulated in differentiated B cells (7–12). Lymphomas with the t(14;18) display inappropriately elevated levels of Bcl-2-Ig fusion RNA for their mature B-cell stage of development (7, 13).

The effect of Bcl-2 on B- and T-cell development has been studied in several transgenic mouse models using various expression constructs (14–16). In transgenic mice where Bcl-2 was also expressed in T cells, the number of T cells in peripheral lymphoid tissues was moderately elevated despite enhanced T-cell survival in vitro (14, 15). Some (16) but not all of these transgenic lines developed autoimmune disease (14, 15).

In mouse bcl-2 transgenic lines, an increased number of B lymphocytes (2- to 5-fold) was observed in the spleen, lymph nodes and bone marrow and B cells demonstrated an extended survival in vitro (14–16). These findings provided consistent evidence that the accumulation of B cells after Bcl-2 overproduction results from prolonged cell survival and not increased cell cycling. Bcl-2 transgenic lines showed follicular hyperplasia but did not develop B-cell lymphomas. Mice with elevated Bcl-2 expression in B cells also displayed hypergammaglobulinemia with ~2-fold elevations of serum IgM, IgG and IgA (16).

Deregulated Bcl-2 expression in some Bcl-2 transgenic mice resulted in the development of autoimmune disease
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(16). This disease is similar to systemic lupus erythematosus; in that, the mice developed serum antibody to nuclear double-stranded (ds) DNA and their kidneys showed evidence of immune complex glomerulonephritis (16). Analysis of B-cell development in bcl-2 transgenic mice demonstrated that selection of memory B cells in germinal centers was perturbed, consistent with a model of selection where competition of B cells for an antigen-mediated survival signal is required for entry into the memory compartment (17).

In contrast, the stringent selection of high-affinity bone marrow antibody-forming cells was not influenced by the bcl-2 transgene, consistent with a selective process requiring the germinal center B cell to exceed an affinity threshold. Analysis of autoantibody production in bcl-2 transgenic mice revealed that autoantibodies did not originate from anti-dsDNA B cells rendered tolerant in the bone marrow but rather from somatic mutation in B cells that transited through a germinal center (18).

The effect of Bcl-2 over-expression on hybridoma production was also studied using transgenic mice (19). Serum antibody titers in immunized mice were shown to be similar in transgenic versus wild-type animals. However, Bcl2 over-expression resulted in increased numbers of hybridomas after fusion with myeloma cells. In addition, an increased number of hybridomas generated with bcl-2 transgenic mice produced antigen-specific IgG. Bcl-2 over-expression in hybridomas also increased sub-cloning efficiency.

The rat is an important biomedical experimental model that benefitted from the recent development of new transgenic and knockout models and techniques (20–23). In addition, rats are routinely used for the generation of monoclonal antibodies. In this study, we assessed effects of Bcl-2 over-expression in transgenic rats. We also investigated whether Bcl-2 over-expression would allow a more efficient generation of stable hybridomas expressing mAbs. Over-expression of Bcl-2 in rats resulted in increased B-cell numbers in lymphoid organs, elevated production of Igs and increased survival of B cells in vitro. Bcl-2 transgenesis in splenocytes did not result in increased hybridoma generation following fusion with myeloma cells. On- and off-rates of mAbs generated with bcl-2 transgenic and wild-type animals were similar. Nevertheless, hybridomas over-expressing Bcl-2 showed increased Ig production.

Materials and methods

Construction of the SV40-Eμ-hBcl2 transgene and generation of transgenic rats

The construct used to generate the transgenic rats (kindly provided by Professor J. Adams, Melbourne, Australia) contained the human Bcl-2 cDNA under the regulatory control of the Ig heavy chain enhancer and the SV40 promoter and has been previously used to generate bcl-2 transgenic mice (16). The 2.7 kb fragment containing the Eμ-SV40-Bcl2 insert was microinjected using Sprague–Dawley rats as described (24). All procedures complied with the institutional ethical guidelines and were in accordance with the guidelines for animal experiments of the French Veterinary Services.

Founder animals were identified by PCR using the following primers: 5’-GTGTGGAGAGCGTCAACCG-3’ (forward) and 5’-CCGTACAGTTCCACAAAGGCAT-3’ (reverse). The standard amplification profile consisted of 2 mn at 50°C and then 10 mn at 95°C followed by 40 cycles of 15 s at 95°C, 1 mn at 60°C, 15 s at 77°C and 15 s at 80°C. Offsprings were obtained by crossing founders with wild-type rats. Transgene zygosity was determined by quantitative PCR (25).

Southern blot analysis

Genomic DNA from tail biopsies was purified as described (24). Transgene integration and copy numbers were determined by Southern blot analysis as described (24) using 10 μg of DNA digested with EcoRI for line 70815 and BstXI for line 70818. Transgenes were visualized with enhanced chemiluminescence and quantified using a Fuji LAS 4000 (Fujifilm) imaging system and Multi Gauge V3.0 software (Fujifilm).

ELISA for serum Igs

Serum Ig concentrations were determined as described (26) by a quantitative ELISA. Purified rat monoclonal antibodies of IgM, IgG, IgA and IgE isotypes (from Abd Serotec, Jackson ImmunoResearch, BD Biosciences) were added at different concentrations and used as standard curves. Serum Ig concentrations were determined by extrapolating absorbance values of sera dilutions in the linear range of the dilution curves to those of isotype standard curves and multiplied by the dilution factor.

ELISA for anti-beta-galactosidase antibodies

ELISA plates were coated with 1.0 μg 100 μl⁻¹ per well of beta-galactosidase (Roche) in carbonate coating buffer overnight at 4°C. Binding was blocked with 3% skimmed milk powder in PBS–Tween (pH 7.4) at 100 μl per well for 1 h at room temperature. Hybridoma tissue culture supernatant and rat monoclonal controls were added at 100 μl per well. Immune serum was diluted 1/2000 in tissue culture medium and added at 100 μl per well. Subsequently, plates were incubated for 1 h at 37°C. Bound antibody was detected with 100 μl per well HRP-conjugated goat anti-rat IgG (H + L) (Pierce), diluted 1/5000 in PBS–TWEEN. Following incubation for 1 h at 37°C with shaking, TMB buffer (BioFX®) was added at 50 μl per well and incubated in the dark at room temperature. The color reaction was stopped with 50 μl 1 M HCl per well after 10 min and read at Optical Density 450 nm.

ELISA for dsDNA autoantibodies

For anti-ds DNA quantification in sera by ELISA, we used a previously described technique (27). Briefly, ELISA plates were coated with 10 μg ml⁻¹ of salmon sperm DNA in PBS (1 h at 37°C and overnight at 4°C). Plates were then saturated with PBS–0.5% Tween 20–2% BSA (1 h) and washed in PBS–0.5% Tween 20 before incubation with sera (90 min at 37°C). Bound antibodies were revealed, after washing, using peroxidase-coupled anti-rat Fcγ (Jackson Immunoresearch) and TMA/TMB substrate. Each serum was tested in duplicate and was assessed at four different dilutions. Sera were titrated by comparison with a reference curve built with a pool of sera from BN rats injected with HgCl2. Results are
with 10% FCS (Thermo Fisher Scientific), 2 mM glutamine, 100 µM amino acids, 1 mM sodium pyruvate, 200 µg ml⁻¹ streptomycin, 200 u ml⁻¹ penicillin, 5 × 10⁻⁵ M 2-mercaptoethanol and 1 µM staurosporin (all from Sigma–Aldrich). Live, apoptotic, late apoptotic and necrotic cells were determined by a double labelling with antigen-presenting cells (APC)-conjugated annexin-V (BD Biosciences) and DAPI followed by flow cytometry analysis.

**Flow cytometry analysis**

Antibodies used for flow cytometry analysis were APC-conjugated mouse anti-rat marginal zone B cells, PE-conjugated mouse anti-rat CD45R, FITC-conjugated mouse anti-rat CD45RA/B220 (clone OX-33), FITC-conjugated mouse anti-rat TCRαβ, PE-conjugated mouse anti-rat CD90 (clone OX-7), FITC-conjugated mouse anti-rat CD3 (clone G4.18) and FITC-conjugated mouse anti-rat CD11b/c (all purchased from BD Biosciences). The following antibodies were obtained from Abd Serotec (Oxford, UK): APC-conjugated mouse anti-rat IgG (clone MARD-3), APC-conjugated purified mouse anti-rat CD43 (clone W3/13). The FITC-conjugated mouse anti-rat IgM chain was bought from Jackson ImmunoResearch Laboratories and the APC-conjugated mouse anti-rat dendritic cells (clone OX-62) and FITC-conjugated mouse anti-rat CD161 (clone 3.2.3) were non-commercial antibodies.

The different B-cell subsets in the spleen, bone marrow, lymph nodes and thymus were identified using one and two color flow cytometry as previously described (26). Single-cell suspensions (3.10⁶) were incubated with different combinations of antibodies specific for cell surface markers at the appropriate dilutions in 25 µl of PBS–1% BSA 0.1% azide for 30 min on ice. Cells were then washed twice with PBS–1% BSA 0.1% azide and then fixed with PBS–1% BSA 0.1% azide–1% formol. For each sample, 30 000 cells were analyzed by flow cytometry using a BD FACS CANTO II system (BD Biosciences). Further analysis was performed with FlowJo software (Tree Star).

**Generation and analysis of hybridomas**

Hybridomas were generated at Aragen Bioscience, ImmunoPrecise and Maine Biotechnology Services, Inc. Bcl-2 transgenic and wild-type Sprague–Dawley rats were immunized with beta-galactosidase (Roche). Primary immunization was done with 100 µg beta-galactosidase in CFA, followed by booster immunizations in incomplete Freund's adjuvant. Splenocytes were fused with rat (YB2/0) or mouse (SP2/0 or X63) myeloma cells using standard procedures. Hybridoma supernatants were screened for anti-beta gal antibodies by ELISA. Antibody-producing hybridomas were sub-cloned by limiting dilution. Analyses of IgG levels were done in cell cultures at 3.1 and 6 × 10⁵ ml⁻¹ after 72 h of culture. Expression of Bcl-2 in hybridomas was analyzed by western blot.

**Binding kinetics of anti-beta galactosidase mAbs** were determined using surface plasmon resonance (Biacore). Polyclonal anti-rat IgFc was coupled to CMS sensor chips to yield 10 000 response units on all four flow cells. Rat mAbs were captured in the range of 30–100 response units for antibodies with moderate to high binding activities.

**mAb isotyping**

ELISA plates were coated with an antigen at 1.0 µg 100 µl⁻¹ per well in carbonate coating buffer overnight at 4°C. Binding was blocked with 3% skimmed milk powder in PBS (pH 7.4) at 100 µl per well for 1 h at room temperature. Hybridoma tissue culture supernatant and rat monoclonal controls were added at 100 µl per well. Immune serum was diluted 1/2000 in tissue culture medium and added at 100 µl per well. Subsequently, plates were incubated for 1 h at 37°C with shaking. Bound antibody was detected with 100 µl per well goat anti-rat IgG1, goat anti-rat IgG2a, goat anti-rat IgG2b or goat anti-rat IgG2c (all from Bethyl Laboratories) conjugated to HRP and diluted 1/5000 in PBS–Tween. Following incubation for 1 h at 37°C with shaking, TMB buffer (BioFX®) was added at 50 µl per well and incubated in the dark at room temperature. The color reaction was stopped with 50 µl 1 M HCl per well after 10 min and read at OD₄₅₀ nm.

**Western blot analysis**

Western blots were performed as previously described (26) using a mouse anti-human Bcl2 mAb (Abd Serotec) followed by incubation with HRP-conjugated goat anti-mouse IgG + IgM (H + L) antibodies (Jackson ImmunoResearch). As a control, expression of GAPDH was also assessed. The binding was visualized with enhanced chemiluminescence and quantified using a Fuji LAS 4000 (Fujifilm). Western blots were performed as previously described (26) using a mouse anti-human Bcl2 mAb (Abd Serotec) followed by incubation with HRP-conjugated goat anti-mouse IgG + IgM (H + L) antibodies (Jackson ImmunoResearch). As a control, expression of GAPDH was also assessed. The binding was visualized with enhanced chemiluminescence and quantified using a Fuji LAS 4000 (Fujifilm). Western blots were performed as previously described (26) using a mouse anti-human Bcl2 mAb (Abd Serotec) followed by incubation with HRP-conjugated goat anti-mouse IgG + IgM (H + L) antibodies (Jackson ImmunoResearch). As a control, expression of GAPDH was also assessed. The binding was visualized with enhanced chemiluminescence and quantified using a Fuji LAS 4000 (Fujifilm). Western blots were performed as previously described (26) using a mouse anti-human Bcl2 mAb (Abd Serotec) followed by incubation with HRP-conjugated goat anti-mouse IgG + IgM (H + L) antibodies (Jackson ImmunoResearch). As a control, expression of GAPDH was also assessed. The binding was visualized with enhanced chemiluminescence and quantified using a Fuji LAS 4000 (Fujifilm).
Subsequently, antigen was run over the chip. Each kinetic experiment used a series of five concentrations of β-galactosidase ranging from 2 μM to 125 nM.

Histopathology

Kidney samples from homozygous transgenic animals ranging from 12 to 16 months of age were fixed in phosphate-buffered formaldehyde (10%) and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin and analyzed under a light-phase microscope by a pathologist.

Statistical analysis

Results are presented as the means ± standard deviation. Statistical analysis was performed by a Mann–Whitney test using GraphPad Prism 4 software (GraphPad Software). Differences associated with probability values of \( P < 0.05 \) were considered statistically significant.

Results

Generation of E\(_\mu\)-hBcl2 transgenic rats

A construct containing the E\(_\mu\) enhancer and the SV40 promoter controlling expression of human Bcl-2 was
microinjected to generate transgenic rats (Fig. 1A). Mice with the same transgene have been described previously and showed expression in B cells (16). We generated six transgenic rat lines. F1 offspring from all lines were analyzed for IgM and IgG expression in serum and two lines (70815 and 70818) with elevated IgM/G expression were identified (data not shown). These two lines were further analyzed.

Southern blot analysis demonstrated integration of one and five copies of the transgene into the genome of line 70815 and 70818, respectively (Fig. 1B).

Western blot analysis with an anti-human Bcl-2 mAb revealed expression of the transgene in the spleen of both lines. Expression appeared to be 10- to 20-fold higher in line 70818 compared with line 70815 (Fig. 1C and D, respectively) and it was higher than the one observed in a human lymphoid cell line (data not shown).

Analysis of Bcl-2 expression at the cellular level by cytofluorimetry in homozygous animals from line 70818 revealed that Bcl-2+ cells comprised 47.4 ± 10.8 and 20.0 ± 6.3% of spleen CD45R+ B cells and TCR+ T cells, respectively (n = 6 and 4, respectively, P < 0.05) and 74.9 ± 4.7% of CD45R+ bone marrow cells (n = 3, P < 0.05 versus spleen B cells) (Fig. 1E).

Increased levels of serum Ig in Eμ-hBcl2 transgenic rats

Levels of Ig in sera from transgenic and wild-type animals were analyzed by ELISA (Fig. 2). In line 70818, significantly higher (~2- to 5-fold) levels of IgM, IgG, IgA and IgE were observed in transgenic compared with non-transgenic littermate animals. A comparison of homozygous and heterozygous animals revealed no significant differences. In line 70815, expression levels of IgM and IgG were significantly increased (~2- to 3-fold) compared with wild-type, while levels of IgA and IgE were similar. Analysis of four homozygous animals revealed IgM expression levels that were similar to those observed in heterozygous transgenic animals. However, IgG levels in homozygous transgenics were significantly higher than those observed in heterozygous transgenic animals (P = 0.006). IgG expression in homozygous 70815 and heterozygous/homozygous 70818 animals was similar.

Analysis of autoantibodies directed against dsDNA in line 70818 revealed slightly elevated anti-dsDNA levels in old (10-15 months) but not young (<9 months) animals, but the difference was not statistically significant (Supplementary Figure 1 is available at International Immunology Online).
Animals of the 70815 line did not show increased anti-dsDNA levels in old or young rats (data not shown). Thus, bcl-2 transgene expression resulted in elevated Ig levels but not in autoantibody production.

**Analysis of cell subtypes in spleens**

The spleen of Eμ-hBcl2 transgenic rats was modestly increased, whereas the size of the lymph nodes and thymus was normal (data not shown). Detection of Bcl-2 by immunohistology showed higher transgene expression in line 70818 compared with line 70815. Bcl-2 expression was highest in B-cell areas but also present in T-cell areas (Fig. 3A). Compared with non-transgenic littermates, the CD45R+ B-cell areas in spleens of transgenic rats were larger, whereas there were no differences for marginal B-cell zones (Fig. 3A). The analysis of TCR+ cells showed normal T-cell areas in spleens of both lines compared with non-transgenic littermates (Fig. 3A).

In conclusion, analysis of spleen histology showed that in accordance with bcl-2 transgene expression in B-cell areas, the B-cell areas were enlarged, whereas T-cell areas appeared normal.

In bcl-2 transgenic mice, an increased number of B lymphocytes were observed in spleen and bone marrow (14, 16). Therefore, we analyzed several markers of B-cell development in spleen and bone marrow by FACS.

The total number of splenocytes from 70818 homozygous (373 ± 41.5 x 10^6, P < 0.05, n = 4) but not heterozygous (337.5 ± 79.5 x 10^6, n = 4) animals was significantly higher compared with littermate non-transgenic animals (251.6 ± 41.5 x 10^6, n = 4). The absolute number of bone marrow cells from 70818 homozygous (139.9 ± 7.7 x 10^6, P < 0.05, n = 4) but not heterozygous (130.5 ± 16.6 x 10^6, n = 4) animals was also significantly increased compared with littermates (112.8 ± 18.7 x 10^6, n = 4). Total numbers of lymph node and thymus cells were comparable between 70818 homozygous and heterozygous transgenic rats versus littermates (data not shown).

Flow cytometric analysis of single-cell suspensions from whole spleens, lymph nodes and femurs revealed a significant increase of populations expressing two B-cell markers, CD45R (P = 0.029) and IgM (P = 0.028) as well as of the early lymphoid differentiation marker, CD90 (P = 0.028) in homozygous rats of transgenic line 70818 compared with littermates (Fig. 3B). In heterozygous 70818 animals, cell numbers were also increased but not significantly (Fig. 3B).

A similar significant increase of IgM+ or CD45R+ B cells was observed in the lymph nodes of transgenic animals compared with littermates (data not shown).

In line 70815 numbers of CD45R+*, IgM* and CD90+* cells were also increased but the difference between Eμ-hBcl2 transgenic and non-transgenic littermates did not reach statistical significance (Fig. 3B).

No differences were observed in the total number of TCR+ T cells for either of the transgenic lines (Fig. 3B). Analysis of Bcl-2 expression in major T-cell subsets of homozygous animals of line 70818 showed similar expression in CD4+CD25+Foxp3+ Treg cells versus CD4+CD25+Foxp3- cells (Supplementary Figure 2 is available at International Immunology Online) or CD4+CD25+ Treg versus CD4+CD25- cells (data not shown). No significant differences were observed between homozygous animals of line 70818 versus littermates for the total numbers of CD4+CD25- cells (34.6 ± 7.8 x 10^6 versus 27.8 ± 4.5 x 10^6, P > 0.05, n = 4) or CD4+CD25+ Treg (8.9 ± 1.0 x 10^6 versus 8.1 ± 0.7 x 10^6, respectively, P > 0.05, n = 4).

**B-cell subsets in spleen and bone marrow**

To better define B cells, we performed double staining using CD45R (B220) and IgM or IgM and IgD. With these markers, several rat B-cell populations can be differentiated, such as pre-B and B cells in bone marrow and pre-B/transitional, follicular and marginal zone B cells in spleen (28, 29). In the spleen, using CD45R and IgM as markers, the absolute number of follicular and marginal zone B cells in heterozygous (P = 0.03) and homozygous (P = 0.03) line 70818 animals was twice as high as in non-transgenic littermates (Fig. 4A). Using IgM and IgD as markers, follicular but not marginal zone B cells in homoygous but not heterozygous 70818 animals were twice as high as in non-transgenic littermates (P = 0.03) (Fig. 4B). In transgenic line 70815 rats, the numbers of different B-cell populations in the spleen were also elevated, but compared with non-transgenic littermates, the differences were not significant (data not shown).

In bone marrow, using CD45R and IgM as markers, absolute number of B cells in homozygous but not heterozygous 70818 animals was increased and pre-B cells were comparable versus non-transgenic littermates (Fig. 4C). Using IgM and IgD as markers, B cells in homozygous 70818 animals were twice as high as in non-transgenic littermates (P = 0.03) (Fig. 4D). In transgenic line 70815 rats, the numbers of pre-B and B cells in bone marrow as defined with all these markers were comparable to non-transgenic littermates (data not shown).

In conclusion, only animals from the bcl-2 transgenic line 70818 showed higher numbers of B cells both in the spleen and in the bone marrow as compared with non-transgenic littermates.

**Increased survival of splenic B cells and bone marrow cells from Eμ-hBcl2 transgenic rats**

To assess the effect of Bcl-2 on B-cell survival, B cells were purified from the bone marrow and spleen, cultured in vitro and spontaneous apoptosis and necrosis as well as that induced by sub-optimal concentration of the apoptosis inducer staurosporin. The percentages of living cells, apoptotic, late apoptotic and necrotic were defined using colabelling with annexin V and DAPI. B cells from bone marrow of homozygous line 70818 showed resistance to spontaneous apoptosis and necrosis at days 1, 4 and 7 as compared with wild-type B cells (Fig. 5A and B). B cells from spleen of heterozygous line 70818 as well as spleen and bone marrow from homozygous but not heterozygous line 70815 also showed resistance to apoptosis induced by staurosporin (data not shown).

Thus, B-cell resistance to apoptosis was increased in bcl-2 transgenic rats and correlated with levels of transgenic
Fig. 3. Cellular composition of Eμ-hBcl2 transgenic rat spleens. Spleens from transgenic homozygous (Tg homozygous) rats of lines 70818 and 70815 or non-transgenic littermates (LM) (8 months old) were harvested and analyzed by immunohistology and flow cytometry. (A) Hematoxylin–eosin counterstained immunoperoxidase staining of spleen cryostat sections from rat spleens labeled with anti-hBcl2, anti-CD45R, anti-marginal zone B cells (clone His57) and anti-TCR mAbs, respectively. One representative spleen from four analyzed. (B) Spleen cell suspensions from transgenic heterozygous or homozygous (Tg heterozygous or homozygous, respectively), as well as non-transgenic littermates (LM) were labeled with monoclonal antibodies and quantified by flow cytometry (upper) line 70818 and (lower) line 70815. The data (mean ± SD) were derived from four animals of each group and represent the absolute number of cells. For CD45R, non-transgenic littermates versus Tg homozygous $P = 0.029$; for IgM, non-transgenic littermates versus Tg homozygous $P = 0.028$; for CD90, non-transgenic littermates versus homozygous $P = 0.028$. 

**Effects of BCL-2 over-expression on B cell**
Fig. 4. Effect of \( hBcl2 \) on B-cell development in \( E_{\mu}\cdot hBcl2 \) transgenic rats. Transgenic heterozygous or homozygous (Tg heterozygous or homozygous, respectively) of line 70818 as well as non-transgenic littermates (LM) were analyzed by flow cytometry for B-cell populations. (A) Upper panel, histograms from one representative animal of four in which spleen cells were labeled with anti-IgM (ordinates) and anti-B cell-specific CD45R mAb (abscissas). The windows represent follicular B cells (CD45R\( ^+ \) IgM\(^+ \)) and marginal zone B cells (MZ) (CD45R\( ^+ \) or CD45R\( ^- \)IgM\(^{++} \)). Lower panel represents absolute numbers of follicular and marginal zone B cells per spleen (mean ± SD, \( n = 4 \), *\( P < 0.05 \)). (B) Upper panel, histograms from one representative animal of four in which spleen cells were labeled with anti-IgD (ordinates) and anti-IgM mAb (abscissas). The windows represent follicular B cells (CD45R\( ^+ \) IgM\(^+ \)IgD\(^+ \)) and marginal zone B cells (MZ) (IgM\(^{++} \)IgD\(^{low} \)). Lower panel represents absolute numbers of follicular and marginal zone B cells (mean ± SD, \( n = 4 \), *\( P < 0.05 \)) per spleen. (C) Upper panel, histograms from one representative animal of four in which bone marrow cells were labeled with anti-IgM (ordinates) and anti-B cell-specific CD45R mAb (abscissa). The windows represent pro-B (CD45R\( ^+ \)IgM\(^- \)) and B cells (CD45R\( ^+ \)IgM\(^+ \)). Lower panel represents absolute numbers of pro-B or B cells (mean ± SD, \( n = 4 \), *\( P < 0.05 \)) per femur. (D) Upper panel, histograms from one representative animal of four in which bone marrow cells were labeled with anti-IgD (ordinates) and anti-IgM mAb (abscissas). The window represents B cells (IgM\(^+ \)IgD\(^- \) or IgD\(^+ \)) since with these markers, it is not possible to define early stages of B-cell differentiation. Lower panel represents absolute numbers of B cells (mean ± SD, \( n = 4 \), *\( P < 0.05 \)) per femur.
Bcl-2 expression in the two transgenic lines as well as in heterozygous versus homozygous transgenic animals.

**Effect of Bcl-2 expression on hybridoma production, in vitro proliferation, Ig expression and antibody affinity**

For the generation of mAbs, bcl-2 transgenic and non-transgenic rats were immunized with beta-galactosidase in the laboratories of three different service providers specialized in rat hybridoma technology. No significant differences in anti-beta-galactosidase antibody titers were observed in immunized transgenic or wild-type animals (data not shown).

Twelve fusions with splenocytes from six transgenic and six wild-type Sprague–Dawley rats resulted in the generation of 46 anti-beta-galactosidase-specific mAbs. Differences in fusion efficiency between animals were large and no consistent differences with splenocytes from transgenic and non-transgenic animals were observed. Four fusions with splenocytes from two Sprague–Dawley rats and two bcl-2-transgenic animals did not result in the detection of antigen-specific antibody-producing hybridomas. The remaining eight fusions resulted in the generation of 22 \( (n=22) \) stable hybridomas with splenocytes from bcl-2 transgenic animals and 24 \( (n=24) \) stable hybridomas with wild-type

**Fig. 5.** Inhibition of apoptosis in Eµ-hBcl2 transgenic bone marrow and B cells. For the analysis of apoptosis, B cells were purified (>95%) from bone marrow of homozygous animals from line 70818 or littermates (LM) and were cultured at \( 1.5 \times 10^6 \) cells per well in 24-well plates and apoptosis and necrosis was determined at different time points by flow cytometry analysis using annexin-V and DAPI colabelling. (A) Dot plot analysis of double labelling with annexin-V and DAPI from one representative animal of three analyzed. Annexin ‑ DAPI ‑ living cells; annexin ‑ DAPI ‑ apoptotic cells; annexin ‑ DAPI ‑ late apoptotic cells; annexin ‑ DAPI ‑ necrotic cells. (B) Cell number and mean ± SD of each the four cell populations \( (n=3, P<0.05) \).
spleenocytes. Hybridomas were sub-cloned by limiting dilution. No differences in sub-cloning efficiency were observed between hybridomas generated with bcl-2 transgenic or wild-type splenocytes (data not shown). Isotyping of antibodies produced by 14 hybridomas generated with bcl-2 transgenic spleens showed that 3 (21%) produced IgG1, 5 (36%) produced IgG2a and 6 (43%) produced IgG2b. Isotyping of 24 hybridomas generated with wild-type spleenocytes showed that 3 (12.5%) produced IgG2b and 21 produced IgG2a (87.5%) but none produced IgG1.

Binding kinetics of 39 mAbs were determined by Biacore measurements. Fifteen of these mAbs had been generated with bcl-2 transgenic animals and 24 with wild-type animals. There was no significant difference in binding kinetics between the two groups (Fig. 6A).

Proliferation in tissue culture was compared using 13 hybridomas generated with bcl-2 transgenic splenocytes and the 24 hybridomas generated with wild-type splenocytes. No differences in mid-log or peak cell densities were observed (data not shown).

Thirteen hybridomas generated with bcl-2 transgenic spleens were analyzed for Bcl-2 expression by western blot. Four of the 13 hybridomas did not express detectable levels of Bcl-2. The remaining nine hybridomas expressed various levels of Bcl-2 ranging from a Bcl-2/GAPDH ratio of 0.2–1.2 (Fig. 6B and data not shown). IgG concentrations in culture supernatants were determined daily by ELISA. Hybridomas expressing detectable levels of Bcl-2 produced significantly higher amounts of IgG (Bcl-2+ versus Bcl-2−, \( P = 0.02 \)) (Fig. 6C). When we evaluated IgG production in the nine hybridomas expressing various levels of Bcl-2, no significant correlation in IgG production were observed (data not shown).

In conclusion, Bcl-2 expression was associated with increased Ig production by hybridomas in culture but had no effect on fusion efficiency, hybridoma growth or mAb binding kinetics.

**Discussion**

With the goal to optimize rat mAb production and to analyze the effect of Bcl-2 over-expression in rat B cells, we generated bcl-2 transgenic rats with a DNA construct containing the mouse 5’ IgH enhancer and the SV40 promoter. This DNA construct had previously been used for the generation of transgenic mice over-expressing Bcl-2 in B cells (16, 30).

Six transgenic rat lines were generated and two lines with increased Ig expression were selected for further characterization. Line 70815 had a single copy of the transgene integrated in its genome, line 70818 had about five copies. Line 70818 animals expressed higher levels than line 70815 animals. Higher copy numbers of the transgene resulted in expression of Bcl-2 at higher levels by splenic and bone marrow B cells versus T cells.

Consistent with observations made in mice (16, 30), over-expression of Bcl-2 in rats resulted in increased numbers of B cells in the spleen, lymph node and bone marrow and normal numbers of T cells. Higher expression of Bcl-2 in line

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**Fig. 6.** Hybridoma characterization. (A) Binding constants (nanomolar) of mAbs generated with spleenocytes from non-transgenic littermates (LM) and transgenic line 70818 animals (Bcl2) as determined by Biacore analysis (log scale nanomolar). (B) Western blot analysis of Bcl-2 expression in hybridomas from bcl-2 transgenic animals (each line represents one hybridoma). The positive control corresponds to a lysate from human Jurkat cells. The ratio of Bcl-2/GAPDH for each hybridoma is depicted under the respective lines. (C) Concentration of IgG in tissue culture supernatants at day 3 of culture (cells between 3.1 and 6 \( \times 10^3 \) ml\(^{-1} \)) measured by ELISA. Each point represents the values of one hybridoma supernatant and the horizontal bar the mean of each group of values \( *P < 0.05 \) hybridoma Bcl-2− \( (n = 4) \) versus hybridoma Bcl-2+ \( (n = 9) \).
70818 had a greater effect on B-cell development compared with line 70815 rats.

Similar to observations made in bcl-2 transgenic mice (14, 16, 30), bcl-2 transgenic rats expressed higher levels of all Ig isotypes compared with non-transgenic littermates. Line 70818 animals expressed higher levels of Ig than line 70815 animals.

Bcl-2 has been shown to protect lymphocytes from certain forms of death by neglect, including growth factor withdrawal (6). This inhibition of apoptosis resulted in prolonged in vitro survival of cells expressing Bcl-2 (19,31–33). Similarly, B lymphocytes from transgenic mice displayed prolonged cell survival (14–16, 30). Consistent with these observations, B cells from bcl-2 transgenic rats were more resistant to apoptosis. This effect appeared to correlate with higher Bcl-2 expression since cells from homozygous and heterozygous line 70818 animals showed better survival than cells from line 70815 animals.

Histological analysis of eight animals older than 10 months revealed lymphoma development in only one heterozygous animal from line 70815 (data not shown). Bcl-2 transgenic mice generated with the same bcl-2 construct as the one used here showed modest follicular hyperplasia but did not develop lymphoma (16) or development of disseminated aggressive lymphomas required crossing with transgenic mice expressing myc (34). Other bcl-2 transgenic mice generated with a construct containing the mouse 5’ IgH promoter and enhancer did not develop lymphomas (14, 35). Thus, the results obtained for our bcl-2 transgenic rats mimic those for bcl-2 transgenic mice.

Bcl-2 transgenic mice generated with the same construct as the one used in this study developed serum antibody to dsDNA, and their kidneys showed evidence of immune complex glomerulonephritis resembling systemic lupus erythematosus (16). In contrast, bcl-2 transgenic mice generated with the construct containing the mouse 5’ IgH promoter and enhancer despite increased levels of B cells did not show autoimmunity (14, 15). At least in one of these transgenic lines (14), absence of autoimmunity was shown to be due to Bcl-2 expression, not only by B cells but also by CD4+CD25+Foxp3+ Tregs, which resulted in increased numbers of Tregs and protection against autoimmunity (36). In our bcl-2 transgenic rats, Bcl-2 was expressed equally by CD4+CD25+Foxp3+ Tregs and CD4+CD25+Foxp3-non-Treg cells and no increase in the number of CD4+CD25+Foxp3+ Tregs was observed. In agreement with this, anti-dsDNA autoantibodies were not significantly increased. Furthermore, anatomopathological analysis of kidneys from 12- to 16-month-old homozygous transgenic rats of both lines revealed no evidence of kidney disease (data not shown) or significant proteinuria (data not shown) and all transgenic rats appeared healthy, reproduced normally and displayed normal mortality. The basis of the difference with transgenic mice is not yet clear but might be due to differences in the genetic background. Further studies will be necessary to exclude the possibility that the absence of autoimmunity in bcl-2 transgenic rats is associated with Bcl-2 over-expression in regulatory T cells.

Bcl-2 transgenic mice have also been used for the generation of hybridomas and bcl-2 transgenic mice showed better performances compared with wild-type B cells. In B6 immunized mice, similar antigen-specific antibody titers were observed in transgenic animals and non-transgenic littermates but transgenic mice had increased fusion efficiencies and higher number of antigen-specific antibody-producing hybridomas as compared with wild-type mice. In contrast, bcl-2 transgenic rats versus littermates did not reveal differences in serum antibody titers and no differences were observed in fusion efficiency or in the number of antigen-specific antibody-producing hybridomas produced in 12 fusions. However, we cannot exclude the possibility that bcl-2 transgene expression in rat strains with different genetic backgrounds would have resulted in better hybridoma generation, as observed with different strains of mice (19).

The majority of mAbs generated with transgenic or wild-type splenocytes were IgG2a or IgG2b isotypes (≥79%). Antibodies generated with bcl-2 transgenic or wild-type splenocytes bound antigen with similar on- and off-rates. These results suggest that constitutive Bcl-2 expression in transgenic rats had little effect on affinity maturation of antigen-specific B cells. Cellular proliferations rates of rat hybridomas varied widely and no differences correlating with Bcl-2 expression were observed. However, Bcl-2 expressing hybridomas expressed significantly higher amounts of antibody in vitro compared with hybridomas with undetectable expression of Bcl-2 and since most hybridomas expressed Bcl-2, these transgenic rats will be of value to generate hybridomas producing more antibody. These differences are consistent with observations in mice where the effect of Bcl-2 expression on hybridoma proliferation and Ig production varied widely (31, 37). The most likely explanation for such variations appeared to be genetic differences between hybridomas.

In summary, our results are consistent with previously reported results generated with transgenic mice (14–16, 30) and zebrafish (38). Bcl-2 over-expression in transgenic rats using a promoter with predominant B-cell expression inhibited B-cell apoptosis and resulted in increased numbers of immature B cells in lymphoid organs and elevated expression of Igs. Bcl-2 transgenic rats will be a useful tool for the generation of hybridomas producing higher quantities of mAbs. They will also be useful for the analysis of B cells in different pathophysiological models, such as autoimmunity, cancer or organ transplantation (20, 22, 23, 39). In addition, these transgenic rats will be useful for the study of B-cell biology, i.e. the effect of Bcl-2 over-expression on other genes involved in B-cell function, such as those implicated in the generation of rats deficient in B cells (26).

Supplementary Data
Supplementary data are available at International Immunology Online.

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