B-cell overexpression of Bcl-2 cooperates with p21 deficiency for the induction of autoimmunity and lymphomas

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Abstract

Genetic abnormalities predisposing to autoimmunity generally act in a cooperative manner affecting one or several mechanisms regulating immunological tolerance. In addition, many of these genetic abnormalities are also involved in the development of lymphoproliferative diseases. In the present study, we have determined the possible cooperation between deficiencies in members of the Cip/Kip family of cell cycle regulators (p21^{WAF1/Cip1} or p27^{kip1}) and the overexpression of human Bcl-2 in B lymphocytes in the induction of autoimmune and lymphoproliferative diseases in non-autoimmune C57BL/6 (B6) mice. Unlike single mutant mice, B6.p21^{-/-} mice transgenic for human Bcl-2 in B cells developed a lethal autoimmune syndrome characterized by the production of autoantibodies, the prominent expansion of memory B and CD4⁺ T cells and the development of severe glomerular lesions resembling IgA nephropathy. Furthermore, these mice presented a high incidence of B-cell lymphoproliferative disorders. Such genetic cooperation in the induction of autoimmunity was not observed in B6.p27^{-/-} mice transgenic for human Bcl-2 in B cells. Altogether, what we have demonstrated here is the existence of preferential interactions among particular regulators of the G₁/S transition of the cell cycle and B cell survival in the induction of systemic autoimmune and lymphoproliferative diseases.

Key words: Systemic lupus erythematosus, B-cell lymphomas, p21^{WAF1/Cip1}, p27^{kip1}, Bcl-2.

Abbreviations: Transgenic mice (Tg), human Bcl-2 (hBcl-2), systemic lupus erythematosus (SLE), C57BL/6 (B6), p21^{WAF1/Cip1} (p21), p27^{kip1} (p27), autoantibodies (autoAbs), lymphoproliferative disorders (LPD), B6-SV40-E μ -*hbcl*-2-22 Tg mice (B6-hBcl-2-B Tg), B6.129S2-Cdkn1a^{tm1Tyj}/J (B6.p21^{-/-}), B6.129S4-Cdkn1b^{tm1Mlf}/J (B6.p27^{-/-}), carboxyfluorescein diacetate succinimidyl ester (CFSE), bromodeoxyuridine (BrdU), gp70 anti-gp70 immune complexes (gp70 IC), wild type (wt), polymorphic LPD (PLPD), activation-induced cell death (AICD).

1. Introduction

With rare exceptions, the development of autoimmune diseases requires the contribution of multiple genetic, epigenetic and environmental factors. These factors generally act in a cooperative manner affecting one or several mechanisms that regulate the induction and maintenance of immunological tolerance [1]. Among the best characterized genetic alterations involved in the development of autoimmunity, are those that affect the regulation of lymphocyte survival. Using a line of transgenic (Tg) mice overexpressing human Bcl-2 (hBcl-2) in B lymphocytes, we and others have demonstrated that Tg mice bearing a pro-autoimmune genetic background develop a lethal autoimmune lupus-like syndrome that is dependent on the activity of CD4⁺ lymphocytes [2, 3]. However, the overexpression of hBcl-2 in B cells from C57BL/6 (B6) mice, which do not have a genetic predisposition to developing the disease, fails to promote autoimmune manifestations [2], indicating that the inhibition of B lymphocyte apoptosis by itself is not sufficient to trigger a severe autoimmune reaction. Similarly, B6 mice deficient in *bim* or *Fas* fail to develop a fatal autoimmune pathology in contrast to the severe systemic lupus erythematosus (SLE) observed in mixed (129/Sv x B6)-*bim*^{-/-} or MRL.*Fas*^{lpr} mice, respectively [4-6].

The deregulation in the control of lymphocyte proliferation is also associated with the development of autoimmune diseases. In this sense, mice deficient in p21^{WAF1/Cip1} (p21; a member of the Cip/Kip family of cell cycle inhibitors) in a line of mice with a mixed (129/Sv x B6) genetic background (derived from 2-3 intercrosses) develop a fatal lupus-like syndrome that affects females more than males [7]. Similarly, the administration of a peptidyl mimic of p21 into (NZB x NZW)F₁ females significantly reduces the severity of the spontaneous SLE in these animals [8]. Again, the severity of the autoimmune syndrome in p21^{-/-} mice appears to be modulated by the

genetic background of the mice analyzed. Thus, B6 and BXSB mice deficient in p21 or an additional line of p21^{-/-} mixed (129/Sv x B6) mice, derived from an unknown number of intercrosses, are largely protected from the development of SLE [9-12]. Another member of the Cip/Kip family acting as a negative regulator of cell cycle is p27^{kip1} (p27). Studies have shown that p27 plays an important role in the induction and maintenance of clonal anergy of T lymphocytes induced after inhibition of co-stimulation [13]. In anergic CD4⁺ T lymphocytes, p27 inhibits the phosphorylation of Smad-3 resulting in an increase of the inhibitor of Cdk, p15. Consequently, the deficiency in p27 prevents the induction of clonal anergy in T lymphocytes [14]. However, p27^{-/-} mice do not develop evident autoimmune manifestations [15].

In addition to their ability to promote autoimmunity in the context of a particular genetic predisposition, the cell cycle and apoptotic regulators mentioned above may be also involved in tumor development. In this regard, Bcl-2 was initially identified as a gene implicated in the t(14:18) chromosomal translocation found in the majority of follicular B-cell lymphomas [16] and Tg mice overexpressing Bcl-2 in B cells spontaneously developed plasmacytomas or immunoblastic B-cell lymphomas albeit with a low incidence [17, 18]. Similarly, an association between Burkitt's lymphomas and mutations in p21 that diminish the functional activity of this cell cycle regulator has been reported in humans [19], and p21^{-/-} mice have a higher incidence of different types of tumours, including B-cell lymphomas, in aged but not young mice [20]. More recently, it has been shown that p27^{-/-} mice overexpressing Bcl-2 in T lymphocytes spontaneously develop T-cell lymphomas with a high incidence [21].

Based on these findings, in the present study we have determined the possible cooperation between p21 or p27 deficiency and the B-cell overexpression of Bcl-2 in the development of both lupus-like autoimmune disease and B-cell malignancies in

normal B6 mice. Our results show that B6.p21^{-/-} mice transgenic for hBcl-2 in B cells (B6.p21^{-/-}-hBcl-2-B Tg) developed a lethal lupus-like syndrome characterized by the production of autoantibodies (autoAbs), the increase in memory B and CD4⁺ T cells and the presence of severe glomerular lesions resembling an IgA nephropathy. In addition, these mice exhibited a high incidence of B-cell lymphoproliferative disorders (LPD). On the other hand, the deficiency in p27 failed to promote autoimmune responses in B6.hBcl-2-B Tg mice, indicating the existence of preferential interactions among particular regulators of the G₁/S transition of the cell cycle and cell survival for the control of B-cell tolerance.

2. Materials and methods

2.1. Mice

B6 mice were obtained from Harlan Iberica (Barcelona, Spain). B6-SV40-Eμ-*hbcl-2-22* Tg mice (B6-hBcl-2-B Tg) [3] overexpressing hBcl-2 in B cells, B6.129S2-Cdkn1a^{tm1Tyj}/J (B6.p21^{-/-}) [22] and B6.129S4-Cdkn1b^{tm1Mlf}/J (B6.p27^{-/-}) mice [15] were purchased from The Jackson Laboratories (Bar Harbor, ME). The selection of hBcl-2-B Tg mice was performed by flow cytometry using peripheral blood mononuclear cells and a specific mAb against hBcl-2 (clone 6C8; BD Biosciences, Madrid, Spain), as described previously [23]. B6.p21^{-/-}-hBcl-2-B Tg and B6.p27^{-/-}-hBcl-2-B Tg mice were obtained by intercrossing F1 progeny of B6.p21^{-/-} and B6.p27^{-/-} mice with B6-hBcl-2-B Tg mice. The p21 and p27 genotyping of the different strains of mice was performed by PCR. Male and female mice of each mouse strain were analyzed together. The mice were bled every two months from the retro-orbital plexus, and the resulting sera were stored at -20°C until used.

All studies with live animals were approved by the Universidad de Cantabria Institutional Laboratory Animal Care and Use Committee.

2.2. Cell death and *in vitro* proliferation assays

To analyze the possible modulation of hBcl-2 anti-apoptotic activity in B cells by p21 or p27 deficiency *in vivo*, 2 month-old mice of the different strains used here were injected ip. with 2 mg of dexamethasone sodium phosphate (American Regent Laboratories, Shirley, NY). The numbers of pre-B (B220^{low}IgM⁻) and immature B (B220^{loww}IgM⁺) cells in the bone marrow were analyzed 48 hrs later by flow cytometry.

Purified B cells (more than 95% purity in all cases) from different strains of mice were obtained from the spleen after *in vitro* treatment with anti-Thy-1.2 mAb (clone AT-83, a rat IgM anti-mouse Thy-1.2 mAb) and complement (Cedarlane Laboratories, Hornby, ON, Canada). The effects of hBcl-2 overexpression on B cell survival *in vitro* in the different lines of hBcl-2 Tg mice and non-Tg controls were assessed using purified cell populations cultured with RPMI medium supplemented with 2mM L-glutamine, 10⁻⁵ M 2-mercaptoethanol and 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT), as described previously [24].

B-cell labelling with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes Europe, Leiden, The Netherlands) was performed according to the manufacturer's instructions. For *in vitro* proliferation assays, 2 x 10⁵ CFSE-labelled B cells from the different strains of mice were stimulated during 48 hrs with LPS or an affinity-purified F(ab')₂ goat anti-mouse IgM polyclonal Ab (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), at concentrations of 20 µg/ml. Cell division was analyzed by flow cytometry according to the CFSE intensity dilution.

2.3. Flow cytometry studies

The number of total B220⁺, B220⁺IgM⁻IgD⁻CD138⁻ (memory phenotype), naïve CD4⁺CD44^{low}CD62L^{high}, CD4⁺CD44^{high}CD62L^{low} (memory phenotype), naïve CD8⁺CD44^{low}CD62L^{high} and CD8⁺CD44^{high}CD62L^{low} (memory phenotype) cells in the spleen of the different strains of mice was evaluated by flow cytometry at 2 and 8 months of age. Single cell suspensions were stained with different combinations of fluorescein isothiocyanate-, phycoerythrin-, peridinin chlorophyll protein- and biotin-conjugated mAbs (BD Biosciences) followed by allophycocyanin-conjugated streptavidin. A total of 5×10^4 viable cells were analyzed in a FACSCanto II flow cytometer using FACSDiva software (BD Biosciences).

2.4. Serological studies

Serum levels of total IgA and IgG were determined by ELISA as described previously [2, 25]. Results were expressed in mg/ml in reference to a standard curve obtained with a mouse reference serum (ICN Biomedicals Inc.). The presence of IgG and IgA anti-DNA autoAbs was determined in sera by ELISA as described [2, 25] and the results expressed in TU in reference to a standard curve obtained from a serum pool from 6-8 months old MRL *lpr/lpr* mice. Serum levels of IgG gp70 anti-gp70 immune complexes (gp70 IC) were quantified by ELISA [26] and the results expressed as μg of Ab-bound gp70/ml of serum.

2.5. Histopathology

Samples of all major organs were obtained at autopsy. Organs were included in paraffin and stained with hematoxylin-eosin or Masson trichrome for pathological studies. Glomerulonephritis was scored on a 0 to 4 scale as previously described [27].

Grades 3 and 4 glomerulonephritis were considered significant contributors to clinical disease and/or death. The incidence of B-cell LPD in the different mouse strains was analyzed at 8-10 and 18 months of age by histology, immunohistochemistry and flow cytometry. All histological preparations were analyzed blinded by one (kidney sections) or two (B-cell LPD) pathologists.

Tissue-bound IgG and IgA antibodies were studied by immunofluorescence on kidney cryosections using FITC-conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch) or FITC-conjugated goat anti-mouse IgA antibody (Cappel Laboratories, Cochranville, PA).

2.6. Statistical analysis

Statistical analysis of differences between groups of mice was performed using the Mann-Whitney test. Probability values <0.05 were considered significant.

3. Results

3.1. Lack of the effect of p21 deficiency on the survival and proliferation of B cells overexpressing hBcl-2

We first determined whether p21 deficiency modulated the anti-apoptotic activity of hBcl-2 in B cells from B6.p21^{-/-}-hBcl-2-B Tg mice, as p21 can act as a survival factor in several cell types including T lymphocytes [reviewed in 28]. After the viability of purified B cells in culture had been assessed during a period of 10 days, B cells from both B6.hBcl-2-B Tg and B6.p21^{-/-}-hBcl-2-B Tg mice exhibited a similarly improved survival as compared to B cells from B6-wt and B6.p21^{-/-} mice (Figure 1A). The lack of the effect of p21 deficiency on B-cell survival was confirmed *in vivo* after induction of apoptosis of pre-B and immature B cells with glucocorticoids. The

injection of dexamethasone into B6-wt and B6.p21^{-/-} mice induced the elimination of about 85% of B220^{low}IgM⁻ pre-B and 80% B220^{low}IgM⁺ immature B cells in the bone marrow (Figure 1B). These two B-cell precursors were, however, largely preserved in dexamethasone-treated B6.hBcl-2-B Tg mice and this protection was not modified in B6.p21^{-/-}-hBcl-2-B Tg mice (Figure 1B).

It has been reported that the overexpression of Bcl-2 has an anti-proliferative effect in lymphocytes [29, 30]. Accordingly, the *in vitro* proliferation of B lymphocytes from B6.hBcl-2-B Tg mice 48 hrs after stimulation with anti-IgM antibodies or LPS was lower than that observed with B cells from B6-wt mice (Figure 1C). Although an increased *in vitro* anti-IgM- or LPS-induced B-cell proliferation was observed in B6.p21^{-/-} mice, the p21 deficiency was not sufficient to rescue the proliferative defect induced by Bcl-2 overexpression in B cells from B6.p21^{-/-}-hBcl-2-B Tg mice (Figure 1C).

3.2. Effects of B-cell overexpression of hBcl-2 in p21^{-/-} mice in the development of autoimmunity

We next compared the development of a lupus-like autoimmune disease between B6-wt, B6.p21^{-/-}, B6.hBcl-2-B Tg and B6.p21^{-/-}-hBcl-2-B Tg mice by analyzing the levels of circulating autoAbs at 6 months of age. A significant increase in serum levels of IgA and IgG anti-ssDNA autoAbs was observed in B6.hBcl-2-B Tg mice in comparison to B6-wt and B6.p21^{-/-} mice ($p < 0.01$ in all cases), while levels of nephritogenic gp70 IC were essentially identical in these three strains of mice (Figure 2A). In contrast, B6.p21^{-/-}-hBcl-2-B Tg mice exhibited 2-4-fold higher levels of anti-ssDNA and gp70 IC than B6.hBcl-2-B Tg mice (Figure 2A; $p < 0.005$ in all the cases). In addition, analysis of serum levels of IgA and IgG also revealed significant increases of

IgA in B6.hBcl-2-B Tg mice, as compared with B6-wt and B6.p21^{-/-} mice ($p<0.01$), and of both IgA and IgG in B6.p21^{-/-}-hBcl-2-B Tg mice, as compared with B6.hBcl-2-B Tg mice ($p<0.005$), (Figure 2B).

We compared by flow cytometry the distribution of B and T cell populations in spleens between B6-wt, B6.p21^{-/-}, B6.hBcl-2-B Tg and B6.p21^{-/-}-hBcl-2-B Tg mice at 2 and 8 months of age. As expected, mice overexpressing hBcl-2 in B cells displayed a marked accumulation of B cells in B6.hBcl-2-B and B6.p21^{-/-}-hBcl-2-B Tg mice (Table 1). The size of B cells with a memory phenotype (B200⁺IgM⁻IgD⁻CD138⁻) was greatly enlarged in 2 and 8 month-old B6.hBcl-2-B Tg mice ($p<0.01$ and $p<0.001$, respectively) and modestly increased in B6.p21^{-/-} mice at 8 months of age ($p<0.05$; Table 1). The combination of Bcl-2 overexpression and p21 deficiency appeared synergistic as the number of B200⁺IgM⁻IgD⁻CD138⁻ B cells in B6.p21^{-/-}-hBcl-2-B Tg mice was highly increased ($p<0.05$ at 2 months and $p<0.001$ at 8 months; Table 1). As reported in mixed (129/Sv x B6) mice deficient in p21 [7], an age-dependent expansion in CD4⁺ T cells with a memory phenotype (CD4⁺CD44^{high}CD62L^{low}) was observed in B6.p21^{-/-} mice ($p<0.05$; Table 1). Such an expansion was more pronounced in B6.p21^{-/-}-hBcl-2-B Tg mice ($p<0.005$) and already evident at 2 months of age, which was not the case in B6.hBcl-2-B Tg mice (Table 1). No differences were observed in the numbers of naïve and memory CD8⁺ T cells among these strains of mice.

In correlation with the production of autoAb and the expansion of B200⁺IgM⁻IgD⁻CD138⁻ B and CD4⁺CD44^{high}CD62L^{low} T cells, B6.p21^{-/-}-hBcl-2-B Tg mice had a reduced lifespan in comparison to B6-wt, B6.p21^{-/-} and B6.hBcl-2-B Tg mice (Figure 3). In fact, 50% of B6.p21^{-/-}-hBcl-2-B Tg mice died at around 16 months of age ($p<0.001$). When examined for the cause of death, 52% (22/42) of B6.p21^{-/-}-hBcl-2-B Tg mice ($p<0.001$) showed at the time of autopsy a severe glomerulonephritis (Figure

4A) characterized by an intense mesangial matrix expansion, secondary to collagen accumulation (stained in blue with the Masson trichrome) that completely collapsed the capillary spaces (glomerulosclerosis) (Figure 4B). By direct immunofluorescence, prominent deposits of IgA, but not of IgG, were detected in the glomerular mesangium of these animals (Figure 4B). None of the B6-wt, B6.p21^{-/-} and B6.hBcl-2-B Tg mice examined showed severe glomerular lesions at 14-16 months of age (Figure 4A and B). Altogether, our results indicated the development of an IgA nephropathy associated to SLE in B6.p21^{-/-}-hBcl-2-B Tg mice. Unlike mice deficient in p21 in a mixed (129/Sv x B6) genetic background which developed a lupus-like syndrome that affects females more than males [7], no differences in the levels of circulating autoAbs, memory B and CD4⁺ T cell expansion, development of glomerulonephritis and mortality rate were observed between both sexes of B6.p21^{-/-}-hBcl-2-B Tg mice (data not shown).

3.3. Absence of a severe SLE in B6.p27^{-/-}-hBcl-2-B Tg mice

We further explored whether the deficiency of p27, another regulator of the G₁/S transition of cell cycle, also cooperates with hBcl-2 overexpression in B cells for the induction of autoimmunity mice. As observed in p21^{-/-} mice, the deficiency in p27 failed to modify the survival of B cells and the anti-apoptotic activity of Bcl-2 *in vitro* and *in vivo* (Figure 1A and B). In addition, the analysis for the effects of p27 deficiency in the proliferation of hBcl-2 overexpressing B cells showed that the reduced proliferation of B cells in mice overexpressing hBcl-2 was not restored by p27 deficiency (Figure 1C).

When the production of autoAbs in B6.p27^{-/-} mice at 6 months of age was analyzed, only the levels of circulating total IgA and IgG were slightly increased or reduced, respectively, in comparison to B6-wt and B6.p21^{-/-} mice (Figure 2; $p < 0.02$ in all cases). The levels of circulating IgA and IgG anti-ssDNA autoAbs in B6.p27^{-/-}-hBcl-

2-B Tg mice were significantly elevated as compared to B6.p27^{-/-} mice ($p<0.005$ in all cases), but not higher than those of B6.hBcl-2-B Tg mice and much lower than those of B6.p21^{-/-}-hBcl-2-B Tg mice (Figure 2; $p<0.002$ in all cases). The pool of B200⁺IgM⁻IgD⁻CD138⁻ B cells was increased in the spleen of 8 month-old B6.p27^{-/-} and 2 and 8 month-old B6.p27^{-/-}-hBcl-2-B Tg mice in comparison to B6-wt mice, but was still lower than that of B6.p21^{-/-}-hBcl-2-B Tg mice at both 2 and 8 months of age (Table 1; $p<0.01$ in both cases). Furthermore, B6.p27^{-/-} and B6.p27^{-/-}-hBcl-2-B Tg mice also displayed an increase in the number of CD4⁺CD44^{high}CD62L^{low} T cells at levels similar to that observed in B6.p21^{-/-}-hBcl-2-B Tg mice (Table 1). The analysis of renal histopathology at 14 months of age in B6.p27^{-/-} and B6.p27^{-/-}-hBcl-2-B Tg mice revealed that only 13% of B6.p27^{-/-} (3/22) and B6.p27^{-/-}-hBcl-2-B Tg mice (3/23) developed a severe glomerulonephritis (Figure 4A and C).

3.4. Development of B-cell LPD in B6.p21^{-/-}-hBcl-2-B Tg mice

Since almost half of B6.p21^{-/-}-hBcl-2-B Tg mice did not show severe glomerular lesions at the time of autopsy, we explored for the presence of additional causes of death in these animals. In fact, B6.p21^{-/-}-hBcl-2-B Tg mice presented an elevated incidence of B-cell LPD, evaluated by histological analysis (Figure 5 and Table 2) and immunohistochemistry and/or flow cytometry (data not shown). These malignancies were classified into two histological categories; polymorphic LPD (PLPD) composed of B cells at different stages of development/activation (small cells, immunoblasts and plasma cells) (Figure 5A and B) and monomorphic LPD resembling immunoblastic B-cell lymphomas (Figure 5C and D) showing signs of high proliferative activity such as abundant aberrant mitosis (Figure C) and a starry sky pattern (Figure 5D), indicative of the presence of macrophages phagocytosing dying cells. These lymphomas usually

formed solid peritoneal tumours and infiltrated other organs such as the liver or kidneys (Figure 5D). Some animals displayed only PLPD but the majority of mice with B-cell lymphomas also had PLPD usually in the infiltrating organs (Figure 5D and E).

To analyze in greater detail the development of LPD in B6.p21^{-/-}-hBcl-2-B Tg mice in comparison to B6-wt, B6.p21^{-/-} and B6.hBcl-2-B Tg mice, groups of these animals were sacrificed at 8-10 months and 18 months of age. At 8-10 months of age 31% and 44% of B6.p21^{-/-}-hBcl-2-B Tg mice presented PLPD and B-cell lymphomas, respectively, in contrast to 7% of B6.p21^{-/-} mice presenting PLPD (Table 2). None of B6-wt and B6.hBcl-2-B Tg mice had B-cell malignancies at this age. Among the B6.p21^{-/-}-hBcl-2-B Tg mice that survived up to 18 months of age, 15% and 70% of animals presented PLPD and B-cell lymphomas, respectively (Table 2). In contrast, the respective incidences were 19% and 2% in B6.p21^{-/-} mice, and 0% and 17% in B6.hBcl-2-B Tg mice.

4. Discussion

The control of cell cycle and survival is essential for the maintenance of lymphoid homeostasis and self-tolerance. Disruption in the regulation of any of these processes in mice can lead to autoimmunity and/or LPD [1]. However, the development of pathology in these animals, particularly autoimmune diseases, is largely influenced by the genetic background of the strains of mice studied [2, 9-12]. Due to the polygenic nature of autoimmune diseases, we have analyzed here the possible complementation between genetic defects affecting the regulation of lymphocyte survival (Bcl-2 overexpression) and proliferation (p21 or p27 deficiency) in the development of autoimmunity and LPD in mice with a non-autoimmune genetic background. We have demonstrated that the deficiency in the cell cycle regulator p21

and the overexpression of Bcl-2 in B cells in non-autoimmune B6 mice promote a lethal lupus-like syndrome characterized by autoAb production and an IgA nephropathy. This lupus-like syndrome associated to IgA nephropathy resembles that of (NZW x B6)F1 mice overexpressing hBcl-2 in B cells [2], supporting the importance of B-cell Bcl-2 deregulation in the pathogenesis of this particular form of experimental glomerulonephritis. In addition, B6.p21^{-/-}-hBcl-2-B Tg mice present a high incidence of B-cell LPD. In contrast, such cooperation for the development of autoimmunity is not observed in p27^{-/-} mice overexpressing Bcl-2 in B cells.

Our present results confirm the importance of the pro-autoimmune genetic predisposition for the complete manifestation of autoimmunity in mice deficient in p21. Recently, it has been shown that B6.p21^{-/-} mice develop mild lupus-like serological and glomerular abnormalities that do not modify the lifespan of these animals in comparison to mice deficient in p21 in an autoimmune-prone mixed (129/Sv x B6) genetic background [7, 9]. This contrasts with the lack of development of even a mild lupus-like syndrome in our B6.p21^{-/-} mice, and the reasons for this discrepancy may be attributed to differences in animal housing conditions or to the origin of B6 colonies used in both studies. However, the fact that B6.p21^{-/-}-hBcl-2-B Tg mice develop a severe lupus-like syndrome clearly supports the idea that p21 deficiency acts as an autoimmunity enhancer that cooperates with hBcl-2 overexpression in B cells, thereby triggering severe autoimmune responses in normal mice. The beneficial effects of the treatment with a peptidyl mimic of p21, which inhibits the interaction between cyclin-dependent kinase 4 and type D cyclins, on the development of SLE in (NZB x NZW)F₁ females [8] further support this idea. In contrast, other studies in BXSB mice or in a line of p21^{-/-} mixed (129/Sv x B6) mice, derived from an unknown number of intercrosses, claim that p21 deficiency may have irrelevant or even protective effects on disease development

[10-12], suggesting the existence of unknown strain-specific genetic interactions that modulate the accelerator activity of this genetic abnormality in autoimmunity.

There are multiple evidences indicating that p27 is an important regulator of clonal anergy of T lymphocytes [13, 14]. In this sense, p27 is required for transplantation tolerance induced by costimulatory blockade [31]. However, p27^{-/-} mice have failed to develop autoimmune diseases even in a (129/Sv x B6) genetic background [15]. Accordingly, we have been unable to demonstrate the presence of high titres of circulating autoAbs and glomerulonephritis in almost all B6.p27^{-/-}-hBcl-2-B Tg mice analyzed here, despite an expansion in memory B and T cells being observed in these animals. Recent studies have shown the presence of increased serum levels of autoAbs and mild glomerular lesions in B6.hBcl-2-B Tg mice deficient in the G₁/S cell cycle regulators E2F1 or E2F2, although the survival of these animals is similar to that of non-Tg controls [32, 33]. Altogether, these and our present results indicate that the regulators of the G₁/S transition of the cell cycle are hot spot targets for the control of lymphocyte tolerance. However, not all the regulators of this critical cell cycle check point appear to influence lymphocyte tolerance to the same extent, there existing a certain hierarchy in which each genetic defect of these regulators exhibits different potentials to promote systemic autoimmune diseases in cooperation with genetic abnormalities that prolong B cell survival (at least Bcl-2 overexpression). In this hierarchy, the pro-autoimmune potential of p21 deficiency in cooperation with B-cell hBcl-2 overexpression is higher than that of E2F1 or E2F2 deficiency, which in turn is superior to that of p27 deficiency.

In addition to its anti-apoptotic activity, Bcl-2 is also involved in the control of cellular proliferation. In particular, the overexpression of Bcl-2 in lymphocytes or fibroblasts significantly delays the cell cycle entry of these cells after activation [29,

30]. On the other hand, p21 may act as a survival factor in several cell types, including T lymphocytes [28]. Thus, epistatic interactions between p21 deficiency and Bcl-2 overexpression affecting the survival and/or proliferation of B cells can be involved in the induction of severe autoimmunity in B6.p21^{-/-}-hBcl-2-B Tg mice. However, the anti-apoptotic and anti-proliferative effects of hBcl-2 overexpression in B cells from B6.hBcl-2-B Tg mice are not modified by the presence of the *p21* null mutation, indicating a dominant role of overexpressed Bcl-2 over p21 deficiency. Although p27 has been proposed as a critical mediator of the anti-proliferative function of Bcl-2 [30], our experiments clearly show that the proliferative defects observed in B cells overexpressing hBcl-2 are not restored by p27 deficiency. These results are fully in agreement with a recent report showing a similar cell cycle delay in anti-CD3 stimulated T cells from hBcl-2-T Tg and p27^{-/-}-hBcl-2-T Tg mice [21]. However, as our studies have been carried out on total B-cell populations, we cannot exclude the possibility that the interaction between p21 deficiency and Bcl-2 overexpression implicated in cell cycle progression and survival may be restricted to some minor subsets of activated/memory B cells (see below).

Consistent with a previous finding [7], our B6.p21^{-/-} mice exhibited an age-dependent enlargement of memory CD4⁺ T-cell subpopulation, and this is more pronounced in B6.p21^{-/-}-hBcl-2-B Tg mice developing a severe SLE. However, we observed that non-autoimmune B6.p27^{-/-} and B6.p27^{-/-}-hBcl-2-B Tg mice had increased numbers of CD4⁺CD44^{high}CD62L^{low} T cells even at 2 months of age and at levels comparable to those of B6.p21^{-/-}-hBcl-2-B Tg mice. These data strongly suggest that the expansion of these memory T cells in B6.p21^{-/-}-hBcl-2-B Tg mice by itself is not sufficient to promote the disease.

It has been demonstrated that Bcl-2 overexpression extends the lifetime of memory B cells [34]. Accordingly, high numbers of B220⁺IgM⁻IgD⁻CD138⁻ memory B cells are found in B6.hBcl-2-B Tg mice even at 2 months of age. Parallel to the increase in memory CD4⁺ T cells, memory B cells are also expanded in B6.p21^{-/-} and B6.p27^{-/-} mice. However, whereas the size of this B cell population in B6.p27^{-/-}-hBcl-2-B Tg mice is similar to that of B6.hBcl-2-B Tg mice, a synergistic interaction between p21 deficiency and Bcl-2 overexpression seems to regulate the number of memory B cells in B6.p21^{-/-}-hBcl-2-B Tg mice. This synergistic interaction may operate at two levels. First, and as in T cells [9], p21 can affect the proliferation of memory, but not naïve, B cells that survive activation-induced cell death (AICD) processes. Second, hBcl-2 overexpression can modulate the pro-apoptotic activity of Bim regulating the survival of memory B and autoAb-forming cells during AICD *in vivo* [35, 36]. In this scenario, the abnormal expansion and/or survival of these B cells together with the increased number of memory CD4⁺ T cells may be the cause for the induction of SLE in B6.p21^{-/-}-hBcl-2-B Tg mice.

A careful analysis of B6.p21^{-/-}-hBcl-2-B Tg mice at the time of autopsy indicates that in addition to the development of a lupus-like autoimmune syndrome associated with an IgA nephropathy, these mice present B-cell LPD with a much higher incidence than B6.p21^{-/-} or B6.hBcl-2-B Tg mice. Histologically, these LPD can be classified as PLPD and immunoblastic B-cell lymphomas. The coexistence of both types of LPD in many animals strongly suggests that these lesions may represent different stages of the same B-cell malignancy. Despite the absence of autoimmunity in B6.p27^{-/-}-hBcl-2-B Tg mice (present study), we have observed the development of B-cell LPD in these animals that shortened their lifespan (Santiuste I. et al, manuscript in preparation). In this regard, it has been shown that p27^{-/-} mice overexpressing Bcl-2 in T lymphocytes

spontaneously develop T-cell lymphomas with a high incidence [21]. At present, the mechanisms responsible for the development of B-cell LPD in B6.p21^{-/-}-hBcl-2-B Tg mice are unknown. One possibility is that the same epistatic interaction between p21 deficiency and Bcl-2 overexpression promotes LPD in B6.p21^{-/-}-hBcl-2-B Tg mice independently of the autoimmune syndrome, as both B6.p21^{-/-} or B6.hBcl-2-B Tg mice develop with age B-cell malignancies without overt autoimmune diseases [16, 17, 19]. Additionally and/or alternatively, the development of B-cell LPD in B6.p21^{-/-}-hBcl-2-B Tg mice may be closely linked to the autoimmune process, secondary to the chronic activation of autoreactive B lymphocytes by non-tolerized CD4⁺ T cells. In this regard, the spontaneous development of B-cell lymphomas is common in different lupus-prone mice, such as NZB, Fas or FasL deficient and interleukin-12 receptor beta2 deficient mice [37-40]. Experiments are in course to clarify this important question.

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Figure Legends

Figure 1. Survival and proliferation of B cells from B6 mice overexpressing hBcl-2 in B cells and deficient in p21 or p27. **A)** Purified B cells from different strains of mice were cultured in triplicates in 96-well flat-bottomed plates at a concentration of 10^6 cells/ml in RPMI 10% FCS. From day 1 to 10, viability of spleen B cells was assessed by trypan blue exclusion. Results are representative of two separate experiments. **B)** 2 month-old mice were injected ip with PBS (upper panels) or 2 mg of dexamethasone (lower panels). The elimination of B220^{low}IgM⁻ pre-B and B220^{low}IgM⁺ immature B cells was evaluated by flow cytometry 48 hrs after treatment. The mean $\pm$ SD of the percentages of B220^{low}IgM⁻ pre-B (upper left) and B220^{low}IgM⁺ immature B cells (bottom right) in the different experimental groups (3 mice/group) are indicated in each panel. Statistic differences between hBcl-2 Tg and non-Tg dexamethasone-treated animals are indicated as follow: * $p < 0.01$, ** $p < 0.001$. Results are representative of three separate experiments. **C)** CFSE-labelled B cells (2×10^5) from the different strains of mice were stimulated during 48 hrs with 20 μ g/m LPS or 20 μ g/ml of anti-IgM antibody. Cell division was analyzed by flow cytometry according to the CFSE intensity dilution. Results, representative of two separate experiments, are expressed as the mean percentage $\pm$ SD (3 mice/group) of proliferating cells.

Figure 2. Production of autoAbs in B6 mice overexpressing hBcl-2 in B cells and deficient in p21 or p27. **A and B)** Serum levels of IgA and IgG anti-DNA autoAbs and gp70 IC (**A**) and of total IgA and IgG (**B**) in the mentioned mouse strains at 6 months of age. Values of individual mice are expressed. Bars represent the mean value of each examination.

Figure 3. Mortality curve of B6 mice overexpressing hBcl-2 in B cells and deficient in p21.

Figure 4. Development of glomerulonephritis in B6 mice overexpressing hBcl-2 in B cells and deficient in p21 or p27. **A)** Glomerulonephritis score in the different strains of mice at the time of autopsy (B6.p21^{-/-}-hBcl-2-B Tg mice) or at 14-16 months of age (other groups). Values of individual mice are expressed. Bars represent the mean value of each group. **B)** Representative histological appearance of glomeruli (x40) from B6-wt, B6.p21^{-/-} and B6.hBcl-2-B Tg mice at 14-16 months of age and from B6.p21^{-/-}-hBcl-2-B Tg mice at the time of autopsy, stained with hematoxylin-eosin or Masson trichrome (two upper panels). Presence of IgG and IgA deposits (two lower panels) in the glomeruli of B6-wt, B6.p21^{-/-} and B6.hBcl-2-B Tg mice at 14-16 months of age and from B6.p21^{-/-}-hBcl-2-B Tg mice at the time of autopsy (x40). **C)** Representative histological appearance of glomeruli (x40) from 14 month-old B6.p27^{-/-} and B6.p27^{-/-}-hBcl-2-B Tg mice stained with hematoxylin-eosin or Masson trichrome.

Figure 5. Development of LPD in B6 mice overexpressing hBcl-2 in B cells and deficient in p21. **A)** PLPD in a lymph node from a 10 month-old B6.p21^{-/-}-hBcl-2-B Tg mice (x40). **B)** PLPD in the lymph node of a 10 month-old B6.p21^{-/-} mice (x40). **C)** Immunoblastic B-cell lymphoma in a 14 month-old B6.p21^{-/-}-hBcl-2-B Tg mice (x100) showing an aberrant mitosis (red arrow and squared amplification in the bottom left corner). **D)** Starry sky pattern of the immunoblastic B-cell lymphoma showed in B (x20). **E)** PLPD infiltrates in the kidney of an 18 month-old B6.p21^{-/-}-hBcl-2-B Tg mice (x40). **F)** Immunoblastic B-cell lymphoma in the lymph node of a 18 month-old B6.hBcl-2-B Tg mice (x40) showing areas with PLPD (red arrows).

Table 1: Distribution of naïve and memory B and CD4⁺ T cell populations in p21 and p27 deficient mice in the context of hBc-2 B-cell overexpression.

Mice	Age	B Cells		CD4 ⁺ Cells		CD8 ⁺ Cells	
		B220 ⁺	memory	naïve	memory	naïve	memory
B6-wt	2	36.4 ± 8.9	2.3 ± 0.5	10.8 ± 4.9	5.3 ± 1.1	7.9 ± 3.2	1.8 ± 0.3
	8	57.1 ± 10.5	4.9 ± 2.4	11.9 ± 5.3	8.1 ± 1.5	11.6 ± 5.9	2.1 ± 1.8
B6.p21 ^{-/-}	2	44.1 ± 17.1	3.9 ± 1.9	11.3 ± 4.8	8.3 ± 4.1	7.9 ± 3.1	1.9 ± 1.5
	8	35.6 ± 15.4	7.1 ± 3.1	6.4 ± 2.8	14.1 ± 3.9	6.4 ± 2.7	2.2 ± 1.0
B6.hBcl-2-B Tg	2	166.8 ± 56.5	16.8 ± 6.8	14.1 ± 3.2	6.5 ± 1.0	13.1 ± 3.2	2.1 ± 0.9
	8	138.8 ± 66.2	22.8 ± 8.4	14.8 ± 5.4	10.1 ± 3.7	14.5 ± 6.3	2.8 ± 1.8
B6.p21 ^{-/-} -hBcl-2-B Tg	2	206.4 ± 42.8	31.1 ± 9.3	12.2 ± 5.6	15.1 ± 7.7	11.1 ± 3.3	3.9 ± 1.1
	8	220.6 ± 38.5	41.5 ± 10.1	7.6 ± 3.7	22.5 ± 7.8	7.8 ± 4.2	3.4 ± 1.4
B6.p27 ^{-/-}	2	66.2 ± 12.9	4.4 ± 2.2	19.9 ± 8.2	18.1 ± 8.2	23.1 ± 7.1	5.9 ± 3.4
	8	104.5 ± 39.3	17.3 ± 6.1	7.2 ± 3.4	18.4 ± 5.7	8.3 ± 3.7	5.4 ± 2.3
B6.p27 ^{-/-} -hBcl-2-B Tg	2	137.7 ± 50.1	16.4 ± 7.5	23.4 ± 4.6	21.9 ± 8.1	28.4 ± 6.3	6.2 ± 1.8
	8	116.6 ± 32.8	29.2 ± 7.1	5.1 ± 2.7	23.8 ± 8.9	6.0 ± 2.3	6.7 ± 2.7

Total B220⁺, B200⁺IgM⁺IgD⁻CD38⁻ memory, CD4⁺CD44^{low}CD62L^{high} naïve, CD4⁺CD44^{high}CD62L^{low} memory, CD8⁺CD44^{low}CD62L^{high} naïve and CD8⁺CD44^{high}CD62L^{low} memory cells were evaluated in the spleen of 2 and 8 month-old mice by flow cytometry. Results from 6-8 mice/group are expressed as the mean ± SD of the number of cells of each population.

Table 2: Increased incidence of B-cell malignancies in B6.p21^{-/-}-hBcl-2-B Tg mice.

Mice	Type	8-10 months	18 months
B6-wt	PLPD	0/16 (0 %)	0/12 (0 %)
	B-cell lymphoma	0/16 (0 %)	0/12 (0 %)
B6.p21 ^{-/-}	PLPD	1/14 (7.1 %)	8/43 (18.6 %)
	B-cell lymphoma	0/14 (0 %)	1/43 (2.3 %)
B6.hBcl-2-B Tg	PLPD	0/18 (0 %)	0/24 (0 %)
	B-cell lymphoma	0/18 (0 %)	4/24 (16.7 %)
B6.p21 ^{-/-} -hBcl-2-B	PLPD	5/16 (31.3 %)	4/26 (15.4 %)
	B-cell lymphoma	7/16 (43.8 %)	18/26 (69.2 %)

Groups of the different mouse strains were sacrificed at 8-10 and 18 months of age. Sections of major organs were included in paraffin and stained with hematoxylin-eosin. The presence of LPD, either PLPD or B-cell lymphoma, was evaluated by two pathologists. Under the PLPD group, we included animals that presented only this type of LPD. Under the B-cell lymphoma group, we included animals that presented this type of LPD independently of the coexistence of PLPD.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5