The **BCL-6** proto-oncogene controls germinal-centre formation and Th2-type inflammation

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Structural alterations of the promoter region of the **BCL-6** proto-oncogene represent the most frequent genetic alteration associated with non-Hodgkin lymphoma, a malignancy often deriving from germinal-centre B cells. The **BCL-6** gene encodes a zinc-finger transcriptional repressor normally expressed in both B cells and CD4⁺ T cells within germinal centres, but its precise function is unknown. We show that mice deficient in **BCL-6** displayed normal B-cell, T-cell and lymphoid-organ development but have a selective defect in T-cell-dependent antibody responses. This defect included a complete lack of affinity maturation and was due to the inability of follicular B cells to proliferate and form germinal centres. In addition, **BCL-6**-deficient mice developed an inflammatory response in multiple organs characterized by infiltrations of eosinophils and IgE-bearing B lymphocytes typical of a Th2-mediated hyperimmune response. Thus, **BCL-6** functions as a transcriptional switch that controls germinal centre formation and may also modulate specific T-cell-mediated responses. Altered expression of **BCL-6** in lymphoma represents a deregulation of the pathway normally leading to B cell proliferation and germinal centre formation.

The **BCL-6** proto-oncogene was identified by virtue of its involvement in chromosomal translocations in diffuse large cell lymphoma (DLCL), the most common form of non-Hodgkin lymphoma (NHL).⁴-⁶ Subsequent studies have demonstrated that rearrangements of the **BCL-6** gene can be found in 30-40% of DLCLs and in a minority (5-10%) of follicular lymphomas (FL).⁷-⁹ These rearrangements juxtapose heterologous promoters, derived from other chromosomes, to the **BCL-6** coding domain, causing its deregulated expression by a mechanism called promoter substitution.⁰ The 5' non-coding region of the **BCL-6** gene can also be altered by somatic point mutations that are detectable, independent of rearrangements, in approximately 70% of DLCLs, in 45% of FLs and in AIDS-associated NHL.¹¹,¹² Taken together, rearrangements and mutations of the **BCL-6** promoter region represent the most frequent genetic alteration in human B-cell malignancies, suggesting that they may be important for tumorigenesis.¹³

The **BCL-6** gene encodes a nuclear phosphoprotein characterized by six Krüppel-type C-terminal zinc-finger (ZF) motifs that have been shown to recognize specific DNA sequences in vitro, and by an N-terminal POZ motif shared by various ZF molecules, including the Drosophila developmental regulators Traumatomy and Broad-Complex, human KUP, ZIF, and PLZF, proteins, by some proteins of FOX viruses and by the actin-binding Drosophila oocyte protein, Kelch. **BCL-6** functions as a potent transcriptional repressor of promoters linked to its DNA target sequence. Recent results suggest that **BCL-6** can bind the Stat-6 DNA binding site and repress Stat-6-mediated IL-4 signaling (Chang, C.-C. et al., pers. comm.).

**BCL-6** mRNA is found at low abundance in multiple tissues; high levels of protein expression are found only in the lymphoid system. In the B-cell lineage, **BCL-6** protein is found only in B cells within germinal centres (GC), but not in pre-B cells or in differentiated progenies such as plasma cells. In the T lineage, **BCL-6** protein is detectable in cortical thymocytes and in CD4⁺ T cells within the GC in addition to those scattered in the peri-follicular area.

This study elucidates the biological role of **BCL-6** by analysing the phenotype of mice in which **BCL-6** has been inactivated. It shows that **BCL-6** is necessary for the development of GC and a normal T-cell-dependent antibody response. In addition, **BCL-6**-deficient mice display a characteristic inflammatory response, which implicates **BCL-6** in the control of Th2-mediated responses. Thus, our results identify **BCL-6** as a gene important in regulating both B- and T-cell responses and have direct implications for the role of deregulated **BCL-6** expression in lymphomagenesis.

**Disruption of the **BCL-6** gene in the mouse germ-line**

Disruption of the **BCL-6** gene was obtained by homologous recombination in embryonic stem (ES) cells using two targeting vectors. The first generated a truncated **BCL-6** allele (**BCL-6A**) lacking sequence (exon 8-9) coding the COOH-terminal half of the **BCL-6** protein corresponding to four of six zinc-fingers of its DNA-binding domain (Fig. 1a). This allele encodes a truncated protein (**BCL-6AZF**) that cannot bind DNA (not shown) and localizes mainly in the cytoplasm (Fig. 6), consistent with the observation that DNA binding and nuclear localization are dependent upon the COOH-
**Fig. 1** Targeted disruption of the BCL-6 gene in the mouse germ line. a, Schematic representation of the mouse BCL-6 locus before (top) and after (bottom) homologous recombination with the targeting vector (middle) generating the BCL-6<sup>−/−</sup> allele. Expected restriction fragments and probes used for Southern blot analysis are also indicated. BCL-6 exons are indicated by boxes (shaded for coding domain; dotted for region between POZ and zinc fingers; filled for zinc finger domain; empty for non-coding exons). The BCL-6 coding sequence was disrupted by insertion of a cassette containing the neomycin-resistance gene under the control of the pGK promoter (for the positive selection of transfected cells) and the thymidine kinase (TK) gene also under the control of the pGK promoter (negative selection against random integration). Restriction sites: B, BglII; H, HindIII; R, EcoRI; X, XbaI. Below: Southern blot analysis of XbaI-digested tail DNA from offspring generated by mating BCL-6<sup>−/−</sup> mice showing the wild-type 7.8-kb and the mutated 4.6-kb fragments. b, Schematic representation of the mouse BCL-6 locus before (top) and after (bottom) homologous recombination with the targeting vector (middle), generating the BCL-6<sup>−/−</sup> allele. Southern blot analysis of EcoRI-digested tail DNA of offspring generated by mating BCL-6<sup>−/−</sup> mice shows the wild-type 11-kb and the mutated 8.9-kb fragments.

The second construct was targeted to BCL-6 exons 4–10, corresponding to virtually the entire (92.5%) BCL-6 coding domain. It produced a BCL-6 'null' allele with no coding capacity (BCL-6<sup>−/−</sup>) (Fig. 1b; see also Fig. 5). Genotypic analysis of the offspring (Fig. 1) indicated that normal (BCL-6<sup>−/+</sup>), heterozygous (BCL-6<sup>−/ΔΔ</sup> or BCL-6<sup>−/−</sup>) and homozygous (BCL-6<sup>−/ΔΔ</sup> or BCL-6<sup>−/−</sup>) animals were born at expected mendelian frequencies (not shown). The offspring generated from four independent ES clones (3 BCL-6<sup>−/−</sup>; 1 BCL-6<sup>−/−</sup>) indicated that both types of heterozygous animals were phenotypically indistinguishable from wild-type, while homozygous BCL-6<sup>−/ΔΔ</sup> and BCL-6<sup>−/−</sup> mice displayed an identical pathologic phenotype (see below). Thus, similar to the fully-deleted BCL-6<sup>−/−</sup> allele, the truncated BCL-6<sup>ΔΔ</sup> gene is functionally 'null'.

**Bacterial infections in BCL-6 null mice**

At birth, all heterozygous and homozygous mice were indistinguishable from wild-type littermates in morphology and weight. However, while wild-type and heterozygous mice remained indistinguishable during growth of BCL-6<sup>−/ΔΔ</sup> or BCL-6<sup>−/−</sup> mice derived from all four ES lines was significantly retarded (BCL-6<sup>−/ΔΔ</sup>; 51.7 ± 5.3% the weight of wild-type age-matched littermates; BCL-6<sup>−/−</sup>; 42.8% ± 19%). No gross developmental abnormalities were noted and the weights of
most organs were comparable in wild-type, heterozygous and BCL-6 null mice after normalization for body weight; the spleen weight was more variable in BCL-6 null mice showing hyperplasia due to infiltration by non-lymphoid cells in some animals (see below) and occasional hyperplasia in very young mice (<2 weeks old); the thymus was smaller in some mice, particularly in those with early infections (see below). As no specific developmental abnormality was detectable at birth, growth retardation was attributed to the infectious and inflammatory diseases developed later by these animals (see below), and in particular to oesophagitis, which may impair their capability to intake food.

Approximately half of the BCL-6 null mice (BCL-6Δ/Δ, 30/70; BCL-6Δ/+, 8/18), but none of the wild-type (0/50) or heterozygous (0/27) mice, displayed infections within the first three months of life. These infections had a variable clinical course ranging from subclinical (that is, detectable at autopsy) to lethal, and they most frequently involved the oesophagus, but also various other mucosal sites in the upper respiratory and digestive tracts (oribut, ear and nose) as well as the lung. Pathologic examination of these lesions showed infiltrates of granulocytes and monocytes occasionally leading to frank abscesses. Histochemical staining and testing for anti-viral antibodies (see Methods) showed that these infections were due to bacteria (Fig. 2); infections were explained by the impaired antigen-specific antibody response of BCL-6 null mice (see below).

BCL-6 null mice develop a Th2-type inflammatory disease

A second disorder of BCL-6 homozygous mutant mice was represented by a systemic inflammatory disease involving multiple organs most frequently including the myocardium, spleen, gut, liver and skin. Prominent signs of inflammation in one or more organs were detectable in 61 of 70 (87%) BCL-6Δ/Δ mice and in 14 of 18 (77%) BCL-6Δ/+ mice (features were macroscopically and microscopically indistinguishable between two types of mice), but in none of the heterozygous (0/27) or wild-type (0/50) animals. The pathologic picture was characterized by prominent eosinophilic infiltrates (Fig. 3a), which were first detectable as intraepithelial infiltrates in the gut of young animals at three months. These infiltrates were often accompanied or followed by a lympho-monocytic component (Fig. 3a). The presence of eosinophilic infiltrates in lung, heart and mediastinal lymph nodes was invariably associated with a particular mouse disease of the lung, called acidophilic macrophage pneumonia10, and characterized by the infiltration of macrophages filled by degradation products of eosinophils (Fig. 3a). In these animals, the bone marrow showed a modest increase in eosinophilic precursors, while no peripheral eosinophilia was observed. The involved organs tested negative for bacteria and parasites and did not show pathologic signs of viral infection (data not shown; see Methods).

Since BCL-6 is not expressed in eosinophils in humans25 and mice (Cattoretti, G., et al., pers. comm.), their altered regulation suggested a Th2-mediated inflammatory reaction31. To examine this possibility, we investigated whether BCL-6 null mice also had abnormal distributions of IgG1 and IgE, features also typical of Th2-mediated responses31. By immunohistochemical analysis, a dramatic increase in IgE in eosinophil-absorbed form was detected in the gut (Fig. 3b) and a significant number of IgE-expressing B cells was detectable in various organs including the spleen and liver in BCL-6Δ/Δ and −/− mice but not in control littermates (Fig. 3b). Markedly increased number of IgG1-bearing plasmacytoid cells, identified by double-staining with antibodies for IgG1 and the mature B-cell marker syndecan, was also detectable in the perilobular areas of the spleen of BCL-6 null mice (Fig. 3b). The number of IgG2a- and IgG2b-positive cells was also increased, although less dramatically. No alteration in the expression of other immunoglobulins (IgA and IgG3) was detectable by immunohistochemical analysis on the same tissue sections. Taken together, the organ infiltrations by eosinophils and an increase in IgG1- and IgE-bearing B cells are consistent with a Th2-mediated inflammatory response in BCL-6 null mice.

Normal B and T cell populations and lymphoid organ development in BCL-6 null mice

The presence of bacterial infections and inflammatory disease as well as the fact that BCL-6 is normally expressed at high levels in lymphoid cells25 prompted a detailed investigation of the development of the lymphoid tissues in the BCL-6 mutant mice. Upon histologic examination, both BCL-6Δ/Δ and −/− mice displayed normal development of the bone marrow, spleen, thymus, lymph nodes and Peyer’s patches (not shown).

Flow cytometric analysis of bone marrow, spleen, thymus, lymph nodes and peritoneal cavity cells indicated that BCL-6 null mice had normal numbers and distributions of B cells (pro-B: IgM+, B220+, CD43+; pre-B: IgM+, B220+, CD43-; immature B: IgM+, IgD+, CD23-, B220+, CD54+, CD43-; resting mature B: IgMhi, IgDhi, CD23-, B220hi, CD54-, CD43+; B1 cells: IgM+, IgD+, B220+, CD5-, Mac-1+) as well as T cells (pro-thymocytes: CD30+, CD4+, CD8-; cortical thymocytes: CD3+, CD4+, CD8+; peripheral T cells: CD3+, CD4+, CD8+). These results show that BCL-6 is not required for lymphoid subpopulations32,33. These results show that BCL-6 is not required for lymphoid subpopulations32,33.
Reduced antigen-specific T-cell-dependent Ig response and lack of affinity maturation in BCL-6 null mice

We analysed the ability of BCL-6 null mice to produce antibodies and to mount an antigen-specific immune response. Basal serum levels of most Ig subtypes were modestly decreased in naive healthy BCL-6 null mice (Fig. 4a). The presence of all Ig isotypes in serum and in tissues (immunohistochemical analysis using anti-IgA, IgG1, IgG2a, IgG2b, IgG3 and IgE antibodies; not shown) suggested that these mice were able to undergo Ig isotype switching. In order to directly assess their ability to mount an antigen-specific antibody response, mice were immunized with either a T-cell-independent (NP-Ficoll) antigen or a T-cell-dependent antigen (NP-KLH). No significant difference was detectable in NP-specific Ig serum levels in mice immunized with the T-cell-independent antigen (not shown). On the contrary, the T-cell-dependent antibody response of most Ig isotypes was consistently lower in BCL-6 null mice (Fig. 4b), ranging from a modest reduction in IgM level (2-fold compared with wild-type) to more significant decreases (4-20-fold) for all IgG subtypes. Thus, the lack of a functional BCL-6 molecule caused a selective deficiency in T-cell-dependent antibody responses.

Affinity maturation is thought to be associated with the development of GCs. To determine whether affinity maturation takes place in BCL-6 null mice, we hyperimmunized wild-type and BCL-6+/− mice with NP-KLH and analysed the serum for the presence of high affinity antibodies (that is, those able to bind to a low haptenated protein, NP3-BSA). The results demonstrate that while the class-switched IgG1 anti-NP specific antibodies in wild-type mice showed progressive affinity maturation in the secondary (2nd) and tertiary (3rd) responses, there is no detectable maturation in the BCL-6 null mice even following a third immunization (Fig. 4c). Thus, while the absence of BCL-6 results in a partial reduction in the IgG response, it leads to a complete lack of affinity maturation.
Lack of germinal centre formation in BCL-6 null mice

Antibody response to T-dependent antigen is largely dependent on the ability of the B cell to undergo clonal expansion and affinity maturation within GC, while T-independent responses are typically GC-independent \[14,25\]. Thus, the reduced antigen-specific Ig response of BCL-6 null mice prompted us to examine their ability to form GC. Morphologic examination as well as immunohistochemical staining for the GC B-cell marker peanut agglutinin \[26\] and for the BCL-6 protein showed that non-immunized BCL-6 \[\alpha/\alpha\] mice and \[\alpha/\delta\] mice had not spontaneously formed GC in the spleen, although these structures were readily detectable in age-matched wild-type littermates (Fig. 5, top panels; note the absence of BCL-6 protein in BCL-6 \[\delta/\delta\] mice). Upon immunization with the T-dependent antigen, both types of BCL-6 null mice failed to form GC in the spleen, lymph nodes and Peyer’s patches, while these structures developed normally in immunized heterozygous and wild-type littermates (Fig. 5, bottom panels; note the scattered expression of the truncated BCL-6 \[\delta/\delta\] protein in BCL-6 \[\alpha/\delta\] mice). The architecture of the lymphoid organs in BCL-6 \[\alpha/\alpha\] null mice indicated that primary follicles formed, including mantle and T cell zones, but appeared distinctly ‘empty’ of GC even after repeated immunizations (Fig. 5). The lack of GC formation in these mice is consistent with their impaired ability to mount an antigen-specific Ig response to T-dependent antigens and with their complete lack of affinity maturation (Fig. 4b–c).

Mechanism of germinal centre defect: follicular B cells fail to proliferate and differentiate into GC

We investigated the mechanism by which BCL-6 null mice fail to form GC, and in particular whether the defect was due to lack of proliferation, differentiation or increased death of B cells. Standard in vitro proliferation assays using purified spleen IgM \[B\] cells and various combinations of mitogens and activators (LPS, IL-4 and CD40 ligand) did not identify any significant difference between BCL-6 null and control mice (not shown). However, these assays could not adequately inform on the proliferative response of BCL-6-expressing cells because cells corresponding to the GC fraction are quite rare among total splenic B cells (<5%; unpublished data, C.G.); in addition, BCL-6-expressing cells cannot be purified from BCL-6 null animals, since they lack GC markers.

To circumvent these problems, we exploited the fact that the truncated protein (BCL-6 \[\delta/\delta\]F) produced by the BCL-6 \[\alpha/\delta\] mice is recognized by an antiserum raised against the amino-terminal portion of BCL-6, and can therefore be used to track in vivo the fate and phenotype of cells in which BCL-6 gene expression is induced. Initial studies, using double immunohistochemical staining for IgD and BCL-6, showed that the expression of these two molecules is mutually exclusive in B cells of wild-type mice (mantle zone B cells: IgD+ /BCL-6+; GC B cells: IgD+/BCL-6−; ref. 25), while the same molecules are co-expressed in sparse cells in the primary follicles of BCL-6 \[\delta/\delta\] mice (data not shown). Triple-immunofluorescent staining for BCL-6

Fig. 4 Serum antibody levels in BCL-6 null mice. a, Resting serum antibody levels in the BCL-6 null mice. Serum samples were collected from 11–13-week-old mice and analysed by ELISA. P-values derived by Student’s T test on the group means (indicated as short horizontal bars) of wild-type versus homozygous groups are given below each subclass in the graph. b, Reduced antigen-specific antibody responses in BCL-6 null mice. A litter of 11-week-old mice were immunized with NP-KLH and sacrificed 11 days later for immunohistological (Fig. 5) and ELISA analysis. The NP-specific titers for each subclass are relative. c, Lack of affinity maturation in BCL-6 null mice. Sibling wild-type or BCL-6 \[\delta/\delta\] mice were immunized with NP-KLH on days 0, 21 and 42 and bled 5 days after each immunization. Sera were tested for the binding of NP-specific IgG1 antibodies to NP-BSA and NP3-BSA coated plates. Higher NP/NP3 binding ratios indicate the presence of higher-affinity (affinity-matured) antibodies.
The number of cells undergoing apoptosis was found to be comparable in mutant and wild-type mice by an ISEL (in situ end-labeling) assay, although apoptotic cells appeared to cluster within GC in BCL-6+/+ and +/−/− mice, while they were uniformly dispersed in Δ/Δ mice (data not shown). Thus, the phenotype of a normal B cell that expresses BCL-6 is IgD+/PNA+/PCNA+ within the GC, while the phenotype of the B cell that expresses the inactive BCL-6ΔZF protein is IgD+/PNA+/PCNA− in the periphery of the primary follicle. Collectively, these results indicate that BCL-6 is required for follicular B cells to acquire the GC phenotype (IgD+/PNA+) and to proliferate.

**Lack of GC formation and type-2 inflammation as a lymphoid-specific cell-autonomous defect**

Antibody-mediated immune responses within GC are dependent upon the specific cell-cell and paracrine interactions among various cell types, including B cells, T cells and follicular dendritic cells (FDC). Thus, the abnormalities detected in BCL-6 null mice, including infections and inflammatory infiltrates, could be due to defects in the function of lymphoid cells and/or other cell types. To address this issue, we generated BCL-6 null ES cells by high-dose neomycin selection of BCL-6−/Δ ES cells in vitro, and injected these cells into the blastocysts of RAG−/− mice, which lack both mature B and T cells.

The resulting chimeric [BCL-6−/Δ/RAG−/−] mice contained lymphoid cells derived exclusively from BCL-6−/Δ ES cells (Fig. 7a). These mice appeared identical to control mice [BCL-6+/+/RAG−/−], obtained by injection of wild-type ES cells into RAG−/−blastocysts) with respect to the development of lymphoid organs and the number and distribution of B and T cells (not shown). However, as with BCL-6Δ/Δ mice, BCL-6Δ/Δ/RAG−/− mice failed to produce any detectable GC upon immunization with the polyclonal activator sheep red blood cells (SRBC), while control mice produced prominent GC in most lymphoid organs (Fig. 7b). Upon histopathologic examination, two of six [BCL-6Δ/Δ/RAG−/−] mice showed signs of infection. Thus, the defect in GC formation in BCL-6-deficient mice is due to an intrinsic defect in the lymphoid compartment rather than to a defect in FDC

6, the B-cell marker B220, and PNA (Fig. 6a) showed that BCL-6ΔZF-expressing B cells are dispersed in the follicular area of the spleen in BCL-6Δ/Δ mice and fail to express PNA. In addition, triple-immunofluorescent staining for BCL-6, the B-cell marker B220 and the proliferation-associated marker PCNA (Fig. 6b) showed that BCL-6ΔZF expressing B cells fail to express PCNA and therefore do not proliferate. In the same tissue sections, the or in soluble molecules produced by non-lymphoid cells. In addition, all [BCL-6Δ/Δ/RAG−/−] animals displayed intraepithelial eosinophilic infiltration of the gut, an early sign of Th2-type inflammation in BCL-6−/− animals. Thus, the abnormal inflammatory response also reflects a lymphoid-restricted defect consistent with the hypothesis that it may be due to the lack of BCL-6 function in lymphoid cells.
Fig. 6 B cells expressing the inactive BCL-6ΔZF protein fail to proliferate and acquire the germinal-centre phenotype. Spleen sections from BCL-6+Δ and +/+ mice stained for BCL-6, the B-cell marker B220 and the GC marker PNA (a) or the proliferation marker PCNA (b). The triple stainings show that most B cells express BCL-6, PNA and PCNA in the GC of control mice. Conversely, BCL-6ΔZF expressing B cells lack both PNA and PCNA expression in BCL-6ΔΔ mice. Note the abnormal cellular distribution of the BCL-6ΔZF protein.

Discussion

In an attempt to understand the biological role of BCL-6 in lymphomagenesis we studied the phenotype of mice lacking a functional BCL-6 protein. In general, our results indicate that the main function of BCL-6 is in the lymphoid system as no primary pathologic feature was detectable in other tissues and all abnormalities were shown to be lymphoid-derived in the RAG-1ΔΔ complementation study (Fig. 7). Based on the results obtained from the study of four independent BCL-6 null mouse lines, a lack of functional BCL-6 appears to lead to two major phenotypes: a systemic inflammatory disease, suggesting an unexpected role of BCL-6 in the control of Th2-dependent responses and a specific defect in GC formation, with direct implications for a role of BCL-6 in the pathogenesis of B-cell lymphoma.

Role of BCL-6 in Th2-type inflammation

The lymphoid-dependent phenotype of RAG-1ΔΔ/BCL-6ΔΔ mice suggests that the inflammatory response of BCL-6 null mice is due to the function of BCL-6 in T and/or B cells. The pathologic features of the inflammatory disease, including organ infiltration by eosinophils and hyper-production of IgG1/IgE-bearing B cells, are typical of Th2-mediated hyper-responses. Combined with the fact that BCL-6 is expressed in a subset of CD4+ T cells, this phenotype suggests that BCL-6 modulates Th2 cell differentiation or function. Th2 cells are the critical regulators of allergic responses because they produce IL-4, which causes the preferential Ig switch of B cells toward the IgG1 and IgE isotypes, as well as IL-5, which leads to the stimulation of eosinophils. In strong support of a role for BCL-6 in Th2 responses, we recently observed that BCL-6 can bind to the DNA target sequences of Stat-6, repress Stat-6-activated transcription and therefore cause the repression of Stat-6 mediated IL-4 responses (Chang et al., manuscript in preparation), the main effectors of Th2 function and IgG1/IgE isotype switch in B cells. Thus, it is possible that the inflammatory phenotype of BCL-6 null mice may be due to a lack of BCL-6 modulation of IL-4 signalling, which, in turn, would cause a
and lymphotoxin-α, as well as in mice lacking the B-cell surface molecule CD19 (ref. 49), the signal transducer Lyn50, the IκB-type molecule BCL-3 (ref. 51), and the B-cell-specific transcription coactivator OCA-B52,53. However, in all these cases the GC defect was part of, and probably secondary to, broader defects in B-cell lineage development (CD19, OCA-B and Lyn), the result a general disturbance in lymphoid organ development (in the mice lacking lymphotoxin-α, type-I TNF receptor or BCL-3), or was only partial, with scattered clusters of cells displaying a typical GC phenotype (in mice lacking CD40L and CD40L, unpublished data, C.G.). In contrast, BCL-6−/− mice displayed a selective and complete defect in GC formation in the absence of qualitative or quantitative alterations of lymphoid organ or overall B-cell lineage development. This phenotype, together with the specific pattern of expression of BCL-6 in GC B cells25, suggests that BCL-6 may trigger a signal essential for GC formation. Because BCL-6 downregulates IL-4 signaling (Chang, C.-C., pers. comm.) and is downregulated by CD40 activation26, it is conceivable that it may play a central role in integrating various signals that regulate GC formation and GC-dependent B-cell-mediated immune responses.

The precise mechanism by which BCL-6 controls GC formation remains to be elucidated. Since BCL-6 is normally expressed in T cells as well as in B cells within GC27, the lack of GC may be due to inadequate T-cell priming and/or decreased B-cell responsiveness. This question can be addressed by analysing GC formation in chimeric mice obtained by injection of BCL-6−/− null ES cells into the blastocysts of mice lacking either the B or T cell lineage34,55. Regardless of the cellular components involved, the fact that the B cell expressing the inactive BCL-6 molecule does not acquire PNA and PCNA markers demonstrates that BCL-6 controls both proliferation and differentiation of B cells into GC.

Implications for lymphomagenesis

Our data provide direct insights into the role of BCL-6 in the pathogenesis of DLCL, tumors thought to derive from GC B cells56. Of the DLCLs in which BCL-6 function is perturbed, approximately 30% carry BCL-6 translocations which deregulate BCL-6 expression by promoter substitution10. In most of the remaining fraction of DLCL and in 45% FL, the BCL-6 promoter region is affected by mutations that, at least in some cases studied, have also been shown to deregulate its expression (our unpublished results). Therefore, in most B-cell lymphomas, the switch off of BCL-6 expression normally associated with exit from GC failed to occur. The observation that BCL-6-deficient B cells fail to form GC because they fail to undergo activation and rapid proliferation, implies that a B cell carrying a deregulated BCL-6 gene may be altered in these functions. Thus, alterations of BCL-6 expression by chromosomal translocation may contribute to lymphomagenesis by triggering an uncontrolled clonal expansion of the GC precursor cell.

Methods

Targeted Disruption of the BCL-6 Gene in the Mouse Germline. The murine BCL-6 genomic locus was cloned from a genomic library constructed from mouse strain 129/Sv DNA using a human BCL-6 cDNA clone as a probe. The exon-intron organization was determined by restrict-
tion endonuclease and DNA sequencing analysis. The first targeting vector was constructed from plasmid pU6NT2 by inserting a 6.5-kb EcoRI-BgII BCL-6 genomic fragment upstream and a 2.8-kb HindIII fragment downstream of the pGKNeo cassette which, after homologous recombination, replaces the BCL-6 exons 8–9 coding four of six BCL-6 zinc-fingers (Fig. 1a). The second pU6NT2-derived targeting construct contained a 2.0-kb Hind III-EcoRI fragment containing the BCL-6 exon 3 region and a 6.9-kb SacI fragment downstream of the BCL-6 encoding region. This construct replaced BCL-6 exons 4–9 as well as the coding region of exon 10 (Fig. 1b). The targeting vectors were linearized and electroporated into C57 ES cells as described before. G418- and gancyclovir-resistant clones were screened for homologous recombination by Southern blot analysis using diagnostic digestions and probes as shown in Fig. 1. Generation of chimeras and breeding of mutant mice were essentially as described. Genomic DNA was extracted from mouse tail tissue, digested with XbaI, and hybridized to the 1.1X.2 probe shown in Fig. 1a, or digested with EcoRI and hybridized to the SacII probe shown in Fig. 1b.

Histology and immunohistochemistry. Spleen, thymus, lymph nodes (popliteal, axillary, submandibular and mesenteric), liver and Peyer's patches were fixed in 10% buffered formalin overnight at RT and embedded in paraffin. Four µm-thick, dewaxed and antigen-retrieved (0.001M EDTA pH 7.5 for 15 min at 100°C) paraffin sections were stained for H&E and immunostained.

The antibodies used were polyclonal rabbit anti GST-BCL-6 fusion protein (N3; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit (Ab anti-TdT (Serum Lab), Ki-67 (Novacastro Laboratories), rabbit anti-CDS, myeloperoxidase, lysozyme, S-100, mouse anti-PCNA (PC10) (Dako), goat (gt) anti-mouse IgG, IgD (ICN), IgG1, IgG2a, IgM (Southern Biotechnology Associates), goat anti-peanut lectin (Vector), rat anti-mouse CD43/80-B20 (RA3-82b), CD63 (S7) and sycepan (28.1-1) (Pharmingen), gt anti mouse IgA, mouse ascites (MOPC21) (Sigma). Biotinylated Arachis Hypogea lectin (PNA) was purchased from Sigma and used at 5 µg/ml. Primary antibodies were counterlabelled with species-specific biotin- or AP-conjugated antibodies. Avidin-AP and -HRP were purchased from Dako. For single colour immunohistochemistry, the antibodies were revealed with peroxidase-conjugated avidin and AEC (Sigma) brown precipitation. Slides stained for BCL-6 were then restained for PNA in two colour immunostaining and developed in AP with Fast Blue (Sigma) diazonium salts. Double and triple stainings were performed with species-specific secondary antibodies conjugated with FITC, TRITC and biocytin (Southern Biotechnology Associates; these latter counterstained with Avidin-AMCA, Vector).

Bacterial, parasite and viral diagnostics. Bacterial and parasitic infections were evaluated by morphologic microscopic examination of the lesions, the mucosal surfaces (gastrointestinal tract, respiratory mucosa), and the gastrointestinal tract content. In addition, special stains were employed: Acid Fast (to detect mycobacteria), Gram (to detect Gram- and Gram-negative bacteria) and Warthin-Starry silver stain (to detect fusiformer organisms, CAIthecoccus, fungi, pneumocystis and helminthes). Circulating antibodies against 19 murine viral pathogens were tested by Charles River Laboratories (MA).

Flow cytometry analysis. Spleen, thymus, bone marrow and peritoneal cavity lavage (Perc) were obtained from at least two 4-week-old homozygous mice per ES line and from at least three wild-type and three heterozygous littermates. Single-cell suspensions were prepared and stained using standard procedures, with appropriate combinations of fluorochrome and/or biotin-labelled monoclonal antibodies, and analysed on a five-colour FACStar Plus flow cytometer (Becton-Dickinson) with PI exclusion of dead cells as previously described. Fluorochrome-conjugated antibodies for flow cytometry were: FITC-anti-IgM (331.12), biotin-anti-IgD (AP6-12.22), FITC- or APC-anti-B20 (RA3-682), biotin-anti-CD23 (3B8), PE-anti-CD43 (S7), APC-anti-Mac-1 (M1/70), APC-anti-CD5 (53.7.8), biotin-anti-CD3 (2C11), APC-anti-CD4 (GK1.5) and FITC-anti-CD8 (53.7.7). Antibodies to CD23, CD3 and CD43 were purchased from Pharmingen (San Diego). All other antibodies were purified and conjugated as previously described.

Immunization and ELISA. The mice used in the anti-NP response studies were the progeny of (B6x129)F2 inter-crosses. Both the 129 and B6 strains carry the IgH-Cv2b haplotype and are good responders to NP-conjugates. 11-week-old mice were immunized intraperitoneally with NP2-32KLL (100 ng per mouse) in complete Freund’s adjuvant (CFA). Eleven days later, all immunized animals were bled and sacrificed and their spleens removed for immunohistochemical and ELISA analyses. For reconstituted RAG-1 mice, 12-week-old mice were immunized intraperitoneally with 1X 10^5 SBEs in PBS and sacrificed 11 days later for analysis. Resting-level serum immunoglobulins were measured by sandwich ELISA using unlabelled anti-mouse Ig antibodies as capture reagent, AP-labelled anti-mouse Ig subclass-specific antibodies as developing reagents (Southern Biotechnology Associates) and 4 methylumbelliferone phosphate (Sigma, St Louis, MO) as AP substrate. Serum values were measured against control mouse Ig isotype standards. NP-specific serum antibodies were measured similarly except that NP2-32KLL was used as plate coat and the relative titre of NP-specific antibodies are expressed as the relative of dilutions that gave fluorescence counts within linear range of the assay.

Measurement of affinity maturation. Mice were immunized intraperitoneally with 100 µg NP2-32KLL in CFA on day 0 and bled on day 5. On days 21 and 42, the mice were boosted with 10 µg NP2-32KLL in incomplete Freund adjuvant and bled 5 days after each immunization. NP-specific IgG1 antibodies of high and low affinities were assayed by their relative binding to NP2-32KLL and NP3-38KLL-coated plates. Low-affinity antibodies will bind to the highly haptenated protein (NP3-38KLL), but not to the slightly haptenated protein (NP2-32KLL), while high-affinity antibodies bind equally to both the high and low haptenated proteins. Thus, the ratio of binding to NP2-32KLL and NP3-38KLL is a measure of relative avidity of the anti-NP antibodies. Each antisera was titrated on both NP2-32KLL and NP3-38KLL-coated plates. The NP2-32KLL/NP3-38KLL ratio is calculated as the ratio of the amount of antibody (1/serum dilution) required to give equal binding (fluorescence counts) on each protein-coated plate.

**RAG-1**-complementation assay. BCL-6^+/A^ ES cells generated by homologous recombination were subjected to selection with increased neomycin concentration and the resistant clones were screened for BCL-6 status by Southern blot analysis. BCL-6^+/A^ ES cells derived from two distinct BCL-6^+/A^ clones were injected into RAG-1^-/- blastocysts (Jackson Laboratories). The resulting chimeric mice were screened for ES cell-reconstitution by both Southern blot analysis of tail DNA and CD3 staining of circulating T cells (data not shown). Passage-matched BCL-6^+/A^ ES cells were also injected as a positive control for lymphoid reconstitution. To analyse GC formation, 6 BCL-6^+/A^/RAG-1^-/- chimeric animals (derived from 2 BCL-6^+/A^ ES clones) and 3 control BCL-6^+/A^/RAG-1^-/- mice were immunized intraperitoneally with 1X 10^5 SBEs in PBS and sacrificed 11 days later for analysis.

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