

# Lymphomas can develop from B cells chronically helped by idiotype-specific T cells

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**B cell lymphomas have been associated with chronic infections and autoimmunity. However, most lymphomas develop in the absence of any known chronic antigenic stimulation. B cells process their highly diversified endogenous immunoglobulin and present clonally unique variable-region idiotypic (Id) peptides on their major histocompatibility complex (MHC) class II molecules to Id-specific T cells. We show that B cells chronically helped by Id-specific Th2 cells developed into large B cell lymphomas with cytogenetic DNA aberrations. The lymphomas expressed high amounts of Id, MHC class II, CD80/86, and CD40 and bidirectionally collaborated with Th2 cells. Thus, MHC class II-presented Id peptides may represent a chronic self-antigenic stimulus for T cell-dependent lymphomagenesis. Eventually, B lymphomas grew independent of T cells. Thus, T cells do not only eliminate cancers as currently believed. In fact, Id-specific Th2 cells can induce B lymphomas.**

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Abbreviations used: BCR, B cell receptor; CGH, comparative genomic hybridization; H, heavy; IRF-4, interferon regulatory factor 4; V<sub>H</sub>, variable heavy chain.

B lymphocytes proliferate in response to extrinsic antigen and help from T cells, but enter a quiescent memory state once antigen is removed (1). However, if antigen is not removed, B cells could chronically proliferate, accumulating genetic changes in the process, and ultimately become malignantly transformed. Consistent with such an idea, B lymphoma development has been associated with chronic infections caused by EBV, hepatitis C, and *Helicobacter pylori*, autoimmune diseases like rheumatoid arthritis, Sjögren's syndrome, and systemic lupus erythematosus, and allotransplantation as well as graft versus host disease (for review see reference 2, 3). However, most B lymphomas arise in the absence of any known antigenic exposure. Thus, novel sources of antigen relevant to lymphomagenesis should be sought.

Further suggesting a role for chronic antigenic stimulation, human follicular (2, 4, 5) and diffuse large B cell lymphomas (2, 6) are often infiltrated with T cells. However, the specificities

and function of these T cells are unknown. Thus, it is unclear whether the infiltrating T cells attack the lymphoma cells, are inert neutral bystanders, or actually support lymphoma growth through unknown mechanisms. We have investigated if T cells with a defined specificity toward a clonally restricted self-antigen, constitutively produced by B cells, could induce B lymphoma development.

B cells express a receptor for antigen (B cell receptor [BCR]) with variable regions generated by rearrangement of V(D)J gene segments and somatic hypermutation. These genetic mechanisms generate variable regions with unique antigenic determinants, called idiotopes, which mark each clone of B cells. Id is expressed on surface Ig of B cells and may be recognized by antibodies (7). In addition, it has been shown that both B lymphoma (8, 9) and B cells (10) partially degrade their endogenous BCR and display Id peptides on their MHC class II molecules to CD4<sup>+</sup> T cells (reference 11; Fig. 1 A). Supporting evidence comes from the finding that Ig-derived peptides, including Id peptides, can be eluted from class II molecules of B lymphoma cells and EBV transformed B cells (12–15).

Such constitutive B cell presentation of endogenous Id peptides should be of no consequence

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if T cells are uniformly tolerant. However, although T cells are indeed unresponsive to abundant germline-encoded Id peptides shared among large numbers of B cells and serum Ig molecules, they can readily respond to rare Id peptides derived from unique N regions or somatic mutations expressed by very few B cells (16–18). However, the TCR repertoire for a particular rare Id peptide was shown to be severely limited (19), suggesting a rather low frequency of Id-specific T cells.

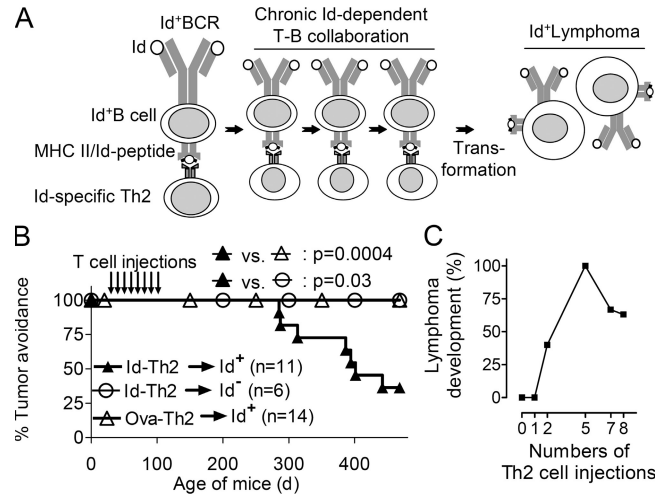
Based on the evidence presented here, it was suggested that infrequent B cells could directly interact with infrequent Id-specific T cells in a process called Id-driven T–B collaboration (20). However, the rarity of matching B and T cells made it difficult to prove that such interactions actually occurred in vivo. This problem was circumvented by making paired Id<sup>+</sup>Ig L chain transgenic mice and Id-specific TCR-transgenic mice, which harbor high frequencies of Id<sup>+</sup> B cells and Id-specific CD4<sup>+</sup> T cells, respectively (21, 22). By transferring B and T cells between such paired strains of mice, it was demonstrated in two independent models by us (10, 23) and others (24) that cognate Id-driven T–B collaboration occurred in vivo. Moreover, such Id-driven T–B collaboration resulted in induction of autoantibodies (23, 24). In the course of these experiments, we incidentally observed that Id<sup>+</sup>  $\lambda 2^{315}$  L chain transgenic mice, which had repetitiously received Id-specific Th2 cells, developed frank lymphomas after a latency period of >5 mo. These results suggested that Id peptides could represent a source of diversified antigen for stimulation of Th2 cells, chronic cognate B cell help, and lymphomagenesis.

## RESULTS

### Id-specific Th2 cells induce tumors in Id<sup>+</sup> mice

Id<sup>+</sup> B cells from Id<sup>+</sup> mice display a BCR with a transgenic Id<sup>+</sup>  $\lambda 2^{315}$  Ig L chain (21). The amino acids 91–101 of this chain correspond to a CDR3 Id peptide that is exceedingly rare in normal mice caused by somatic mutations in positions 94–96 (Tyr<sup>94</sup>Ser<sup>95</sup>Thr<sup>96</sup>→ Phe<sup>94</sup>Arg<sup>95</sup>Asn<sup>96</sup>). The  $\lambda 2^{315}$  L chain is constitutively processed by the Id<sup>+</sup> B cells, and the Id peptide is presented on MHC class II molecules (I-E<sup>d</sup>) (8–10) to Id-specific CD4<sup>+</sup> T cells from TCR transgenic mice (reference 22; Fig. 1 A).

Id<sup>+</sup> mice were injected i.v. with five million cultured Id-specific Th2 cells, derived from TCR transgenic mice, every 10th day for 80 d. About 150 d later, such mice started to develop distended abdomens. Within the next 120 d, more than half of the mice had developed massive splenomegaly (Figs. 1 B and 2 A), greatly enlarged lymph nodes (Fig. 2 B), and often had affection of other organs such as livers (Fig. 2 B). Later experiments demonstrated that only two injections of Id-specific Th2 cells, spaced 10 d apart, sufficed for tumor development (Fig. 1 C). Tumor development was dependent on the Id specificity of the Th2 cells because injections of ovalbumin-specific Th2 cells failed to induce tumors (Fig. 1 B). Old BALB/c mice are predisposed to develop lymphoma; however, none were detected in controls within the time frame of the experiments (Fig. 1 B).

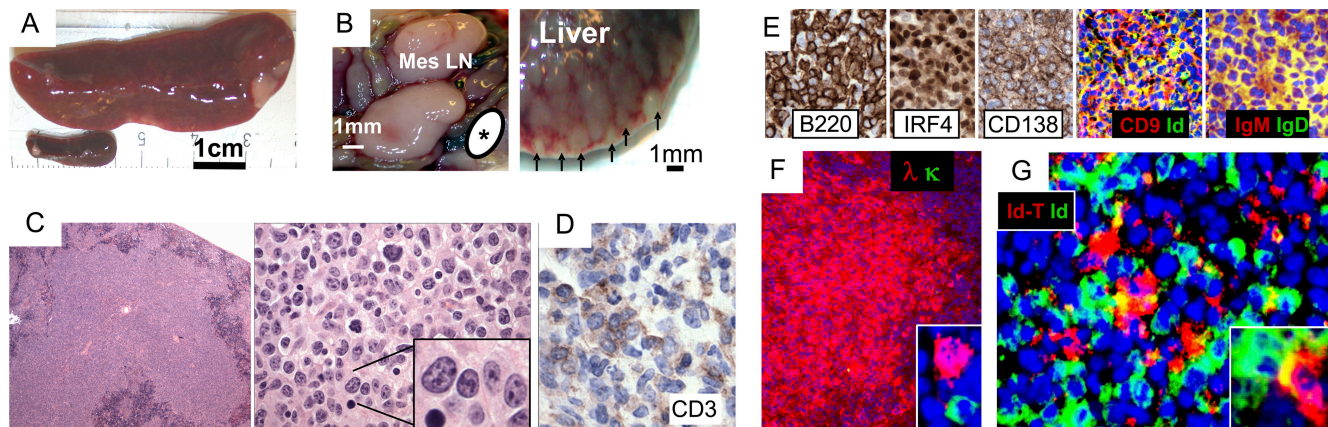


**Figure 1. Id<sup>+</sup> mice repetitiously injected with Id-specific Th2 cells develop lymphoma.** (A) Experimental model. (left) Id<sup>+</sup> B cells from Id<sup>+</sup> mice (17) display a transgenic L chain ( $\lambda 2^{315}$ ) with a rare CDR3 Id peptide (circle). The L chain is processed and the Id peptide is presented on MHC class II molecules (I-E<sup>d</sup>) to Id-specific CD4<sup>+</sup> T cells from TCR transgenic mice. (middle) It was hypothesized that Id-specific Th2 cells might induce chronic proliferation of Id<sup>+</sup> B cells, accumulation of genetic changes, and, finally, B lymphoma development (right). (B) Development of abdominal tumors. Id-specific Th2 cells ( $5 \times 10^6$ ) generated in vitro from TCR transgenic mice were injected eight times, spaced 10 d apart (arrows) into Id<sup>+</sup> mice (Id-Th2→Id<sup>+</sup>) or Id<sup>-</sup> littermates (Id-Th2→Id<sup>-</sup>). As a control, ovalbumin-specific Th2 cells from DO.11.10 TCR transgenic mice were injected into Id<sup>+</sup> mice (Ova-Th2→Id<sup>+</sup>). (C) Number of injections of Id-specific Th2 cells needed for lymphoma development (3–11 mice/group).

### B cell lymphomas were of the large cell type and expressed markers suggesting a relationship to the marginal zone B cell lineage

The normal architecture of the involved tissues was most often effaced by confluent areas of large IgM<sup>+</sup>IgD<sup>+</sup>B220<sup>+</sup> lymphoma cells that mostly had round to ovoid nuclei, distinct nucleoli, and moderate to large amounts of eosinophilic cytoplasm. In cases of lesser disease, only the white pulp was involved (Fig. 3 A). Mitotic figures were observed (Fig. 2 C and Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20061220/DC1>). The lymphomas contained considerable numbers of small, dense lymphocyte-like cells (Fig. 2 C) that stained as CD3<sup>+</sup> T cells (Fig. 2 D). This histopathological examination was consistent with B cell lymphomas of the large type with infiltration of T cells (25).

To remove any confounding effects of normal B cells, lymphomatous spleen cells were transferred into RAG2<sup>-/-</sup> recipients that developed lymphomas within 6–8 wk. The lymphoma cells lacked the follicular B cell marker CD23, the germinal center marker BCL-6 (2), as well as the B1 cell marker CD5 (Fig. 3 A and Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20061220/DC1>). They expressed surface markers suggestive of a relationship to the marginal zone lineage (CD23<sup>-</sup>CD21/35<sup>Hi</sup>CD1d<sup>Hi</sup>CD38<sup>Hi</sup>CD9<sup>+</sup>IgM<sup>Hi</sup>;



**Figure 2. Id<sup>+</sup> diffuse large B cell lymphomas are infiltrated with Id-specific T cells.** (A) Lymphomatous spleen of an Id-Th2→Id<sup>+</sup> mouse (top) compared with spleen from an Ova-Th2→Id<sup>+</sup> mouse (bottom). (B) Photo of enlarged mesenteric LN in an Id-Th2→Id<sup>+</sup> mouse with lymphoma (left). The circled asterisk indicates the normal size of a mesenteric LN in an Ova-Th2→Id<sup>+</sup> mouse. (right) Liver from Id-Th2→Id<sup>+</sup> mouse. Arrows indicate tumor nodules. (C) Low and high power magnification (hematoxylin and eosin) of lymphomatous spleen. (D) Lymphoma of the

liver with infiltrating CD3<sup>+</sup> T cells (brown). (E) Spleen lymphoma were B220<sup>+</sup>, IRF-4/MUM1<sup>+</sup>, and CD138<sup>+</sup> (in about half of the mice) and were IgM<sup>+</sup>IgD<sup>+</sup>. In the rightmost immunofluorescence picture, DAPI stains the nuclei. (F) Low power magnification of splenic lymphoma, Id<sup>+</sup>λ2 L chain (red, 2B6 mAb) versus κ (green). (inset) Large Id<sup>+</sup> lymphoma cell compared with smaller κ<sup>+</sup> B cell. (G) Id-specific T cells (red, TCR clonotype-specific mAb GB113) and lymphoma cells (green, 2B6 mAb) were often in close contact (yellow). See inset for higher magnification of a synapse.

Fig. 3, A and B; and Fig. S2). An equivalent phenotype was found in the lymphoma originator mice (Fig. S2 and not depicted).

The lymphoma phenotype was, however, not completely compatible with the marginal zone B cell subset because: (a) about half the lymphomas expressed low levels of the AA4.1 marker suggestive of a transitional B cell origin (Fig. 3 C). (b) Unlike marginal zone B cells, the lymphoma most often expressed normal amounts of IgD (Fig. 3 B and Fig. S2). (c) Unlike the previously described mouse marginal zone lymphomas (25–27), the present lymphomas were consistently found in extranodal sites, lymph nodes, and the bone marrow (Figs. 2 B, 3 D, S2, and S3, available at <http://www.jem.org/cgi/content/full/jem.20061220/DC1>; and not depicted). (d) No anatomical relationship was found to the marginal zone; however, at the time of analysis extensive tumors may have effaced the splenic architecture. (e) About half of the lymphomas had cells that expressed CD138, especially in the liver and after transfers, suggesting a degree of plasmacytoid differentiation with time (Figs. 2 E and 3 D). Consistent with this, lymphomas were also positive for the interferon regulatory factor 4 (IRF-4) marker (Fig. 2 E), suggestive of some plasmacytoid differentiation. However, the lymphomas lacked a plasma cell phenotype because they secreted little Ig in vivo (unpublished data) and displayed high levels of surface Ig and MHC class II (see below and Fig. 3 G).

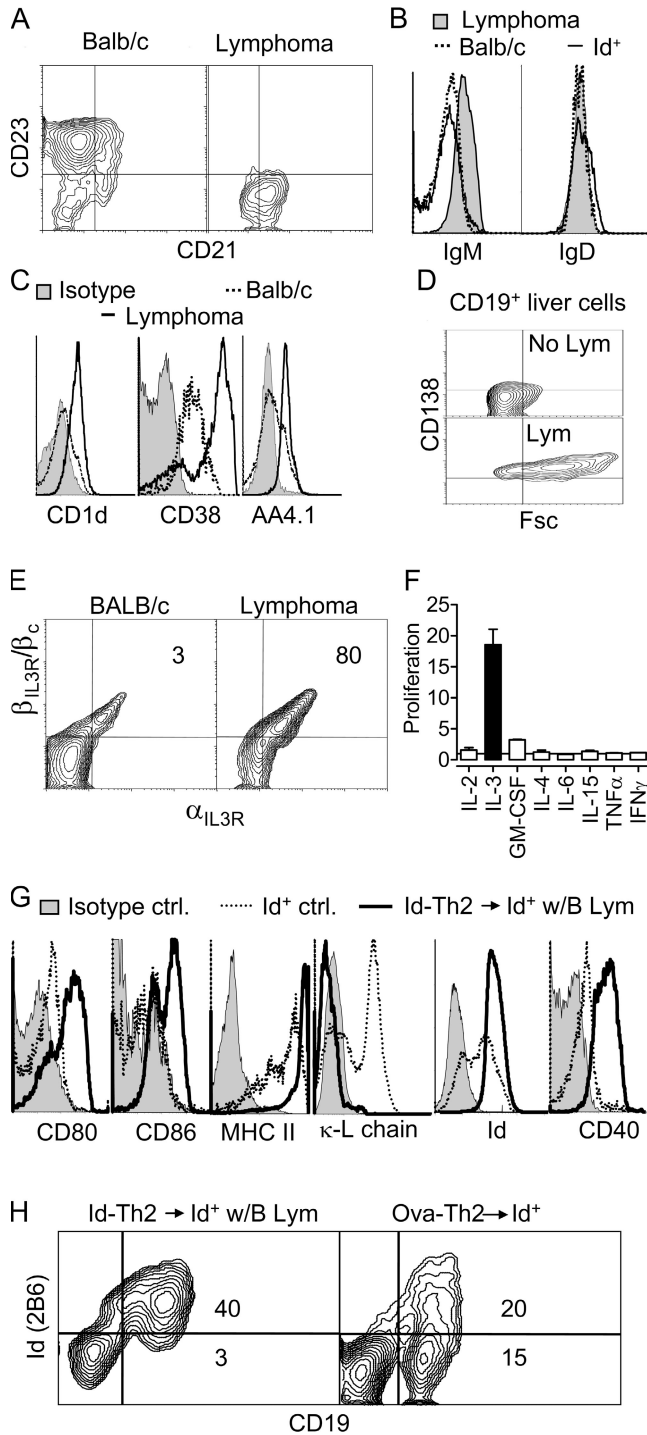
#### The lymphomas were responsive to IL-3 and expressed the IL-3 receptor

A panel of cytokines was added to lymphoma cells ex vivo to augment growth. Surprisingly, IL-3 resulted in 20× increase in lymphoma proliferation. Consistent with this finding, nearly all lymphoma expresses the α (CD123) and β (CD131)

chains of the IL-3 receptor (Fig. 3, E and F). To the best of our knowledge, expression of the IL-3 receptor has not been characterized in the marginal zone B cell compartment. We therefore investigated this issue and found that the IL-3 receptor was expressed on most marginal zone B cell precursors, but less on mature marginal zone B cells (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20061220/DC1>). In conclusion, the functional IL-3 responsiveness, the IL-3 receptor expression of lymphoma cells, and the phenotype described in the preceding section point to a relationship with marginal zone precursor B cells.

#### Lymphoma cells were selected for expression of high levels of Id<sup>+</sup> L chain, MHC class II, and costimulatory molecules

Strikingly, the B lymphoma cells had a phenotype appropriate for potent interaction with T cells. They expressed high levels of MHC class II as well as costimulatory CD80/CD86 molecules, both of which are important for efficient stimulation of CD4<sup>+</sup> T cells (Fig. 3 G). They also strongly expressed the relevant antigen, the λ2 Id<sup>+</sup> L chain (Figs. 2 F and 3 G). In striking contrast, B cells of age-matched Id<sup>+</sup> mice expressed mainly κ, and little λ2, caused by leakiness of allelic exclusion with age (reference 21; Fig. 3 G). The induction of high λ2 Id<sup>+</sup> L chain expression required Id specificity of Th2 cells because injection of Th2 cells with an irrelevant specificity (ovalbumin) failed to increase λ2 expression (Fig. 3 H). These results indicate that injection of Id-specific Th2 cells selectively induced lymphoma cells with a high stimulatory capacity for the injected T cells. Conversely, because lymphoma cells displayed high levels of CD40 (Fig. 3 G), they should be especially receptive to help from activated T cells that are known to express CD40L (1).



**Figure 3.** Id<sup>+</sup> B cell lymphomas resemble late marginal zone precursor B cells and highly express the Id<sup>+</sup> L chain and molecules involved in cognate interaction with T cells. Lymphomatous spleen cells were transferred to RAG2<sup>-/-</sup> mice that developed disease within 6–8 wk. Gated CD19<sup>+</sup> lymphoma cells and BALB/c B cells were compared for CD21 versus CD23 (staining of spleen cells; A), IgM and IgD (spleen cells; B and C), CD1d, CD38, and AA4.1 (spleen cells; C), CD138 versus Fsc (CD19<sup>+</sup> liver cells; D), and CD131 (IL-3R β chain) versus CD123 (IL-3R α chain; E) spleen cells. (F) Proliferation of cultured lymphoma line cells in the presence of cytokines (5 ng/ml), measured as incorporation of <sup>3</sup>HTdR. The y axis shows

**Id-specific T cells collaborate with Id<sup>+</sup> lymphoma in vitro and in vivo**

As suggested by the phenotype analysis above, Id<sup>+</sup> lymphoma cells were excellent stimulators of both naive and memory Id-specific T cells in vitro (Fig. 4 A and not depicted). Vice versa, both naive and memory Id-specific T cells stimulated the proliferation of the CD40<sup>+</sup> Id<sup>+</sup> lymphoma cells. The bidirectional collaboration between B lymphoma cells and T cells was direct and did not require other cells (Fig. 4 B and not depicted). Collaboration was completely inhibited by a combination of antibodies to MHC class II and costimulatory molecules (Fig. 4 C).

Consistent with the bidirectional stimulation observed in vitro, lymphoma tissue was infiltrated by Id-specific T cells that were activated (CD69<sup>+</sup>) and greatly increased in numbers compared with controls (Fig. 5, A–C). Moreover, histological sections revealed that infiltrating Id-specific T cells were frequently in synapse with lymphoma cells, indicating ongoing collaboration in vivo (Fig. 2 G). Note that Id-specific T cells were only expanded in lymphoma-bearing mice, but not in Th2-injected Id<sup>+</sup> littermates that did not develop lymphomas (Fig. 5, A and B). This finding demonstrated prolonged mutual expansion of paired B and T cells exclusively in the mice that had undergone lymphomagenesis.

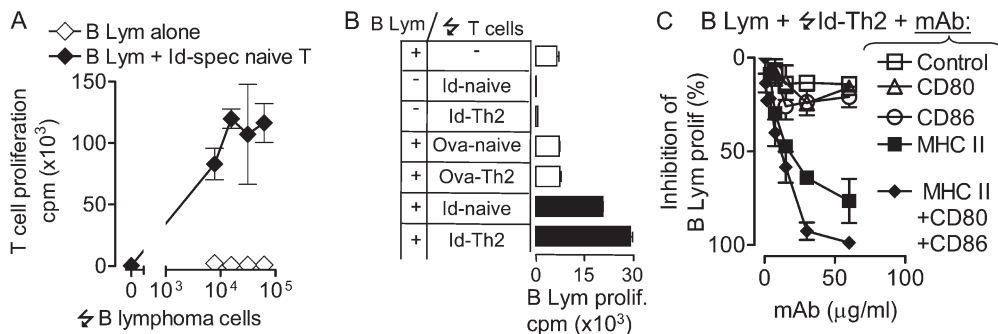
**Id<sup>+</sup> lymphoma eventually expand autonomously, independent of T cells**

It was of interest whether B lymphomas maintained a reliance on Id-specific T cells for their growth. To test this, lymphoma cells were transferred to recipient animals. After only two transfers, hardly any Id-specific CD4<sup>+</sup> T cells were detectable in lymphomas of recipients, suggesting that Id<sup>+</sup> lymphomas had become independent of T cells (Fig. 5 D). In fact, signs of T cell independency were sometimes found already in the original lymphoma mice because lymphoma tissue in kidneys were sometimes devoid of Id-specific T cells (Fig. S3).

**Lymphoma development and reemergence of Id-specific T cells could be monitored in the blood during the latency period**

We have previously described that Id-specific Th2 cells injected into Id<sup>+</sup> mice trigger short-term B cell proliferation, germinal center formation, extra follicular B cell differentiation, and plasma cell differentiation (23). Here, we demonstrate that injected Th2 cells also activate marginal zone B precursors and marginal zone B cells (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20061220/DC1>). However,

time change in cpm compared with proliferation in absence of cytokines, normalized to 1. (G and H) Expression of molecules related to T–B collaboration. (G) Gated B220<sup>+</sup> splenocytes from Id-Th2→Id<sup>+</sup> mouse with lymphoma (thick line) were compared with Id<sup>+</sup> mice (dashed line). (filled histogram) Isotype-matched control. (H) Id<sup>+</sup> L chain expression on CD19<sup>+</sup> splenocytes in Id-Th2→Id<sup>+</sup> mice with B lymphoma compared with Ova-Th2→Id<sup>+</sup> mice. The numbers in the quadrants indicate percentages.



**Figure 4. Id<sup>+</sup> B cell lymphomas bidirectionally collaborate with Id-specific T cells.** (A) Proliferation of naive Id-specific TCR transgenic LN cells ( $10^5$ /well) in response to the indicated numbers of irradiated (♂) B lymphoma cells. (B) Proliferation of an in vitro line of B lymphoma cells

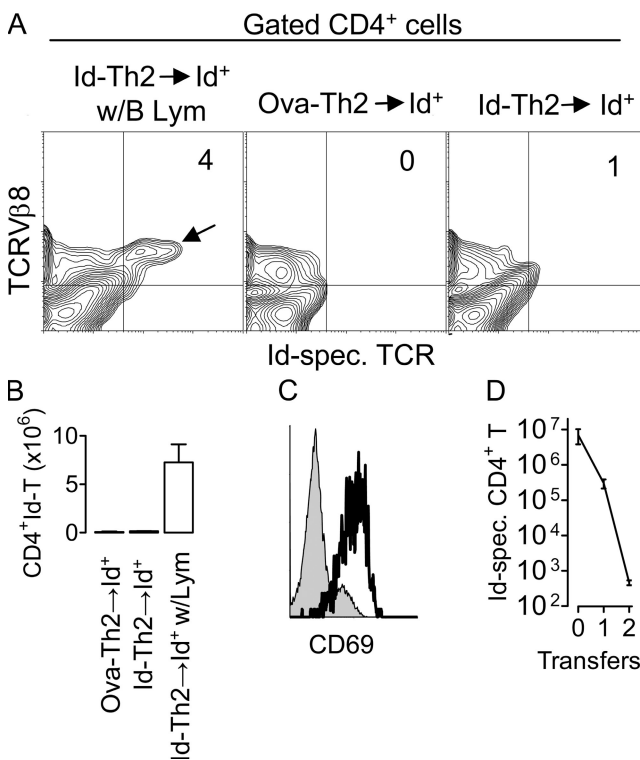
(B Lym) in response to irradiated (♂) T cells. (C) Inhibitory effect of the indicated mAbs on proliferation of B lymphoma line induced by irradiated Id-specific Th2 cells (♂ Id-Th2).

consistent with previous results (10, 23), Id-driven T–B collaboration waned within 7 wk after last T cell transfer, at which time point mice had essentially normal B cell populations and very few Id-specific T cells (< 1%).

It was of interest to detect early signs of lymphoma development during the latency period. However, it is problematic to approach this issue because even if a killed mouse was found to have suspicious changes, we could not be certain if the very same mouse eventually would have developed clinical lymphoma. To circumvent this problem, we resorted to repeated staining of blood lymphocytes during the latency period. Six Id<sup>+</sup> mice injected eight times with Id-specific Th2 cells were followed. Although increased numbers of Id<sup>+</sup>IgM<sup>+</sup>BrdU<sup>+</sup>CD69<sup>+</sup> blasts were found in the blood within the first 35 d after the last T cell transfer, such supposedly polyclonally activated B cells had disappeared by day 50 (Fig. S6, available at <http://www.jem.org/cgi/content/full/jem.20061220/DC1>, and not depicted). However, after another 50 d, two out of the three mice that several months later developed lymphomas had a striking increase in Id<sup>+</sup>IgM<sup>+</sup>CD69<sup>-</sup> large blasts (Fig. S6). The same two mice also had an increase in Id-specific CD4<sup>+</sup> T cells. Thus, occurrence of lymphoma-like B cells and Id-specific T cells in blood correlated significantly ( $P < 0.001$ ) and heralded later lymphoma development (Fig. S6).

#### Id<sup>+</sup> lymphoma were biclonal/monoclonal in terms of variable heavy chain (V<sub>H</sub>) gene usage and expressed few mutations

Id<sup>+</sup> mice have a polyclonal heavy (H) chain repertoire (21, 23, 28). Thus, even though the lymphomas all expressed the transgenic Id<sup>+</sup> L chain, each lymphoma should express distinct H chains that could serve as a clonal marker. We therefore analyzed the VDJ sequence repertoires in two lymphoma mice and their recipients. In one mouse, the sequences obtained from the spleen, liver, and kidney were nearly monoclonal, and this signature was conserved over the course of four consecutive transfers (Fig. 6 and Fig. S7, available at <http://www.jem.org/cgi/content/full/jem.20061220/DC1>). The second



**Figure 5. Id-specific CD4<sup>+</sup> T cells in lymphomatous tissue.** (A) CD4<sup>+</sup> cells were gated from spleens of lymphoma mice and control mice (indicated above plots) and analyzed for expression of the Id-specific TCR (GB113 mAb) versus TCRVβ8. The anti-TCRVβ8 (F23.1) mAb stains the transgenic β chain of both Id- and ovalbumin-specific T cells, as well as Vβ8<sup>+</sup> T cells in the Id<sup>+</sup> recipients. Id-specific T cells are indicated by the arrow. (B) Numbers of Id-specific CD4<sup>+</sup> T cells in spleens of indicated mice ( $n = 4$ /group). (C) CD69 expression on splenic CD4<sup>+</sup> Id-specific T cells (TCRVβ8<sup>+</sup>GB113<sup>+</sup>) from a Th2→Id<sup>+</sup> mouse (thick line) compared with CD4<sup>+</sup>TCRVβ8<sup>+</sup> T cells in an Ova-Th2→Id<sup>+</sup> mouse (filled). (D) Number of CD4<sup>+</sup> Id-specific T cells in spleens of original lymphoma mice and in consecutive recipients after transfers ( $n = 3-5$ ).

lymphoma mouse had a monoclonal VDJ admixed (1:4) with a polyclonal V<sub>H</sub> repertoire (see sequence information in Fig. 6 for the dominant clone). The polyclonality could clearly be seen in a Southern analysis detecting VDJ rearrangements (Fig. S11). Upon transfer, lymphoma sequences had a biclonal pattern, the original clone maintaining dominance (Figs. S8–S10). Consistent with biclonality, two distinct clonal rearrangements were detected by Southern analysis. One of the bands appeared to correspond to one of the two lymphoma clones defined by sequencing (Figs. S9–S11). Isotype-switch variants, as well as a low level of somatic mutations, were found in both of the original lymphoma mice (Fig. 6). Interestingly, with transfers and concomitant loss of Id-specific T cells, unmutated sequences with  $\mu$  constant regions gained dominance (Fig. 6).

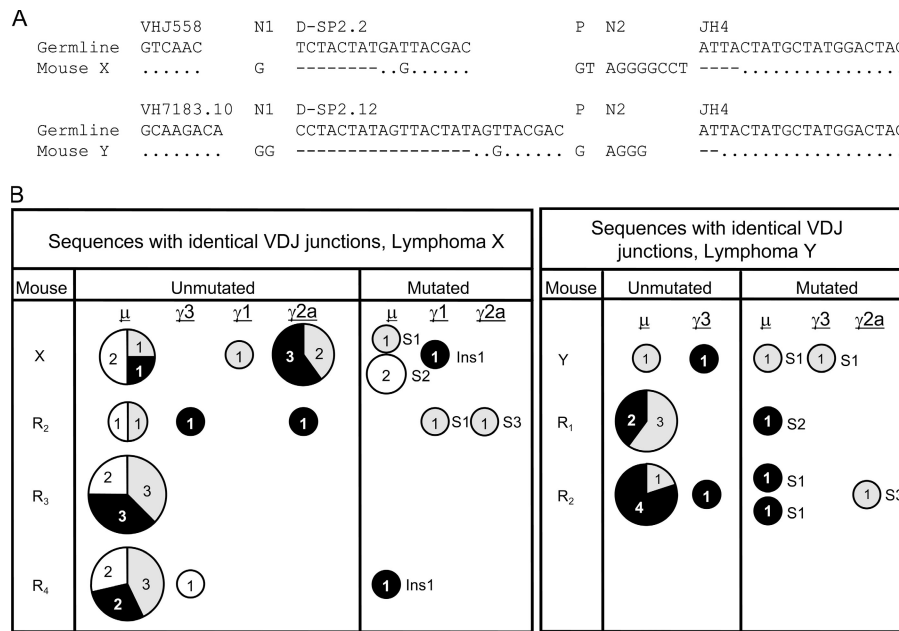
**The Id<sup>+</sup> lymphoma had major cytogenetic aberrations in genomic DNA detected by microarray comparative genomic hybridization (CGH)**

We used high-resolution oligonucleotide-based microarray CGH to investigate whether lymphomas contained genomic aberrations. Although the technique does not give exact information on particular translocations that may exist in the mouse

lymphomas, array CGH allows high-resolution analyses of DNA copy number aberrations genome-wide (29). Interestingly, all of the six independent lymphomas gained chromosomal material and half of them showed gain of whole chromosomes (Fig. S12 and S13, available at <http://www.jem.org/cgi/content/full/jem.20061220/DC1>). Deletions were less extensive and more focal, but some recurrent alterations were found (Fig. S13). Aberrations in copy number included genes for regulation of transcription, signaling pathways, cell cycle and division, as well as apoptosis. Several aberrations were shared by three to five of the six lymphomas (Fig. S13), and some minor chromosomal segments were overrepresented in high copy number (>10; not depicted).

**The Id<sup>+</sup> lymphoma can be rejected by Id-specific T cells in vivo**

Although Id<sup>+</sup> lymphomas were induced by injection of Id-specific Th2 cells, they could nevertheless be susceptible to T cell immunosurveillance mediated by the inflammatory CD4<sup>+</sup> Th1 cell subset previously suggested by us to mediate rejection of Id<sup>+</sup> B lymphomas and myelomas (30, 31). To test this, Id<sup>+</sup> lymphomas (1.25 × 10<sup>6</sup>) were injected s.c. into Id-specific TCR transgenic SCID mice (that only have Id-specific



**Figure 6. Clonal evolution of B lymphomas as indicated by analysis of VDJ<sub>H</sub> sequences.** Two lymphoma-bearing mice, X and Y, were analyzed, as well as recipients (R<sub>1</sub>, R<sub>2</sub>, R<sub>3</sub>, and R<sub>4</sub>) of serially transferred splenic lymphoma cells (the X and Y lymphomas were also analyzed by CGH [Figs. S12 and S13] and by Southern blots using a J<sub>H</sub> probe [Fig. S11]). (A) Repeatedly isolated VDJ<sub>H</sub> lymphoma sequences. Mouse X expressed an ≈90% (14/15) recurrent VDJ sequence, with identical junctions (dots, identical; dashes, nonexisting; N, nontemplate; P, palindromic nucleotides). Mouse Y expressed an ≈20% (4/23) repetitive sequence admixed with a polyclonal V<sub>H</sub> repertoire. (B) Lymphoma sequences, mutations, and isotype switches are summarized for mouse X and its recipients (left) and for Y and

its recipients (right). Sequences with identical VDJ junctions were divided into "mutated" and "unmutated" as indicated. Each of these categories was further classified according to H chain isotype. Each identical sequence with the same H chain is represented as a pie chart. The shade of cake segments indicates organ derivation: spleen (black), liver (gray), and kidney (white). The digits indicate the number of identical sequences obtained from each tissue. For mutated sequences, substitutions (S) and their number and insertions (Ins) and their number are given. For recipients of mouse Y, a minor additional clone emerged in R<sub>2</sub>. See Figs. S7–S10 for complete compilations of sequences. Online supplemental figures are available at <http://www.jem.org/cgi/content/full/jem.20061220/DC1>.

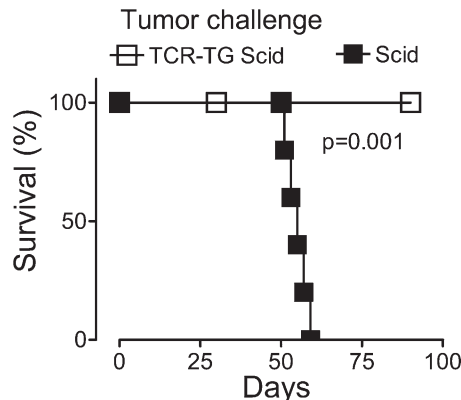
CD4<sup>+</sup> T cells and no CD8<sup>+</sup> or B cells). SCID mice served as controls. Strikingly, no lymphomas were observed in the challenged TCR transgenic mice, whereas all SCID mice developed tumors (Fig. 7), clearly demonstrating the cytotoxicity of Id-specific CD4<sup>+</sup> T cells against the Id<sup>+</sup> lymphoma cells.

## DISCUSSION

Previous papers have suggested that chronic antigenic stimulation of B cells by a variety of mechanisms can induce B lymphomas (2–6, 32–34). In the case of non-Hodgkin's lymphoma associated with hepatitis C, the envelope protein E2 cross-links CD81 thereby chronically activating naive B cells in a polyclonal manner (32). In mucosa-associated lymphoid tissue lymphomas associated with *Helicobacter pylori* gastritis, rheumatoid factor B lymphoma precursors have been suggested to receive chronic stimulation via immune complexes and toll-like receptors (33, 34). In a mouse model, we describe a novel mechanism for lymphomagenesis, which entails two salient features, (a) constitutive MHC class II-restricted presentation of Id peptides by B cells and (b) chronic help delivered by Id-specific Th2 cells. The large B cell lymphomas displayed chromosomal abnormalities, high amounts of the Id<sup>+</sup>Ig L chain, MHC class II molecules, and costimulatory CD80/86 and CD40 molecules, and collaborated bidirectionally with Id-specific T cells. Moreover, the lymphoma cells and T cells were found in close contact in lymphoma tissue. These results suggest that chronic Id-driven T–B collaboration could represent a novel mechanism for lymphomagenesis.

The lymphomagenesis apparently started out as a polyclonal proliferation of Id<sup>+</sup> B cell that later contracted into monoclonal/biclonal lymphomas that eventually gained T cell independence. During this process, lymphoma microheterogeneity, manifested as IgG isotype-switched variants and low levels of somatic mutation, was lost. It might be that the microheterogeneity seen in initial stages of lymphoma development could depend on Id-specific T cells. Moreover, in the absence of T cells, B lymphoma founder cells (IgM<sup>+</sup> and unmutated V<sub>H</sub>) might have a growth advantage. Based on these findings, we would like to suggest a model where Id-specific Th2 cells induce extensive proliferation of Id<sup>+</sup> B cells, rendering them vulnerable to the accumulation of oncogenic mutations.

Although most surface markers of the lymphoma indicated a marginal zone origin (CD9<sup>+</sup>CD1d<sup>Hi</sup>CD38<sup>Hi</sup> IgM<sup>Hi</sup>CD5<sup>-</sup>CD23<sup>-</sup>CD21<sup>Hi</sup>BCL6<sup>-</sup>), some markers did not fit with normal marginal zone B cells (35, 36) or marginal zone lymphomas (25–27). More specifically, most lymphomas were IgD<sup>Hi</sup> and about half expressed low levels of the transitional B cell marker AA4.1. This phenotype is most compatible with a putative intermediate between marginal zone precursor B cells and marginal zone B cells (see summary of surface markers in Fig. S14, available at <http://www.jem.org/cgi/content/full/jem.20061220/DC1>). In addition, we demonstrate that the lymphoma cells express the IL-3 receptor (as do marginal zone B cell precursors). Consistent with this, lymphoma cells proliferated in response to IL-3. Some plasmacytoid differentiation of the lymphomas was seen, especially with



**Figure 7. Th2-induced B lymphomas are rejected by TCR transgenic SCID mice.** Id<sup>+</sup> lymphoma cells ( $1.25 \times 10^6$ ) were injected into Id-specific TCR transgenic SCID mice or SCID mice ( $n = 5/\text{group}$ ) and survival recorded.

time, indicating a degree of terminal differentiation. In this regard, it has been demonstrated that marginal zone B cells are intrinsically capable of very rapidly maturing into plasma blasts (35, 36).

Why did the lymphomas have a late marginal zone precursor phenotype? First, it has been described that expression of the particular Id<sup>+</sup> $\lambda 2^{315}$  L chain in transgenic mice selects against progression into the follicular B cell compartment, thus, the marginal zone B cell compartment is overrepresented in these mice (37, 38). As a possible explanation, the  $\lambda 2^{315}$  L chain might be poorly compatible with BCR specificities for endogenous antigen required for recruitment into the follicular compartment, as suggested in the BCR strength model (36). It has also been reported that marginal zone B cell precursors and B cells express high amounts of costimulatory molecules and are able to stimulate even naive T cells (36, 39). Collectively, (a) overrepresentation of Id<sup>+</sup> B cells in the marginal zone lineage and (b) high stimulatory capacity for Id-specific T cells and responsiveness to IL-3 (produced by Th2 cells) could both contribute to the particular B lymphoma phenotype observed. It should be emphasized that the site of malignant transformation is not known. Although all mice had extensive peripheral involvement, lymphoma cells were also observed in the bone marrow of originator mice. Thus, we have not ruled out the possibility that the lymphomas in fact arose in the bone marrow and thereafter populated the spleen and LN with intact AA4.1 and IL-3 receptor expression as signs of recent bone marrow emigration. Because of this ambiguity, it is difficult to classify the lymphoma according to stages described for marginal zone lymphomas in mice (25, 26).

There is previous circumstantial evidence that supports a role for T cells in B cell lymphomagenesis. First, T cell infiltrates are a common feature of both Hodgkin's and non-Hodgkin's B cell lymphomas (2, 4–6). Second, lymphoma cells attract T cells (including Th2 cells) by secreting chemokines (40, 41). Third, human follicular B lymphoma cells often express CD40, ligation of which may facilitate their growth (42).

Thus, in EBV-associated lymphomagenesis in SCID-Hu models, T cells as well as CD40 ligation were required (43, 44). Fourth, follicular lymphoma cells were helped in vitro by alloreactive T cells in a cell contact-dependent manner (45). Fifth, in Bcl-2 transgenic mice, germinal center hyperplasia and eventual B lymphoma development was dependent on CD4<sup>+</sup> T cells of unknown specificity (46). The present findings extend these previous observations by directly demonstrating that Id-specific Th2 cells can induce lymphomas by chronic cognate interaction with Id<sup>+</sup> B cells. The results suggest that cognate Th2-B cell collaboration could be lymphomagenic also when B cells chronically present other endogenous antigens like viral peptides to Th2 cells.

It is well known that autoimmune diseases like rheumatoid arthritis and systemic lupus erythematosus predispose for B lymphoma development (2, 3). It is therefore interesting to note that transfer of Id-specific Th2 cells into Id<sup>+</sup> mice first induces a transient production of autoantibodies (23, 24) and, only later, B lymphomas. Thus, Id-driven T-B collaboration might possibly represent a mechanism that explains the epidemiological link between autoimmunity and B lymphoma development.

It is surprising that Id-specific Th2 cells in the present experiments caused lymphomas because T cells are currently believed to eliminate rather than induce cancers. In fact, cancer cells have been suggested to be phenotypically sculpted by T cells in a process called immunoediting (47). The latter paradigm certainly applies to cytotoxic CD8<sup>+</sup> and inflammatory CD4<sup>+</sup> Th1 cells (47). As concerns Th2 cells, they have previously been reported to protect mice against ovalbumin-transfected lymphoma cells (48). However, those tumor-challenge experiments (48) were rather different from the present, where the continuous presence of Id-specific Th2 cells over many months induced Id<sup>+</sup> B lymphomas, selected for ability to bidirectionally collaborate with the injected Th2 cells. We would therefore like to suggest that tumor-associated Th2 cells may initially nurture B lymphoma cells in a symbiotic relationship that we call T cell-dependent lymphomagenesis. Whether this process involves a selection of preexisting Th2 and B cells, or long-term mutual shaping events that facilitate potent interaction, remains to be determined.

It should be stressed that even with these Th2-induced lymphomas, immunosurveillance still operates because the lymphomas were promptly rejected when injected into Id-specific TCR-transgenic SCID mice, most likely caused by generation of cytotoxic Th1 cells (30). Thus, Id-specific CD4<sup>+</sup> T cells with an identical TCR appear capable of either inducing or eradicating lymphoma cells, depending on T cell phenotype and the experimental situation.

Because few normal individuals develop lymphomas, severe constraints (a-e) must operate on Id-driven T-B collaboration and lymphoma development. (a) Id peptides must have the ability to bind MHC class II molecules of the individual. (b) T cells are tolerant to germline-encoded Id peptides and can only respond to rare Id peptides that express somatic mutations or N region diversity (16-18). (c) Id<sup>+</sup> B and Id-specific

T cells are both infrequent, reducing the chance that matched cells encounter each other during lymphocyte recirculation (17-19, 23). (d) Id-dependent T-B collaboration could be controlled by peripheral tolerance mechanisms (49-51). (e) The accumulation of secondary genetic changes required for B lymphoma development might be quite rare.

Some previous observations could, if reinterpreted, support a role for Id-driven T-B collaboration in B cell tumorigenesis in patients (52-55). Thus, Id-specific CD4<sup>+</sup> T cells have previously been described in a B lymphoma patient (52), in B cell chronic lymphocytic leukemia patients (53), as well as in patients with monoclonal gammopathy of unknown significance (54) or multiple myeloma (55). In these cases, Id-specific T cells have been considered to fight the tumor. The present results suggest an alternative explanation, i.e., that Id-specific T cells could rather have promoted tumor development in these patients. Clearly, further studies on the dichotomous lymphomagenic versus lymphomatotoxic potential of Id-specific CD4<sup>+</sup> subsets (Th1 and Th2) are needed. This issue is particularly relevant to Id vaccination of patients, which conceivably could result in expansion of Id-specific T cells with tumor-enhancing instead of tumor-eradicating properties.

## MATERIALS AND METHODS

**Mice.** Mice transgenic for the  $\lambda 2^{315}$  Ig L chain (Id<sup>+</sup> mice; reference 21) were on a BALB/c background. Offspring from BALB/c  $\times$  hemizygous Id<sup>+</sup> mice were used, with the transgene-negative littermates serving as negative controls. Id-specific TCR transgenic mice (17) were on a C.B-17 *scid/scid* background. BALB/c, BALB/c RAG2<sup>-/-</sup>, and C57BL/6 RAG2<sup>-/-</sup> mice were obtained from Taconic M&B. Ovalbumin-specific DO.11.10 TCR transgenic mice were purchased from JaxMice. All experiments were approved by the Norwegian Animal Research Committee.

**Induction and transfer of lymphomas.** Short-term cultured polarized Th2 cell lines were generated from Id-specific TCR transgenic SCID or ovalbumin-specific TCR transgenic mice as described previously (23), and  $5 \times 10^6$  cells were injected i.v. every 10th day the indicated number of times into Id<sup>+</sup> or Id<sup>-</sup> littermates, starting at 6 wk of age. Mice were monitored for >250 d. The following signs of pathology were monitored: abdominal distension, lethargy, anemia, and weight loss. Splenic Id<sup>+</sup> lymphomas were further transferred into recipients (RAG2<sup>-/-</sup> BALB/c [H-2<sup>d</sup>] or RAG2<sup>-/-</sup> C57BL/6 [H-2<sup>b</sup>]). In some experiments, BrdU was provided as an i.p. injection (1 mg) 2 d before analysis.

**Antibodies and flow cytometry.** The following mAbs were affinity purified and biotinylated or FITC conjugated in our laboratory: transgenic TCR clonotype-specific GB113 (17), anti-MHC class II (TIB-120 and M5/114.15.2; American Type Culture Collection), 2B6 (anti- $\text{C}\lambda 2/3$ ), and anti- $\kappa$  (187.1). The following antibodies (FITC, phycoerythrin, biotinylated, or allophycocyanin conjugated) were purchased from BD Biosciences: anti-CD1d (1B1), anti-CD4 (RM4-5), anti-CD5 (53-7.3), anti-CD8 (53-6.7), anti-CD9 (KMC8), anti-CD11b (M1/70), anti-CD19 (1D3), anti-CD21/CD35 (7G6), anti-CD23 (B3B4), anti-CD38 (90), anti-CD40 (3/23), anti-CD45R/B220 (RA3-6B2), anti-CD69 (H1.2F3), anti-CD80 (16-10A1), anti-CD86 (GL1) anti-CD138 (281-2), anti-CD123 (5B11), anti-CD131 (JRO50), Ly-6G [Gr-1] (RB6-875), anti-TCRV $\beta$ 8 (F23.1), anti-IgM<sup>s</sup> (DS-1), anti-IgD (11-26c.2a), anti-immature B cells (AA4.1, anti-C1qR<sub>p</sub>/CD93), and anti-BrdU (Becton Dickinson). Streptavidin-Cy-chrome and streptavidin-peridinin chlorophyll-a protein (SAV-PerCP) were purchased from BD Biosciences. Quadruple stainings were performed with FITC-, PE-, PerCP-, and APC-conjugated mAbs and acquired on a FACS Calibur



(Becton Dickinson), and files were analyzed with CellQuest Pro (BD Biosciences) and WinMDI 2.7.

**B Lymphoma cell and T cell proliferation assays.** Both day 30-cultured lymphoma cell lines and lymphoma cells taken directly ex vivo were tested. Proliferation of B lymphoma cells ( $5 \times 10^4$ /well) in response to Id-specific T cells ( $5 \times 10^4$ /well) was measured by culturing B cells with irradiated T cells (1,000 rad), with a  $^3\text{H}$ -TdR overnight pulse from day 2 to 3, as described previously (23). Conversely, lymphoma cells were irradiated and T cell proliferation was measured. Lymphoma cell proliferation was assayed in the presence of titrated concentrations of anti-MHC class II (TIB-120), anti-CD80, and anti-CD86 mAbs. Recombinant cytokines were obtained from BD Biosciences.

**Immunohistochemistry.** Organs were imbedded in OCT (Tissue-Tek). 5- $\mu\text{m}$  frozen sections were mounted on L-polylysine-coated glass slides, air dried overnight, blocked with 30% heat-aggregated rat serum, stained with biotinylated or FITC-conjugated mAbs (see Antibodies and flow cytometry section) and streptavidin-Cy2 and 3 (GE Healthcare), and counterstained with DAPI (Invitrogen). 5- $\mu\text{m}$ -thick formalin-fixed, paraffin-embedded tissue sections were rehydrated and stained. In brief, slides were soaked in xylene, passed through graded alcohols, and put in distilled water. Slides were then pretreated with 10 mM citrate, pH 6.0 (CD3, B220, and BCL-6), or 1.0 mM EDTA, pH 8.0 (CD138 and MUM1/IRF-4; Zymed Laboratories), in a steam pressure cooker (Decloaking Chamber; BioCare Medical) as per the manufacturer's instructions and followed by washing in distilled water. All further steps were performed at room temperature in a hydrated chamber. Slides were next pretreated with peroxidase block (DakoCytomation) for 5 min to quench endogenous peroxidase activity. Primary rabbit anti-CD3 antibody (DakoCytomation), rat-anti B220 (clone RA3-6B2, BD Biosciences), goat anti-MUM1/IRF-4 (M-17; Santa Cruz Biotechnology, Inc.), rabbit anti-BCL-6 (N-3; Santa Cruz Biotechnology, Inc.), or rat anti-CD138 (clone 281-2; BD Biosciences) was applied in DakoCytomation diluent for 1 h. For B220 and CD138, rabbit anti-rat Ig antibody (DakoCytomation) was applied in DakoCytomation diluent for 1 h. Slides were washed in 50 mM Tris-Cl, pH 7.4, and detected with anti-rabbit Envision+ kit (CD3, B220, BCL-6, and CD138; DakoCytomation) or LSAB+ staining kit (MUM1/IRF-4; DakoCytomation) as per the manufacturer's instructions. After further washing, immunoperoxidase staining was developed using a DAB chromogen (DakoCytomation) and counterstained with hematoxylin.

**Sequencing of lymphoma  $V_H$ .** Variable heavy chains from mouse lymphoma tissue (spleen, liver, and kidney) were cloned by the use of degenerate FR1 region primers, designed to amplify most  $V_H$  mouse sequences, together with C-region primers (56). The recombinant DNA was sequenced and analyzed for VDJ gene segment usage, somatic mutations, and isotype switch. In brief, total RNA was isolated from either mouse lymphoma tissue (spleen, liver, and kidney) frozen in OCT compound or from fresh lymphoma tissue submerged in RNAlater (Ambion) by use of TRIzol reagent (Invitrogen). Reverse transcriptase was performed using a NotI-d(T)<sub>18</sub> primer (First-strand cDNA synthesis kit; GE Healthcare). cDNA was amplified by PCR using Pfu Turbo DNA polymerase (Stratagene) and a mixture of 5' heavy chain FR1-region degeneracy primers together with a mixture of 3' heavy chain constant (C)-region primers without cloning sites (reference 56; Sigma-Aldrich). The PCR products were run on a 1.5% agarose gel, and gel-purified products of predicted size ( $\approx 400$  bp; QIAquick gel extraction kit; QIAGEN) were ligated into pGEM-T Easy vector (Promega) and used to transform JM109 competent cells (Promega). Plasmid DNA was prepared from overnight cultures (Wizard plus SV minipreps DNA purification system; Promega), and colonies found to contain an insert were sequenced using T7 and/or SP6 primer (GATC Biotech AG). At least three colonies were sequenced for each case. Sequence alignments were made using the current IMGT/V-QUEST (<http://imgt.cines.fr>) and NCBI/BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) tools to find the closest matching germline VDJ gene segments. The IMGT/JunctionAnalysis tool was used to identify the

junctions between the rearranged VDJ gene segments, including N (nontemplate) and P (palindromic) nucleotides.

**Statistical analysis.** In survival analyses, p-values were calculated with the Mantel-Haenszel Log rank test. The two-tailed Mann-Whitney test was used to calculate p-values when comparing numerical data.

**$V_H$  sequences.** All nucleotide sequences are available from GenBank/EMBL/DDBJ under accession nos. DQ416684–DQ416716.

**Online supplemental material.** Figs. S1–S14 provide an additional description of the lymphoma phenotype, cytogenetic abnormalities, lymphoma VDJ sequences, IL-3 receptor expression on marginal zone B cells, and marginal zone Id<sup>+</sup> B cell responses to Id-specific T cells. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20061220/DC1>.

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