A Conserved Enhancer Element Differentially Regulates Developmental Expression of CD5 in B and T Cells

Robert Berland, Steven Fiering and Henry H. Wortis

*J Immunol* 2010; 185:7537-7543; Prepublished online 12 November 2010;
doi: 10.4049/jimmunol.1002173
http://www.jimmunol.org/content/185/12/7537

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/11/12/jimmunol.1002173.DC1

References
This article cites 69 articles, 39 of which you can access for free at:
http://www.jimmunol.org/content/185/12/7537.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
A Conserved Enhancer Element Differentially Regulates Developmental Expression of CD5 in B and T Cells

Robert Berland,*1 Steven Fiering,†‡ and Henry H. Wortis*

We previously identified an enhancer element upstream of the mouse cd5 gene that was required in reporter assays for the induction of cd5 promoter activity by BCR cross-linking. This element is highly conserved in placental mammals. To determine its physiological role, we have now generated mice with a targeted deletion of the enhancer. The result is the loss of CD5 expression in peritoneal and splenic B-1a cells of adult mice and an inability to induce CD5 by cross-linking of the BCR on splenic B-2 cells. Surprisingly, CD5 expression on B-1a cells of neonatal mice was only minimally compromised. Cd5 enhancer deletion also had only a modest effect on CD5 expression in the T lineage. Thus, this enhancer provides age- and tissue-specific regulation of CD5 expression and is an example of the utilization of different modes of regulation of expression in T and B cells. The Journal of Immunology, 2010, 185: 7537–7543.

CD5 is a 67-kDa member of the macrophage scavenger receptor superfamily of membrane glycoproteins (1). It is expressed on cells of the T lineage throughout their development (2). In the B lineage of laboratory mice, CD5 is constitutively expressed on the B-1a subset (as reviewed in Ref. 3) as well as on cells of a recently identified CD1dhigh regulatory subset (4). In humans, CD5 is expressed on transitional and prenaive B cells (5, 6) and possibly on a population of B-1a–like cells (7). It is also expressed on malignant chronic lymphocytic leukemia (CLL) B cells (8).

In both T and B lineages, CD5 associates with the Ag receptor complex and becomes phosphorylated in response to Ag receptor cross-linking (9–11). Deletional mutation of cd5 revealed that it negatively regulates Ag receptor signaling in both B and T lineages (12, 13). In developing thymocytes, CD5’s inhibitory activity helps shape the TCR repertoire by raising the threshold of permissible self-avidity during positive selection (2, 12, 14). CD5 inhibits TCR signaling at the immunological synapse (15) and has been shown to play a role in both T and B cell anergy (16, 17).

In addition to functioning as a negative regulator, CD5 can deliver costimulatory or survival signals. In T cells, CD5 ligation enhances proliferative responses to suboptimal CD3 cross-linking (18) and causes increased IL-2 secretion in response to PHA and anti-CD28 treatment (19). In a mouse model of experimental autoimmune encephalitis, CD5 was required for the survival of activated autoreactive T cells (20). In human B cells, CD5 expression resulted in enhanced IL-10 production, leading to increased survival in vitro by an autocrine mechanism (21), and in lymphoma cells from a subset of B cell CLL patients, CD5 ligation enhanced in vitro survival, possibly also by a mechanism involving autocrine IL-10 production (22).

The regulation of cd5 expression is complex. Within the mouse B lineage it is expressed at low levels on anergic B cells (17, 23) and at somewhat higher levels on B-1a cells. Its expression can be induced in vitro on conventional B cells by BCR cross-linking (24, 25). In the T lineage it is expressed at all developmental stages. Its level of expression increases as thymocytes progress from the double-negative (DN) to the single-positive (SP) stage and remains high on mature peripheral T cells (2). The final level of CD5 on a particular T cell is determined during positive selection by the avidity of its TCR for positively selecting self-ligand (2). T cells with high avidity for positively selecting ligand will exhibit higher levels of CD5. The maintenance of surface CD5 in the periphery continues to depend, in part, on ligand-dependent TCR signaling (26).

In laboratory mice CD5 is a defining surface molecule on B-1a cells, a phenotypically and functionally distinct subset of B cells that plays an important role in the early, thymus-independent response to some pathogens (27). B-1a cells are also largely responsible for the constitutive production of natural Ab (28, 29), which plays a crucial role in the early neutralization of pathogens (30–32), in the initiation of an effective thymus-dependent response (33), and in clearance of potentially harmful self products such as apoptotic bodies and low-density lipoprotein (34). B-1a cells are highly enriched in the mouse peritoneum. They are also an important source of gut-associated IgA-producing cells that are important in maintaining symbiosis with intestinal bacteria (35).

A critical step in B-1a development is positive selection by self-Ag (36). This requirement presumably selects into the B-1a population cells with BCR specificities appropriate to the functions of B-1a cells. Several years ago, we undertook a study to identify the molecular events downstream of BCR signaling that are necessary for the induction or maintenance of the B-1a phenotype. Because CD5 expression is a hallmark of B-1a cells, and because CD5 can be induced on splenic B-2 cells by BCR cross-linking (24, 25), we reasoned that determining the events downstream of the BCR that lead to CD5 expression would provide insight into the signals.
responsible for B-1a development or maintenance. Using an in vitro reporter assay, we identified a BCR-responsive NFAT-dependent enhancer upstream of the *cd5* gene (37). This enhancer is highly conserved in placental mammals (Fig. 1). We now report the consequences of targeted deletion of this enhancer on CD5 expression in the B and T lineages.

**Materials and Methods**

*cd5* enhancer knockout mice

A targeting construct was made using the targeting vector pGKneo-loxp2-ΔTA. A 788-bp fragment consisting of nt 2,745 to 1,958 of the *cd5* gene (chromosome 19: 10,816,209–10,815,422) was cloned 5′ of the neomycin resistance cassette and a fragment consisting of nt 1,862 to +10,851 of the *cd5* gene (chromosome 19: 10,815,326–10,802,613) was cloned 3′ of the neomycin cassette. The 5′ fragment was generated by PCR using *Pyrococcus furiosus* polymerase and cloned *cd5* flanking sequences from BALB/c as template. The primers, which introduced a SacII and a NotI site into the 5′ and 3′ ends, respectively, of the product, were ACATCGTTCCGGGCTGCGCTGGAACCTCCGAC and ACATCGTGGCGGCGCTAGCCAAGACCTCA and ACATCGTGGCCGGCTAGCCAAGACCTCA. The fidelity of the PCR reaction was confirmed by sequencing. The 3′ fragment was generated by first cloning a NheI–HindIII fragment from BALB/c genomic DNA containing the region 1,965–2,086 of the enhancer into the pGL3-basic luciferase vector. The *cd5* sequences to +10,851 were then introduced by replacing the SspI–HindIII fragment containing the polylinker region from the targeting vector. The targeted disruption was confirmed by sequencing a PCR product from the targeted allele, whereas KOCREF and cd5enhR amplify a 113-bp product from the wild-type locus.

**Flow cytometry**

Single-cell suspensions of splenocytes, thymocytes, or peritoneal cells were made in staining buffer, which consisted of Dulbecco’s PBS containing Ca²⁺ and Mg²⁺ (Invitrogen, Carlsbad, CA) plus 1% rabbit serum and 0.1% sodium azide, pelleted at 200 × g for 5 min, and resuspended in 50 μl staining buffer plus fluorochrome-conjugated Abs at the appropriate dilution per 1 × 10⁶ cells. After 20 min on ice, cells were washed with 1 ml staining buffer, resuspended in 0.5 ml staining buffer, and analyzed on a BD Biosciences FACSCalibur. In most experiments, propidium iodide was added just prior to analysis to allow identification of nonviable cells. For analysis of thymic DN subsets, in addition to fluorochrome-conjugated anti-CD4 and anti-CD25, cells were stained with a mixture of biotinylated Abs specific for CD4, CD8, CD19, CD49b (DX5), CD11b, Ter119, and Ly-6G (Gr-1), which were all detected by the same fluorochrome coupled to streptavidin. Cells staining with any of the Abs in the mixture were electronically excluded during analysis of the DN subsets.

**In vitro activation of B cells and double-positive thymocytes**

Double-positive (DP) CD4⁺, CD8⁺ thymocytes were purified by sorting on a MoFlo cell sorter. Wells of a 24-well plate were coated with the anti-CD3 Ab 2C11 by overnight incubation at 4°C with 25 μg/ml Ab in 1 ml PBS. After coating, wells were washed three times with PBS, and 4 × 10⁵ purified DP thymocytes were plated per well. After 2 d of stimulation, cells were harvested and stained for flow cytometry. Splenic B cells were purified by complement-mediated lysis and activated with 20 μg/ml soluble F(ab')₂ anti-IgM, as described (37).

**Results**

**Targeted disruption of the *cd5* enhancer**

The *cd5* enhancer region targeted for disruption is indicated in Fig. 1, and the targeting strategy is outlined in Fig. 2. Homologous recombination of the targeting vector with the *cd5* locus results in the replacement of nt 2086 to 1991 with a floxed neo expression cassette. Subsequent breeding to CMV-Cre transgenic mice resulted in the deletion of the neoR cassette. The end result is the replacement of nt 2086 to 1991 of the enhancer with 106 nt of polylinker region from the target vector. The targeted disruption was made in BALB/c embryonic stem cells and was maintained on a BALB/c background.

**Figure 1.** The *cd5* enhancer is highly conserved in placental mammals. Histogram indicates conservation of the mouse enhancer region (chromosome 19: 10,815,430–10,815,286) with other placental mammals. Below the histogram is the sequence alignment for nine representative placental mammals, including mouse, as well as animals that are not placental mammals with no homologous region: tenrec, opossum, chicken, and stickleback. Transcription factor binding sites studied in Berland and Wortis (37) are indicated, as is the extent of the minimal enhancer identified in that study and the region deleted in the current study.
The cd5 enhancer is essential for expression of CD5 on B-1a cells

We originally identified the cd5 enhancer as an element required for the induction of reporter gene activity in response to BCR cross-linking in a transient transfection system (37). To determine whether the identified cd5 enhancer actually plays a role in situ in the expression of CD5 on B-1a cells, we used flow cytometry to compare CD5 expression on peritoneal and splenic B cells from wild-type and enhancer knockout mice. Fig. 3 shows CD5 expression on peritoneal B cells from a representative pair of mice. It is clear that the CD5\(^{+}\) subset is largely absent in the cd5 enhancer knockout mouse. There is a corresponding increase in the fraction of CD5\(^{-}\) B-1 cells, defined as B220\(^{low}\) (Fig. 3B, top panels, lower left region) or as IgM\(^{high}\), IgD\(^{low}\), or Mac-1\(^{+}\) B cells (Supplemental Fig. 1). These cells are apparently B-1a cells lacking CD5 expression, and not an expanded population of the normally CD5\(^{-}\) B-1b population, based on the fact that phosphatidylcholine-reactive B cells, normally present only in the B-1a population, are present in the CD5\(^{-}\) B-1 subset in enhancer knockout mice (data not shown). In addition, naïve enhancer knockout mice have normal serum titers of T15 anti-phosphorylcholine natural Abs that are normally produced by B-1a, but not B-1b cells (data not shown). Similar results were seen in the cd5 gene knockout mouse (40) and reflect the fact that CD5 is not necessary for the development or survival of B-1a cells. We next looked at the effect of enhancer deletion on splenic B-1a cells. These cells can be identified more readily by gating on CD23\(^{+}\) B cells. Fig. 3 (bottom panels) shows CD5 expression on CD23\(^{+}\) B cells from a wild-type and an enhancer knockout mouse. As in the peritoneum, it is clear that CD5\(^{+}\) B-1 cells are largely absent in the spleens of CD5 enhancer knockout mice. Thus, the cd5 enhancer, which we had demonstrated to be a BCR-response element in transient transfection studies, is required for the expression of CD5 on B-1a cells in vivo.

CD5 on thymocytes in enhancer-deficient mice

In addition to being expressed on B-1a cells, CD5 is expressed on cells of the T lineage. Moreover, its expression on thymocytes and T cells is highly regulated and dependent, in part, on pre-TCR and TCR signaling (2). We therefore wished to determine the role of the enhancer in CD5 expression in the T lineage. The earliest stage of thymocyte development is the DN stage. DN thymocytes can be divided into four subsets, DN1–DN4, based on the expression of CD25 and CD44. The developmental progression is from the DN1, which includes both T cell progenitors and other cells, in numerical order through the DN4 stage (reviewed in Ref. 41). Cells progress to the DN4 stage as a result of a successful rearrangement of a TCR \(\beta\)-chain, leading to expression of the pre-TCR, a process termed \(\beta\)-selection (41). CD5 has been reported to be expressed at the DN3 stage and to become upregulated during \(\beta\)-selection as a result of signals through the pre-TCR (2). CD5 expression at earlier stages was not examined in this study. To determine the effect of enhancer deletion on CD5 expression in DN thymocytes, thymocytes from wild-type and enhancer knockout mice were stained with anti-CD44, anti-CD25, and anti-CD5 as well as a mixture of Abs to CD4, CD8, and markers of NK, \(\gamma\delta\) B cell, myeloid, and erythroid lineages. Cells staining with the lineage mixture were electronically gated out during analysis. Fig. 4A shows a representative experiment. The highest levels of CD5 expression were detected on DN1 thymocytes from wild-type mice. cd5 expression in this subset was also the most dependent on the enhancer, with an average reduction of 4.9-fold \(\pm\) 0.6 in enhancer knockout compared with wild-type mice (average ratio of median fluorescence intensities of three experiments \(\pm\) SD). However, the CD5\(^{+}\) cells in the DN1 population are not T cell progenitors based on their failure to express c-kit (data not shown) (42). The effect of enhancer deletion in the other DN subsets was small, with mean decreases of 1.3 \(\pm\) 0.02-, 1.5 \(\pm\) 0.2-, and 2.5 \(\pm\) 0.1-fold, respectively, in the DN2–DN4 populations (average ratios of median fluorescence intensities of three experiments \(\pm\) SD).

We next looked at CD5 expression on CD4/CD8 DP and SP thymocytes in wild-type and enhancer knockout mice. CD5 is upregulated during positive selection as a result of ligation of the TCR by self-peptide/MHC complexes (2). Fig. 4B, left histograms, shows the results of this analysis. As in the DN T cell populations, enhancer deletion had only a modest effect on CD5 expression, with average reductions of 1.4 \(\pm\) 0.2-, 1.5 \(\pm\) 0.1-, and 1.9 \(\pm\) 0.4-fold, respectively, in the DP, CD4SP, and CD8SP populations (average ratios of median fluorescence intensities from five experiments \(\pm\) SD). cd5 is one of a pair of related genes that apparently arose as a result of gene duplication. The other member of the pair, cd6, is located just 50,365 bp upstream of cd5 in the mouse (43). The CD5 enhancer resides in the intergenic region 48,528 bp downstream of CD6. CD6 has been reported to associate with CD5 (44) and, like CD5, is expressed in a regulated fashion throughout thymocyte development (45). Because of the ability of enhancer
elements to act over long distances and bidirectionally, it was possible that the cd5 enhancer might also regulate cd6 expression. We therefore examined CD6 expression on DP and SP thymocytes from wild-type and enhancer knockout mice (Fig. 3B, right histograms). It is clear that loss of the cd5 enhancer had no effect on CD6 expression in these subsets.

CD5 on peripheral T cells in enhancer-deficient mice

The high levels of CD5 expression on SP thymocytes are maintained on T cells in the periphery. This appears to be in part dependent on continued interactions between the TCR and peptide-MHC ligands (26). To see whether the enhancer is required for maintenance of high CD5 levels in the periphery, we examined the effect of deletion of the enhancer on expression on splenic CD4 and CD8 T cells (Fig. 5B, left histograms). The effect is modest, with a 2.5 ± 0.2- and 1.9 ± 0.2-fold decreased CD5 expression, respectively, in CD4+ and CD8+ T cells (average ratio of median fluorescence intensities ± SD of three experiments). Enhancer deletion had no effect on the expression of CD6 on splenic T cells (Fig. 5B, right histograms) or on the CD4/CD8 ratio (Fig. 5A).

The CD5 enhancer is a BCR, but not a TCR response element in vivo induction experiments

Based on our earlier transfection studies, we expected that the CD5 enhancer would act as a BCR response element in B cells. In contrast, its relatively small contribution of CD5 expression in the T lineage suggested that it might not respond to TCR signals. To directly test this, we examined the effect of enhancer deletion on the induction of CD5 by BCR or TCR cross-linking in vitro. Fig. 6A shows that deletion of the enhancer completely abrogates the induction of CD5 by treatment with saturating amounts of anti-IgM. The cells increase in size (data not shown) and induce CD69 expression normally (Fig. 6A).

In vitro TCR cross-linking on purified DP thymocytes has been shown to upregulate CD5 expression, apparently mimicking the increase in CD5 expression that accompanies positive selection (2). Fig. 6B shows a representative experiment in which FACS-purified DP thymocytes from either a wild-type (left panel) or enhancer knockout (right panel) mouse were cultured in vitro with or without saturating amounts of plate-bound anti-CD3 to cross-link the TCR. Consistent with the minimal effect of enhancer loss on CD5 expression in the T lineage, the enhancer was completely dispensable for TCR-mediated induction of CD5 in vitro.

The CD5 enhancer is not required for CD5 expression on B-1a cells of young mice

Studies of cd5 enhancer function in the B lineage were conducted on mice 8 wk or older, whereas our studies of thymocytes were conducted in 6- to 8-wk-old mice. When peritoneal B-1a cells were examined in the younger mice, we found significant expression of CD5 in cells from enhancer knockout mice. This led us to examine the effect of enhancer deletion on CD5 expression on B-1a cells from even younger mice. Fig. 7A shows a representative analysis of a 2.7-wk-old wild-type mouse and a 3-wk-old enhancer knockout mouse. It is clear that a significant fraction of B220+ peritoneal cells expresses CD5 even in the absence of the enhancer. However, compared with the wild-type mouse, the level

**FIGURE 4.** Effect of cd5 enhancer deletion on CD5 expression on thymocytes. A, CD5 expression on DN thymocytes. Thymocytes were stained with Abs to CD44, CD25, CD5, and a mixture of biotinylated Abs reactive with mature T cells and other cell types to be excluded from the analysis (see Materials and Methods). Top panels, Show CD44 and CD25 staining on thymocytes from a representative wild-type (WT) (left panel) and enhancer−/− (right panel) mouse. Cells stained with biotinylated Abs were detected with fluorochrome-conjugated streptavidin and excluded from the analysis. Bottom panels show CD5 expression on wild-type (solid lines) and enhancer knockout (broken lines) mouse. Thymocytes were gated DN1–4 populations, as indicated. B, CD5 and CD6 expression on SP and DP thymocytes. Thymocytes were stained with anti-CD4, anti-CD8, and either anti-CD5 or anti-CD6. B, CD5 or CD6 staining on gated CD4+ or CD8+ lymphocytes from the wild-type (solid lines) or enhancer−/− (broken lines) mouse.

**FIGURE 5.** Effect of CD5 enhancer deletion on CD5 expression on splenic T cells. Splenocytes were stained with anti-CD4, anti-CD8, and either anti-CD5 or anti-CD6. A, CD4 and CD8 expression on gated lymphocytes from a representative wild-type (WT) (left panel) and enhancer−/− (right panel) mouse. B, CD5 or CD6 staining on gated CD4+ or CD8+ lymphocytes from the wild-type (solid lines) or enhancer−/− (broken lines) mouse.
of expression and the frequency of positive cells are reduced. Nonetheless, the effect of enhancer loss on a 3-wk-old mouse is strikingly different from on an adult mouse (Fig. 3A). The effect of enhancer loss in mice of different ages is summarized in Fig. 7B.

Discussion

There is much evidence that self-Ag–driven BCR signaling is required to populate the B-1a compartment. This signaling could be required for B-1a development, survival/self-renewal, or both. As cd5 gene expression is induced on conventional splenic B cells by BCR cross-linking (24, 25, 46), we hypothesized that normal expression in B-1a cells is also a consequence of BCR ligation. We reasoned that BCR-mediated CD5 induction on conventional B cells was likely to involve at least some of the same mechanisms involved in CD5 expression on B-1a cells. Hence, we sought to define the cis-regulatory elements of cd5 that respond to BCR cross-linking on conventional B cells. In an earlier study, we identified a NFAT-dependent enhancer ~2 kb upstream of CD5 as essential for BCR-mediated reporter gene activity in transient transfection experiments (37). We subsequently found that B cell-intrinsic NFATc1 is required for B-1a development and/or survival (47). Consistent with this, Winslow et al. (48) found that B cell-specific deletion of cnb1 encoding calcineurin-b1, a phosphatase that activates NFAT transcription factors downstream of Ag receptor signaling, also resulted in a reduced number of B-1a cells. These results provided support for the idea that the CD5 enhancer regulates CD5 expression in response to the same signals that are important in B-1a cell generation/survival. To definitively establish a role for the enhancer in CD5 expression on B-1a cells in vivo, we generated the cd5 enhancer knockout mice that are the subject of this study.

In vitro, conventional splenic B cells from CD5 enhancer knockout mice failed to induce CD5 expression in response to BCR cross-linking. This indicates that the enhancer we identified as a BCR response element in reporter assays also functions as a BCR response element in situ. In vivo, the enhancer was required for CD5 expression on B-1a cells of adult mice, but, surprisingly, in young mice (<5 wk old) the effect of enhancer deletion was modest, with many cells having significant, although somewhat reduced levels of surface CD5. Numerous studies have suggested that B-1a cells are predominantly generated during the fetal/neonatal period and persist for the life of the animal by self-renewal (49, 50). Thus, the shift in the requirement for the CD5 enhancer is most easily explained if the enhancer is needed for CD5 expression on B-1a cells. These results provided support for the idea that the CD5 enhancer regulates CD5 expression in response to the same signals that are important in B-1a cell generation/survival. To definitively establish a role for the enhancer in CD5 expression on B-1a cells in vivo, we generated the cd5 enhancer knockout mice that are the subject of this study.

In vitro, conventional splenic B cells from CD5 enhancer knockout mice failed to induce CD5 expression in response to BCR cross-linking. This indicates that the enhancer we identified as a BCR response element in reporter assays also functions as a BCR response element in situ. In vivo, the enhancer was required for CD5 expression on B-1a cells of adult mice, but, surprisingly, in young mice (<5 wk old) the effect of enhancer deletion was modest, with many cells having significant, although somewhat reduced levels of surface CD5. Numerous studies have suggested that B-1a cells are predominantly generated during the fetal/neonatal period and persist for the life of the animal by self-renewal (49, 50). Thus, the shift in the requirement for the CD5 enhancer is most easily explained if the enhancer is needed for maintenance of CD5 on peripheral B-1a cells, presumably in response to chronic BCR ligation by self-Ag, but dispensable for CD5 induction during B-1a development, at which time expres-
sion would depend on different CD5 regulatory elements, perhaps the same ones that play a role in CD5 expression on thymocytes.

However, we cannot rule out another possibility. Several studies indicate that B-1a cells can be generated by progenitors in the adult bone marrow, under some conditions quite efficiently (51–56). In one study, ~30% of the B-1a cells in 10-wk-old (unmanipulated) mice were found to have VDJ rearrangements containing N-insertions, indicating that they were the product of adult lymphopoiesis (57). Similar results were obtained using different methodologies (58). Adult-derived B-1a cells containing N-insertions are functionally indistinguishable from N-less B-1a cells (56). Furthermore, the B-1 cells derived from unique progenitors in the bone marrow have been reported to have a fetal repertoire (51). It is therefore possible that there is a wave of fetal-derived B-1a cells that express CD5 independently of the enhancer, and these are replaced by adult-derived B-1a cells in which CD5 expression is enhancer dependent.

The mechanism of CD5 expression is not the only facet of the B-1a phenotype that shifts when animals reach maturity. The fraction of B-1a cells is significantly reduced in young IL-5 or IL-5Rα−deficient mice, but recovers by adulthood (59, 60). Conversely, in notch2 heterozygous mice (homozygous deletion results in embryonic lethality), there is a significant reduction in B-1 cells that is manifested in 12- but not 4-wk-old mice (61).

The cd5 enhancer has been highly conserved among placental mammals, suggesting an important function. It is unlikely that this function is the maintenance of CD5 expression on B-1a cells as CD5-expressing B-1a cells have not been definitively detected in any species other than mouse, and even in mouse, B-1a cells are found in Mus domesticus-derived strains, but not in inbred lines derived from other mouse species (62).

CD5 can be induced by BCR cross-linking even on B cells of mouse strains lacking B-1a cells (62) as well as on bovine B cells (63). It is expressed on anergic B cells (17, 23), presumably as a result of chronic BCR stimulation, and it may play a role in maintaining anergy (17). A role for the enhancer in CD5 expression on anergic cells may explain its persistence over evolutionary time. In humans, CD5 is expressed on 11–49% of peripheral blood B cells, including transitional T1 cells and a population of prenaive B cells (5, 6), and may play a role in the production of IL-10 that enhances B cell survival by an autocrine mechanism (21).

CD5 is also expressed on cells of the T lineage, and its level of expression is determined by pre-TCR and TCR signaling, both in the thymus and periphery (2, 12, 14, 26). It was therefore surprising that the enhancer had a relatively minor role in CD5 expression in the T lineage. In particular, the level of CD5 expression on DP thymocytes is set during positive selection based on the avidity of the TCR for the selecting self-Ag (2, 12, 14). Surprisingly, enhancer deletion had very little effect on the level of CD5 expression on DP thymocytes. Consistent with this, the enhancer was entirely dispensable for in vitro induction of CD5 on purified DP thymocytes by cross-linking with saturating amounts of plate-bound anti-CD3ε. Thus, despite the fact that TCR signaling activates many of the same pathways as BCR signaling (64), including NFAF, induction of CD5 downstream of these Ag receptors involves different cis-regulatory elements in B and T cells. Further support for differential regulation of CD5 in B and T cells comes from reporter assay experiments showing no requirement for the enhancer element in mouse (65) or human (66) T cell lines. An inducible DNase I-hypersensitive site was seen in the vicinity of the enhancer in human T lymphoblasts treated with PMA and a Ca++ ionophore (66), consistent with our finding of some decrease in CD5 expression on T cells in enhancer knockout mice. The most significant effect that we observed was on CD5 expression in peripheral CD4+ T cells, where enhancer deletion resulted in a ~2.5-fold decrease in surface CD5 expression. It is possible that this decrease has functional consequences. CD5 is expressed at particularly high levels on CD25high, CD4+ T regulatory cells (67–70) and on anergic autoreactive T cells in a model of experimental autoimmune encephalitis (16). In the complete absence of CD5, there is an increase in the number of naturally occurring T regulatory cells (68). Thus, CD5 expression may be particularly important for T cell tolerance. However, CD5 enhancer knockout mice develop no obvious pathology and show no defects in the induction of oral tolerance (data not shown).

In summary, we have identified an enhancer element that plays an important role in cd5 gene regulation, primarily in B cells in response to BCR signaling. Given the important functions of CD5 in B cell tolerance and its link to CLL, a detailed understanding of how this enhancer functions may lead to important insights into autoimmunity and CLL pathogenesis as well as a better understanding of B-1a cell development and function.

Acknowledgments
We thank the Dartmouth Transgenic Shared Resource for assistance in generating the mutant mice.

Disclosures
The authors have no financial conflicts of interest.

References
Figure S1. Expression of B-1 cell markers on Peritoneal B cells from adult Cd5 enhancer-deficient mice. A. CD5 and CD11b staining on gated viable B220+ peritoneal lymphocytes from a representative WT (top panel) and Cd5enhancer-/- mouse. B. CD5 and IgD staining on similarly gated peritoneal cells from the same WT and Cd5enhancer-/- mouse. C. CD5 and IgM staining on gated viable IgM+ peritoneal lymphocytes from the same mice.
Berland Figure S1