Signal Transduction through the B Cell Antigen Receptor Is Normal in Ataxia-Telangiectasia B Lymphocytes*

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Peter Speck‡‡, Masato Ikeda‡‡, Akiko Ikeda‡, Howard M. Lederman**, and Richard Longnecker††

From the ‡Department of Microbiology-Immunology, Northwestern University Medical School, Chicago, Illinois 60611, the †Endowed Division of Pediatric Immunology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287, and the §Infectious Diseases Laboratories, Institute of Medical and Veterinary Science, Frome Road, Adelaide 5000, South Australia

The rare human genetic disorder ataxia-telangiectasia (A-T) has multiple consequences including a variable degree of immunodeficiency. Khanna and co-workers (Khanna, K. K., Yan, J., Watters, D., Hobson, K., Beamish, H., Spring, K., Shiloh, Y., Gatti, R. A., and Lavin, M. F. (1997) J. Biol. Chem. 272, 9489–9495) evaluated signaling in Epstein-Barr virus (EBV) immortalized A-T lymphoblastoid cell lines (LCLs), derived from the B cells of A-T patients. They showed that A-T lymphoblastoid cells lack signaling through the B cell antigen receptor and concluded that the fault in A-T encompasses intracellular signaling in B cells. However, it is established that EBV latent membrane protein 2A (LMP2A) blocks signaling in EBV-bearing cells by interaction with cellular tyrosine kinases. To test whether the reported defect in A-T B cells was not inherent in A-T but the result of influence of wild-type EBV, we derived A-T LCLs with wild-type or LMP2A-deleted EBV and studied signaling in these cells in response to cross-linking the B cell antigen receptor. We report that intracellular calcium mobilization and tyrosine phosphorylation in LMP2A-depleted LCLs derived from A-T patients is indistinguishable from that in LMP2A-depleted LCLs derived from normal controls. Further, signaling is blocked similarly in A-T and normal lymphoblastoid cells bearing wild-type EBV. In conclusion there is no evidence of any defect in B cell receptor signal transduction in A-T B cells.

Ataxia-telangiectasia (A-T)† is a rare, autosomal recessive, neurodegenerative disorder with associated endocrine and skin abnormalities and a predisposition for lymphoreticular malignancies. Immunodeficiency is a feature of A-T, with defects in cellular and humoral immunity (2). The gene responsible for A-T, ataxia-telangiectasia mutated (atm), is a large complicated gene that includes 66 exons and encodes a 350-kDa protein. The A-T locus has been mapped to chromosome 11, region q22–23 (3). The atm gene product, ATM, possesses a carboxyl-terminal kinase domain highly homologous to the catalytic domain of the signal transduction protein phosphatidylinositol 3-kinase (PI3-kinase) (4, 5). Other members of the family of atm-related genes include the yeast proteins Rad3, Mec1, and Tel1, the Tor proteins and their mammalian counterpart FRAP, which are involved in DNA repair and metabolism and cell cycle checkpoint control (for review, see Ref. 6). These sequence similarities and the high rate of spontaneous intrachromosomal recombination seen in A-T (7) suggest that ATM functions in DNA repair and cell cycle checkpoint control after DNA damage. Khanna et al. (1) reported that B lymphoblastoid cell lines (LCLs) derived from A-T patients show defective signaling compared with normal control cells in response to cross-linking of the B cell Ig receptor. Their data showed that this defect manifests in several ways: (i) lack of a proliferative response as measured by thymidine incorporation; (ii) reduced intracellular Ca2+ mobilization; (iii) reduced activation of phospholipase C; (iv) lack of tyrosine phosphorylation of the Src-related kinase Lyn, expressed in B cells; and (v) lack of PI 3-kinase activation. These workers used wild-type Epstein-Barr virus (EBV) to immortalize B cells and generate LCLs. Consequently it would be anticipated that any defects in cell signaling which are a usual consequence of the presence of wild-type EBV would be manifest in this system.

Previous reports show that EBV can block signaling through the B cell receptor in LCLs (8–11). This block is mediated by the interaction of tyrosine residues in the EBV-encoded LMP2A amino-terminal cytoplasmic tail with intracellular signaling molecules, including Lyn and Syk (8–11). LCLs bearing LMP2A-deleted EBV transduce signals in a manner indistinguishable from that observed in the EBV-negative B cell line BJAB (12, 13). In contrast, LCLs derived from healthy adults using wild-type EBV show a complete blockade of signaling, resembling the result Khanna and colleagues (1) reported with A-T LCLs bearing wild-type EBV. This suggests that the lack of cell signaling in A-T cells was caused by the action of LMP2A encoded by the wild-type EBV used to immortalize the A-T B cells, rather than an intrinsic fault in A-T B cells. Further, the mode of action of ATM, product of the gene deficient in A-T, has...
not been reported as directly possessing a role in B cell signaling.

Therefore to test the hypothesis that the observed fault in A-T B cells derived from wild-type EBV, we derived LCLs from A-T patients and healthy controls using either wild-type or LMP2A-deleted EBV. We investigated transmembrane signaling events after anti-Ig treatment in each type of LCL and report that intracellular calcium mobilization and tyrosine phosphorylation in LMP2A-deleted LCLs derived from A-T patients are indistinguishable from those in LMP2A-deleted LCLs derived from normal patients. Further, signaling is blocked similarly in A-T and normal lymphoblastoid cells infected with wild-type EBV. We conclude that there is no evidence of a fault in signal transduction through the B cell antigen receptor in A-T B cells.

EXPERIMENTAL PROCEDURES

Patients and Preparation of B Cell Lines—A-T patients were diagnosed according to the multiple element criteria set as described previously (14). 10 ml of peripheral blood was collected into heparinized containers from nine A-T patients and from six healthy controls. Mononuclear cells were purified by density gradient centrifugation using Ficoll-Paque (Amersham Biosciences, Inc.), and derivation of LCLs with virus EBfaV-GFP was carried out as described (15). Stocks of this virus contain a mixture of wild-type (strain B95-8) and mutant virus, in which the EcoRI-SalI region of EBV gene LMP2A (B95-8 sequence locations 2–644) is replaced by a DNA cassette obtained from plasmid pEGFP.N1 (CLONTECH Laboratories, Palo Alto, CA) and which encodes neomycin resistance and enhanced green fluorescent protein (EGFP). 50 μg/ml cyclosporin A was used to enhance the yield of immortalized B cell lines arising from the infection by suppressing the action of donor T cells directed against EBV (16, 17). Infected mononuclear cells were incubated in 96-well plates at 50,000 cells/well with half of the medium changed each week until proliferating colonies of cells were macroscopically evident, typically 5–5 weeks after infection. LCLs were cultured in RPMI 1640 medium containing antibiotics and 10% fetal calf serum.

Determination of EBV Genotypes of LCLs Arising from Infection—Resulting LCLs were evaluated for the genotype of the EBV genome they contained (wild-type or EGFP+; LMP2A-deleted) by visualization of EGFP expression as described (18) and by PCR. DNA samples were extracted from LCLs as described (19). Oligonucleotide primers specific for LMP2A sequences were as described (15), and a standard protocol for PCR was employed (20).

Calcium Flux Assays—Changes in intracellular calcium concentration were measured as described by Miller et al. (21). Briefly, cells were loaded with the ratiometric calcium-binding fluorescent dye indo-1 (Molecular Probes, Eugene, OR) for 30 min at room temperature and loaded with the ratiometric calcium-binding fluorescent dye indo-1 (Molecular Probes, Eugene, OR) for 30 min at room temperature and were examined using a Beckman Coulter Elite ESP flow cytometer using Elite version 4.02 software. This instrument reads fluorescence at 395 and 525 nm and calculates the ratio of these readings, which changes with calcium flux in the cell. Stimulation of cells was by introducing goat anti-human immunoglobulin (IgG+IgA+IgM, H+L, Southern Biotechnologies, Birmingham, AL) to a final concentration of 10 μg/ml in the cell suspension.

Antibodies, Western Immunoblotting, and Tyrosine Phosphorylation Assay—For the phosphotyrosine blot, because visualization of tyrosine phosphorylation is clearer with immunoprecipitates than whole lysates, samples were immunoprecipitated. Cells were treated with goat anti-human immunoglobulin for the indicated times and lysed as described by Fruehling et al. (22). Lysates were immunoprecipitated as described (11) with anti-phosphotyrosine antibody PY20, obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and separated using 8% SDS-PAGE. Transferred protein was blotted with anti-phosphotyrosine antibody RC20 directly conjugated with horse-radish peroxidase (Santa Cruz Biotechnology). For the ATP blot, whole cell lysates were separated on 4–15% gradient polyacrylamide gel (Bio-Rad). The membrane was blotted with mouse anti-ATM (a generous gift from Dr. Y. Shiloh) at 1:1,000 followed by anti-mouse IgG-horseradish peroxidase at 1:2,000. For the PI3-kinase blot, whole cell lysates were separated on 8% polyacrylamide gel, transferred to a membrane that was blotted with rabbit anti-PI3-kinase (1:4,000; Upstate Biotechnology, Inc., Hapaugge, NY) followed by anti-rabbit IgG-horseradish peroxidase (Amersham Biosciences, Inc.) at 1:2,000.

Visualization of horseradish peroxidase-conjugated secondary reagents employed enhanced chemiluminescence (ECL, Pierce).

Flow Cytometry—Flow cytometry was carried out using a FacsCalibur® (Becton-Dickinson, Franklin Lakes, NJ) as described (15, 18). Secondary antibodies were obtained from Jackson Immuno Research (West Grove, PA).

RESULTS

Derivation and Characterization of Lymphoblastoid Cell Lines from A-T Cells Using LMP2A-deleted EBV—Peripheral blood mononuclear cells obtained from A-T patients and normal donors were infected with EBfaV-GFP (15), and the resulting LCLs were scored by EGFP expression and PCR to evaluate the EBV genotype contained. Cells from nine A-T patients were infected with virus EBfaV-GFP and cultured in 96-well plates. Although virus EBfaV-GFP encodes a gene for resistance to the cytotoxic drug G418, drug selection was not applied. LCLs grew from seven of the nine patients, yielding a total of 320 lines. These results are similar to results from normal donors when equal numbers of cells were infected (data not shown). Examination of these lines by fluorescence showed that >95% of the cell lines expressed EGFP. DNA samples from each of these cell lines were examined by PCR, with primers specific for LMP2A which yield a reaction product of 546 bp as described previously (15). LCLs bearing only recombinant LMP2A-deleted EBV were isolated from cells obtained from three of the patients. Fig. 1 shows examples of PCR results obtained from these lines. As an internal control on integrity of the PCR, primers specific for EBV gene BHRF1 and which yield a 239-bp DNA product were included in each reaction. The sensitivity of this PCR to detect EBV LMP2A sequences has been determined previously (15) and is such that a single wild-type EBV genome is detected readily in a background of 10–100 mutant molecules. To confirm the A-T genotype of LCLs derived from A-T patients, Western blotting was performed to examine the expression of ATM, product of the gene mutated in this disorder (Fig. 2). A monoclonal antibody directed against ATM bound to a band of the appropriate size for each of three
LCLs derived from healthy controls (Fig. 2A, LCL1, LCL3, and ES.1). In lysates from A-T LCLs, no band was bound by the ATM antibody (Fig. 2B, V4.1, V4.3, and AZ4.1). Overloading of the gel and overexposure of the horseradish peroxidase-treated blots failed to reveal a band (data not shown). Lack of expression of ATM in the A-T patient LCLs is consistent with the diagnosis and demonstrates the A-T lesion at a molecular level. Panel B, as a check to show that similar amounts of protein were loaded into each well, the membrane was stripped and reprobed with antibodies against PI3-kinase, demonstrating that the absence of reactivity with the ATM protein was not the result of the absence of reactive protein in each lane (Fig. 2B, LCL1, LCL3, ES.1, V4.1, V4.3, and AZ4.1).

**Expression of Surface Immunoglobulin (sIg) Is Similar on A-T and Normal B Cells**—To determine whether differences in intracellular signaling could result from altered expression of sIg on cells, expression levels of sIg on normal and A-T cells, bearing either wild-type or LMP2A-deleted EBV, were measured. Expression levels were assessed by flow cytometry using a Southern Biotechnology goat antibody directed against human immunoglobulin, which was detected using a Cy3-labeled secondary antibody. Examples of flow cytometry plots showing sIg levels are shown in Fig. 3, with panel 1 showing the expression level of sIg on A-T cells bearing wild-type EBV and panel 2 showing an LCL derived from a healthy control and bearing wild-type EBV. These expression levels were indistinguishable from one another. All LCLs used in this study, regardless of origin (healthy control or A-T patient) or EBV genotype (wild-type or LMP2A-deleted) were examined for sIg expression and showed sIg levels indistinguishable from those displayed in Fig. 3 (data not shown). This result agrees with the observations of Khanna et al. (1). It was concluded that sIg levels do not account for any measured differences in intracellular signaling among any of the cell lines examined.

**Anti-Ig-induced Intracellular Calcium Mobilization in A-T LCLs Resembles That in Normal LCLs**—Intracellular signaling in response to antibody stimulation of the B cell receptor was measured using the ratiometric fluorescent dye indo-1. The cell lines examined included six lines derived from three A-T patients which by PCR had been shown to carry wild-type EBV only; and LCLs derived from healthy controls, and which likewise carried either wild-type or mutant LMP2A-deleted EBV. As shown in Fig. 4, intracellular Ca$^{2+}$ mobilization was similarly blocked for control LCLs bearing wild-type EBV (Fig. 4, panels 1 and 2) and A-T LCLs bearing wild-type EBV (Fig. 4, panel 5). LCLs bearing LMP2A-deleted EBV yielded similar changes in intracellular Ca$^{2+}$, regardless of whether derived from controls (Fig. 4, panels 3 and 4) or A-T cells (Fig. 4, panels 6–9). The responses shown in Fig. 4, panels 5 and 6, were obtained from cell lines derived from the same patient and differed only in the LMP2A status of the immortalizing EBV. Using Elite version 4.02 software as supplied with the Beckman Coulter Elite ESP flow cytometer, the proportion of cells responding to stimulation was quantified. Table I lists the calcium response phenotype of LCLs tested. Ca$^{2+}$ flux in A-T cells occurs indistinguishably from that in normal cells. In wild-type and A-T EBV LCLs, $<0.5\%$ of cells show a measurable response to anti-Ig. In contrast, in LMP2A-deleted A-T LCLs, between 31.5 and 61.2% of cells responded. In LMP2A-deleted normal LCLs, this range spanned 29.3–42.6%. We conclude that the calcium responses of A-T LMP2A-deleted LCLs fall within the same range as that of LMP2A-deleted normal LCLs. Comparisons among cell lines derived from the same patient and differing in the LMP2A genotype of transforming virus demonstrate the role that LMP2A is playing in blockage of signaling. For example, KS1.1 and KS1.2 cells, which contain wild-type EBV, do not display calcium signaling in response to sIg cross-linking. In contrast, KS1.7 and KS1.11 cells, derived from the same patient and bearing LMP2A-deleted EBV, display calcium fluxes resembling that of normal controls bearing the same virus. Taken together, these data suggest to us that any apparent lack of signaling in A-T LCLs is the result only of the previously described LMP2A effect of blocking normal B cell signal transduction (8–13) and not any intrinsic fault in A-T B cells.

**Tyrosine Phosphorylation in A-T and Normal Cells Occurs Indistinguishably**—Because phosphorylation of tyrosine residues is a prerequisite for the mobilization of intracellular calcium stores we compared the induction of tyrosine phosphorylation in response to stimulation of sIg in A-T LCLs with that in normal LCLs. As shown in Fig. 5, induction of tyrosine phosphorylation was not observed in LCLs (Fig. 5, LCL1 and LCL3) infected with wild-type EBV. In contrast, induction of phosphotyrosine in response to sIg cross-linking was observed readily in control lines bearing LMP2A-deleted EBV (Fig. 5, ES.1) and in A-T lines bearing LMP2A-deleted EBV (Fig. 5, AZ4.1). A-T cell line AZ4.1 is shown because of the prominence of the tyrosine phosphorylation in these cells. Other A-T LMP2A-deleted LCLs displayed tyrosine phosphorylation similar to the
LMP2A-deleted EBV and derived from A-T or normal B cells. Cells were loaded with the calcium-sensitive dye indo-1 for 30 min at room temperature as described by Rabinovitch et al. (22), stimulated with goat anti-human immunoglobulin at 20 μg/ml, and analyzed by flow cytometry using a Beckman Coulter Elite ESP flow cytometer. This instrument reads fluorescence at 395 and 525 nm, calculates the ratio of these readings, and plots the response of cells over time. Using Elite version 4.02 software as supplied with the cytometer, the proportion of cells responding to stimulation was quantified. Cell lines in which the immortalizing EBV lacks LMP2A showed a responsive phenotype (designated +). LCLs bearing wild-type EBV did not show changes in intracellular calcium (designated –) in response to surface immunoglobulin stimulation. It was concluded that the calcium responses of A-T LMP2A-deleted LCLs fall within the same range as that of LMP2A-deleted normal LCLs.

LMP2A-deleted EBV-transformed cell line shown in Fig. 5 indicating that, like LMP2A-deleted from normal individuals, A-T LMP2A-deleted LCLs also show a range of responses (data not shown). We conclude that phosphotyrosine induction in A-T cells occurs in the same manner and to the same extent as it does in normal cells after B cell receptor activation.

**DISCUSSION**

The product of the ATM gene has multiple functions involving maintaining stability of the genome and signal transduction, and faults within it compromise the immune system. Recent studies suggest that ATM is activated primarily in response to double-strand DNA breaks, the major cytotoxic lesion caused by ionizing radiation, and can directly bind to and phosphorylate c-Abl, p53, and replication protein A (for review, see Ref. 23). The ATM protein is required for phosphorylation of the breast cancer susceptibility gene Brca1 in response to ionizing radiation (24).

Like many aspects of the disorder, the immune deficiency in A-T is pleiotropic in nature, involving multiple aspects of the immune system. In many patients, the thymus is hypoplastic, and numbers of total T cells are reduced. The humoral immune system shows variable deficiency in A-T with whole or partial deficiencies of IgA, IgE, IgG, IgG subclasses and IgM, as well as oligoclonal gammapathy (for review, see Ref. 2). The proportion of total B cells in the peripheral blood of A-T patients is usually normal or elevated (25). A reduction of T cell helper activity has been reported in some patients with A-T (25), but this may be insufficient to account for the Ig absence. One interpretation of these data is that in A-T patients, B cells have a defect in maturation to Ig-producing cells or that the defect relates to abnormal signal transduction after activation (2). Another explanation is provided by the chromosomal instability in A-T which particularly involves chromosomes 7 and 14 in the vicinity of the T cell receptor and Ig genes (for review, see Ref. 2). The humoral immune deficiency in A-T along with the potential importance of the ATM gene product in cell signaling prompted Khanna and colleagues (1) to consider the hypothesis that B cell signaling is intrinsically defective in A-T.

In examining B cell signaling in A-T B cells, Khanna and colleagues (1) to consider the hypothesis that B cell signaling is intrinsically defective in A-T. Signaling through the T cell receptor and Ig genes (for review, see Ref. 2). The humoral immune deficiency in A-T along with the potential importance of the ATM gene product in cell signaling prompted Khanna and colleagues (1) to consider the hypothesis that B cell signaling is intrinsically defective in A-T.

**FIG. 4.** Intracellular free calcium change after sIg cross-linking with representative LCLs bearing wild-type or LMP2A-deleted EBV and derived from A-T or normal B cells. Cells were loaded with the calcium-sensitive dye indo-1 by incubation for 30 min at a concentration of 1 μM, as described by Rabinovitch et al. (22), stimulated with goat anti-human immunoglobulin (Southern Biotechnologies) at 20 μg/ml, and analyzed by flow cytometry using a Beckman Coulter Elite ESP flow cytometer. This instrument reads fluorescence at 395 and 525 nm, calculates the ratio of these readings (displayed on the y axis), which changes with calcium flux in the cell, and plots the changes over time (x axis). Base-line calcium levels were established for about 30–60 s before the addition of the anti-Ig antibody (indicated by a break in the histograms). Panels 1 (LCL1) and 2 (B95-32) are LCLs derived from healthy controls and bearing wild-type EBV. Panels 3 (GFP57) and 4 (GFP75) are derived from healthy controls and bearing LMP2A-deleted EBV. Panel 5 (KS1.2) shows A-T cells bearing wild-type EBV. Panels 6 (KS1.11), 7 (AZ4.1), 8 (V4.1), and 9 (V4.3) show the response of A-T LCLs bearing LMP2A-deleted EBV. LCLs in panels 5 and 6 were derived from the same A-T patient and differ in the LMP2A status of the EBV genome they bear. Cell lines displayed either the responsive phenotype (panels 3, 4, and 6–10) or the nonresponsive phenotype (panels 1, 2, and 5). Calcium responses in A-T LMP2A-deleted LCLs were indistinguishable from responses observed in LMP2A-deleted LCLs derived from normal cells.

**TABLE I**

| Cells                        | Calcium response/– | % Cells responding |
|------------------------------|---------------------|--------------------|
| Healthy controls, wild-type  | EBV                 |                    |
| FS1                          | –                   | <0.5               |
| LCL1                         | –                   | <0.5               |
| LCL3                         | –                   | <0.5               |
| SF1                          | –                   | <0.5               |
| B95–32                       | –                   | <0.5               |
| Healthy control, LMP2A-deleted EBV | |                |
| GFP57                        | +                   | 29.3               |
| GFP75                        | +                   | 42.6               |
| ES.1                         | +                   | 34.0               |
| A-T cells, wild-type EBV     | V4.2                | –                  |
| KS1.2                        | –                   | <0.5               |
| KS1.1                        | –                   | <0.5               |
| AZ4.2                        | –                   | <0.5               |
| A-T cells, LMP2A-deleted EBV | V4.3                | +                  |
| V4.7                         | +                   | 50.4               |
| KS1.7                        | +                   | 31.5               |
| KS1.11                       | +                   | 61.2               |
| V4.1                         | +                   | 40.8               |
| AZ4.1                        | +                   | 36.2               |
| V4.4                         | +                   | 49.4               |
with one important difference. In our current study, we used an EBV recombinant virus deleted for LMP2A to generate our LCLs for subsequent signaling analysis. We, as well as others, have shown that LMP2A expressed by single gene transfer into EBV-negative B lymphoma cells or expressed in EBV-transformed B lymphocytes is able to block normal sIg-mediated signal transduction efficiently after sIg cross-linking (8, 10, 12, 21, 26–31). This is important in preventing the activation of EBV lytic replication after sIg signaling. We have made specific point mutations within LMP2A delineating the interaction of LMP2A with the Src family PTKs and the Syk PTK as essential for the ability of LMP2A to block B cell sIg signal transduction (8, 10, 11). Furthermore, this function of LMP2A in EBV-infected LCLs has prompted the use of LMP2A-deleted EBV to study signal transduction in LCLs generated from patients with mutations in Btk (32).

In the Khanna study, the A-T LCLs generated with wild-type EBV have a phenotype that is entirely expected based on our studies and others illustrating the dramatic effect LMP2A has on normal B cell signal transduction (8, 10–12, 21, 26–31). Namely, we would expect an absence of signal transduction after the cross-linking of sIg. This observation is the result of the presence of LMP2A and not mutation of the ATM gene. What is more difficult to reconcile in the Khanna study is the results with the wild-type EBV-transformed LCLs from normal donors which responded well for a number of cell signaling parameters after sIg cross-linking. Based on our previous studies, we would have expected these control cells to be unresponsive after sIg cross-linking as was observed for the A-T patient LCLs transformed with wild-type EBV. In using the LMP2A-deleted EBV-transformed LCLs, we observed no difference in calcium mobilization or tyrosine phosphorylation after sIg cross-linking in A-T patient or control lines, indicating that signaling was similar. In view of the interrelated nature of the signaling parameters examined by Khanna et al. and the established effect of LMP2 on signaling, it is not necessary for this study to examine each and every parameter addressed by Khanna et al. to show that signaling is normal in A-T B cells.

We do not have an explanation of why Khanna and colleagues observed immunoglobulin-mediated signaling in control wild-type transformed LCLs. Despite this discrepancy between their studies and ours, the most important conclusion in our current study is that calcium mobilization and the induction of tyrosine phosphorylation are not defective in A-T patient LCLs as reported previously in the Khanna study. The results of studies of ATM−/− mice are consistent with the results of this study. ATM−/− mice are defective in their T-dependent antibody responses but normal in T-independent antibody responses. B cells derived from ATM−/− mice proliferate normally in response to stimuli including anti-IgM, lipopolysaccharide, and CD40 ligand (33), suggesting that mouse ATM−/− B cells are functionally normal. Consequently, it is concluded that signaling through the B cell receptor in A-T cells is indistinguishable from that of normal B cells.

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Peter Speck, Masato Ikeda, Akiko Ikeda, Howard M. Lederman and Richard Longnecker

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