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Impaired Germinal Center Maturation in Adenosine Deaminase Deficiency

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Mice deficient in the enzyme adenosine deaminase (ADA) have small lymphoid organs that contain reduced numbers of peripheral lymphocytes, and they are immunodeficient. We investigated B cell deficiency in ADA-deficient mice and found that B cell development in the bone marrow was normal. However, spleens were markedly smaller, their architecture was dramatically altered, and splenic B lymphocytes showed defects in proliferation and activation. ADA-deficient B cells exhibited a higher propensity to undergo B cell receptor-mediated apoptosis than their wild-type counterparts, suggesting that ADA plays a role in the survival of cells during Ag-dependent responses. In keeping with this finding, IgM production by extrafollicular plasmablast cells was higher in ADA-deficient than in wild-type mice, thus indicating that activated B cells accumulate extrafollicularly as a result of a poor or nonexistent germinal center formation. This hypothesis was subsequently confirmed by the profound loss of germinal center architecture. A comparison of levels of the ADA substrates, adenosine and 2′-deoxyadenosine, as well resulting dATP levels and S-adenosylhomocysteine hydrolase inhibition in bone marrow and spleen suggested that dATP accumulation in ADA-deficient spleens may be responsible for impaired B cell development. The altered splenic environment and signaling abnormalities may concurrently contribute to a block in B cell Ag-dependent maturation in ADA-deficient mouse spleens. The Journal of Immunology, 2003, 171: 5562–5570.

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denosine deaminase (ADA)-deficient SCID was the first inherited immunodeficiency for which the underlying molecular defect was identified (1). Although the genetic defects causing ADA deficiency were discovered 30 years ago, the mechanisms of the resulting lymphopenia are not completely clear. A mouse model for the human disease is now available. The model allows studies of lymphoid organs and immune function in ADA deficiency that are not accessible in humans (2). Because ADA deficiency in humans has served as a testing ground for enzyme replacement and gene therapies in humans, the mouse model is key in assessing the in vivo pathophysiological relevance of these therapies. The original version of the null ADA mutation in mice is perinatally lethal, due to liver damage (3). Viable ADA-deficient mice have been generated using an ADA cDNA minigene capable of delivering placental-specific expression. The resulting mice exhibit a phenotype similar to that seen in human patients, including profound immunodeficiency, ribcage abnormalities, and pulmonary difficulties (4).

Studies of ADA deficiency in humans have focused largely on effects on the peripheral blood T cell population, and some gene therapy trials have attempted to replace ADA in T cells only (5, 6).

B cell number or their functional depletion has been assumed to result from lack of CD4+ T cell help, and research on ADA-deficient B lymphocytes has lagged behind that on T cells. Recently, however, measurements of peripheral blood lymphoid cell populations in SCID patients have shown that ADA deficiency leads to the most severe T, B, and NK cell deletion among different types of SCIDs (7). Janus kinase 3-deficient SCID and common cytokine receptor γ-chain-deficient SCID, which have defects in molecules that are known to function in T cell proliferation signaling, show T and NK, but not B, cell depletion. In contrast, ADA deficiency shows depletion of B, T, and NK cells. This broader lymphopenia suggests that ADA deficiency blocks not only T and NK cell development, but also B cell lymphopoiesis. In addition, normalization of B lymphocyte number in an ADA-deficient patient treated with polyethylene-glycolated bovine ADA therapy occurred earlier than the reconstitution of T lymphocytes (8). Thus, a block in B cell development is probably independent of the lack of CD4+ T cell help. Such a block could occur early in the development of a common lymphoid progenitor or at a developmental step that is common to all lymphoid cell types.

Hemopoietic stem cells in bone marrow give rise to most hemopoietic progenitors and precursors, including those of the B cells. After hemopoietic stem cells commit to the lymphoid, and then B cell, lineages, the cells undergo heavy and light chain VDJ/VJ gene rearrangements as pro- and pre-B cells to ultimately express the IgM Ag receptor molecule at their surface (9). Immature B cells exit the marrow and migrate to peripheral lymphoid organs, including the spleen and lymph nodes, where they undergo Ag-dependent maturation. The screening process for the recruitment of germinal center (GC) candidates is initiated by the induction of IgM+ IgD− naive cells to produce polyclonal/low affinity Abs following their primary encounter with Ag. Some of the activated cells will be recruited into GC follicles, where Ag-dependent maturation and selection begin (10–13). Within the GC microenvironment, the B cell maturation program faces a series of
genetic events, including 1) changes in the regulation of cell cycle checkpoint genes that result in the proliferation of Ag-specific B cells, 2) somatic diversification of the IgV domains by the introduction of point mutations, 3) selection of high affinity Ag-specific B cells, and 4) intramolecular switching of the constant Ig regions from IgM into IgG, IgA, or IgE isotypes. The central accomplishment of the GC reaction is the generation of memory B cells that express Ig receptors with high affinity Ag-binding (IgV) domains that are fully able to mount a robust and specific immune response.

Previous reports on ADA-deficient mice have only broadly defined the B cell phenotype. Because B cell maturation in ADA deficiency has not been directly addressed, we sought to examine whether B cell ontogeny and Ag-dependent differentiation are affected in ADA-deficient mice and to determine whether a B cell developmental blockade also occurs. The present study is the first to focus on the impact of ADA deficiency on B cell development in the bone marrow and during Ag-dependent responses within the spleen. In this study, we report a dramatic alteration in GC formation of ADA-deficient mice that prevents Ag-dependent B cell maturation in the spleen, as demonstrated by defects in proliferation and activation signaling. Our findings are consistent with the severe and combined immune deficiency and reveal an intrinsic defect within the B lymphocyte compartment.

Materials and Methods

Mice
ADA-deficient mice were maintained as previously described (2) and were primarily used on days 14–17 before they died of pulmonary insufficiency on day 21. C57BL/6 males were obtained from The Jackson Laboratory (Bar Harbor, ME).

Enumeration and flow cytometric analysis of bone marrow
Bone marrow from 14- to 17-day-old ADA-deficient and littermate control mice was flushed from femurs with HBSS/5% FBS. The cells were pelleted by centrifugation at 300 × g for 5 min at 4°C. Cells were resuspended in 1 ml of PBS/2% FBS, and lymphocyte cell nuclei were counted by suspending 10 µl of cells in 990 µl of 3% acetic acid and visualizing on a hemocytometer. The bone marrow cells were incubated with Fc block (0.25 µg/107 cells) and then stained with anti-Gr-1-FITC (Ly-6G, clone RB6-8C5), Ter119-PE (clone TER-119), and CD11b (Mac-1, clone M1/70) for analysis of myeloid and erythroid lineage cells. Separate aliquots of bone marrow cells were stained with CD19-FITC (clone 1D3), anti-IgM-PE (clone AMS 9.1), B220-APC (clone RA3-6B2), and CD43-biotin (clone S7)/streptavidin-CyChrome (BD PharMingen, San Diego, CA) for analysis of pre- and pre-B cell populations. All Abs were purchased from BD PharMingen unless otherwise noted. Flow cytometry was performed on a FACScan with CellQuest software (BD Biosciences, Mountain View, CA), and appropriate negative isotype control Abs (BD PharMingen) were used in all analyses.

CFU assay
Bone marrow cells were isolated as described above, and 2.5 × 105 cells were suspended in 4.5 ml of complete medium (McCoy’s 5A with penicillin, streptomycin, 15% FCS, sodium pyruvate, MEM nonessential and essential amino acids, sodium bicarbonate, 2 ME, glutamine, asparagine, and MEM vitamins). Ten to 25 µg/ml of IL-7 (Sigma-Aldrich, St. Louis, MO) or 10 µg/ml of LPS (Sigma-Aldrich) was pipetted into triplicate 35-mm petri dishes (Corning, Corning, NY). Heat 3% agar (endotoxin-free; 0.5 ml) was added to the cell suspension. One milliliter of 3% acetic acid was added to the cell suspension. After centrifugation and washing with PBS/FBS, the cell suspension was incubated for 24 h at 37°C in 5% CO2. After incubation, the cell suspension was washed and counted, and 5 × 105 cells/well were plated in 200 µl of 96-well plates. Different stimulants were added to the wells: 1 µg/ml LPS (Sigma-Aldrich), 5 µg/ml mouse anti-mouse IgM Fab’2 (Jackson ImmunoResearch), anti-IgM and 40 ng/ml IL-4 (Sigma-Aldrich), anti-IgM and anti-CD40 (clone HM40-3; BD PharMingen), IL-4 and anti-CD40, IL-4 alone, anti-CD40 alone, and medium alone in control wells. After incubation for 2 days at 37°C in 5% CO2, 1 µCi/well of [3H]thymidine was added, cells were harvested 16 h later with an automatic cell harvester, and incorporated [3H]thymidine was measured. This procedure was repeated using 1–5 µM 2’-deoxycoformycin (gift from Parke-Davis (Detroit, MI) and SuperGen (Dublin, CA)) in the culture medium of normal mouse B splenocytes.

Ab (IgM and IgG) production measurement
B splenocytes were isolated as in the proliferation assay. Cells (5 × 105/well) were incubated in 200 µl of complete RPMI with medium alone, 1 µg of LPS/well (Sigma-Aldrich), or 1 µg of anti-CD40/ well (clone 3-23; BD PharMingen) and 4 µg of IL-4/well (Sigma-Aldrich). After 6 days at 37°C in 5% CO2, Ab production was measured by ELISA, using mouse IgM or IgG (Jackson ImmunoResearch) for standard curves.

Flow cytometric analysis of peritoneal fluid
Peritoneal fluid was collected by peritoneal wash with PBS. Total peritoneal lymphocytes were counted. T cells were removed by addition of anti-Thy1.2 (CD90.2, clone 53-2.1; BD PharMingen) and rabbit complement (Low-Tox-M rabbit complement; Cedarlane Laboratories, Hornby, Canada), followed by Percoll gradient isolation. The interface cells were washed with 10 ml of PBS/FBS, incubated with 0.25 µg of Fc block/105 cells, and then stained with anti-IgM-FITC and CD3-PE (clones AMS 9.1 and 57-7.3, respectively; BD PharMingen).

Splenic activation assay
Spleen cells were isolated as described above, 10 µg/ml goat anti-mouse IgM Fab’2 and cells were incubated for 24 h at 37°C in 5% CO2. Cells were then washed with PBS/FBS, incubated with Fc block and stained with anti-B220-APC and CD86-FITC (B7-2, clone GL1; BD
PharMingen) or with B220-APC and CD69-FITC (clone H1.1F3; BD PharMingen). Cells were then analyzed by flow cytometry.

Measurement of apoptotic B splenocytes

B spleen cells were isolated as described above. Cells (5 × 10^7/well/200 μl) in 96-well plates were stimulated with 1 μg/ml goat anti-mouse anti-IgM (Jackson ImmunoResearch) for 1–3 h at 37°C with 5% CO₂. Cells were then incubated with annexin V-FITC and propidium iodide (kit from BioSource International, Camarillo, CA) for 15 min at room temperature and analyzed with flow cytometry.

Nucleoside isolation and detection

Adenosine and 2'-deoxyadenosine were isolated from bone marrow and spleen samples as described previously (14). Briefly, 850 μl of ice-cold 0.4 N perchloric acid was added to frozen samples, followed by sonicating (30 s at 30 W). After 100 μl was removed for protein determination, the remainder was centrifuged at 12,000 × g for 5 min at 4°C. The supernatant (710 μl) was transferred to a clean tube, neutralized with 356 μl of 0.6 M KHCO₃/0.72 M KOH, and acidified with 111 μl of 0.18 M ammonium dihydrogen phosphate, pH 5.1, and one drop of dilute phosphoric acid. The samples were centrifuged, and the supernatants were stored at −20°C for HPLC (Waters, Millipore Corp., Bedford, MA) analysis on a Partispher bonded phase C₈ column (Whatman, Clifton, NJ) at a flow rate of 1.5 ml/min. The mobile phase was 0.02 M NH₄H₂PO₄, pH 5.1, with a superimposed methanol gradient: 0% for 0–4 min, 0–8% for 4–6 min, 8–20% for 6–8 min, and 20% for 8–18 min.

Nucleotide extraction and detection

Deoxy-ATP was isolated as described previously (15). Bone marrow was flushed from femurs with 10 mM Tris/0.9% NaCl, and the cells were Dounce homogenized (Kontes, Vineland, NJ) in ice-cold 60% methanol and left at −20°C overnight. Spleens were harvested and treated similarly. The cells were then centrifuged at 12,000 × g for 5 min at 4°C, and the supernatant was transferred to a clean tube. The supernatant was evaporated in a Speed-Vac concentrator. Protein determination was performed on the first pellet, which was resuspended in 100 μl of 0.4 N perchloric acid (dCF) and incubated at 37°C. Cell colonies. As seen in wild-type mice, bone marrow cells from ADA-decient mice (data not shown). Although cells can display cell surface lineage-specific markers, the expression alone provides no functional assessment of their maturation status (20); namely, some bone marrow fractions may contain overlapping mixtures of B cells at various differentiation stages (21). Therefore, we conducted CFU assays on ADA-deficient bone marrow to determine whether bone marrow progenitor cells have the capacity to differentiate into pre-B and immature B cell colonies. As seen in wild-type mice, bone marrow cells from ADA-deficient mice formed pre-B and immature B cells after stimulation with IL-7 and LPS (Fig. 1c). Collectively, these data demonstrate that there is no significant defect in B cell ontogeny in ADA-deficient mice. Likewise, there were no significant defects in bone marrow erythocyte, granulocyte, and macrophage development in ADA-deficient mice (data not shown).

To rule out “leaky” ADA expression in the bone marrow as an explanation for the lack of an apparent block in B cell development (5, 16, 17). The developmental parallels showed by B and T cells, including gene rearrangement and selection, suggested that ADA deficiency, with a block in early T cell development, could equally affect the early B cell maturation program. However, in contrast to T cells and as shown in Fig. 1a, the number of total bone marrow cells and the number of bone marrow B220⁺ (B cell-destined) cells were not significantly different between ADA-deficient and normal littermates. Li and Hardy (18, 19) have shown that fractions or subpopulations of B cells developing in the bone marrow, such as pro- and pre-B cells, can be categorized based on the expression of cell surface markers, such as B220, CD19, CD43, and IgM. As shown in Fig. 1b, the proportions of pro- and pre-B cells in ADA-deficient and wild-type mice were not significantly different. Moreover, flow cytometric analysis of CD24, CD19, and IgM expression of B220⁺ bone marrow cells showed no differences between ADA-deficient and normal mice (data not shown).

FIGURE 1. B cell development in bone marrow. a, Total numbers of cells and numbers of B220⁺ cells in ADA-deficient and control (normal) littermate bone marrow, n = 9. b, Pro- and pre-B cell population proportions in ADA-deficient and control bone marrow. The histogram and percentages shown are representative of nine mice (aged 14–17 days) of each type. c, CFU assay with ADA-deficient and control bone marrow cells. The histograms show the number of CFU from pre- and immature B cells after stimulation with IL-7 or LPS, n = 5–6. Values shown represent means. SEs are shown by thin bars above the means.
ontogeny, we conducted zymogram analysis of bone marrow of ADA-deficient mice, which showed no ADA activity (data not shown). Thus, B cell maturation in the bone marrow does not appear to be ADA dependent. Our findings indicate that the B cell lymphopenia consistently observed in SCID-ADA deficiency does not implicate a blockade in the primary maturation program within the bone marrow and strongly suggest that the B cell defect is the result of altered differentiation in the secondary lymphoid organs.

**ADA-deficient mouse spleens are markedly different from those of control littermates in size, cell number, and architecture**

In agreement with our prediction, differences between ADA-deficient and normal spleen were readily detectable in secondary lymphoid organs such as the spleen. First, and as shown in Fig. 2a, a dramatic difference in size between ADA-deficient and control spleens was consistently observed. The total number of cells in ADA-deficient spleens was decreased on day 10 of life and became profoundly less by day 17 (Fig. 2b).

To investigate whether B cells could actually be recruited into GCs, spleen histological preparations were examined. Immunohistochemical analysis of spleens showed dramatic defects in the formation of GC architecture in ADA-deficient mice. As shown in Fig. 3a, H&E staining of spleen sections revealed large nodules of lymphocytes forming white pulp (staining purple) in control mice, but smaller, less organized white pulp areas in ADA-deficient mice. To distinguish between naive and GC B cells, spleen sections were stained with Abs to IgD (Fig. 3b), CD23 (Fig. 3c), and GL7 (early activation marker for B and T lymphocytes; Fig. 3d). IgD, CD23, and GL7 staining of frozen spleen sections showed an almost complete absence of GCs. The few GCs observed were small and had highly abnormal architecture.

Deconvolution and conventional fluorescence microscopy of frozen spleen sections stained with IgM, IgD, and Thy1.2 (Fig. 4) also showed sparse, small, and profoundly altered GCs. Interestingly, T cells were present in the sections, but had not organized within GC follicles. Although B cells and T cells were present in lower numbers in ADA-deficient spleens, they appeared dispersed and without their characteristic organization within germinal centers.

**CD4+ T cells are present in ADA-deficient spleens**

Despite the presence of T cells, their numbers were greatly reduced in ADA-deficient spleens (Fig. 5a). Because a lack of CD4+ helper cells is known to impair effective B cell Ag-dependent humoral responses, we examined whether CD4+ T cells were selectively depleted. Although the total numbers of T cells in ADA-deficient spleens were decreased, CD4+ and CD8+ T cells were present in similar relative proportions in ADA-deficient and normal spleens (Fig. 5b). These results suggest that the profound alterations during secondary immune responses equally affect B and T cell subsets.
and the formation of GCs. Thus, altered GC formation in ADA-deficient mice may indeed impact the capacity of B cells to differentiate in an Ag-dependent manner and may impair the generation of B cell memory.

**B lymphocytes from ADA-deficient mice show profound defects in proliferation and activation, and IgM production is prominent**

Consistent with the hypothesis that the lack of GC formation in ADA-deficient mice may profoundly affect Ag-dependent B cell functions, splenic proliferation assays (Fig. 6a) showed that ADA-deficient B lymphocytes had a markedly decreased ability to proliferate in response to LPS or anti-IgM (with or without IL-4 or CD40). The expression of IgM, as measured by the mean fluorescence intensity, was similar for ADA-deficient and control mouse B lymphocytes (data not shown); therefore, decreased surface IgM levels could not explain the proliferative signaling defect. The signaling defect was due solely to the lack of ADA in the cells, because normal mouse B lymphocytes that were cultured in deoxycoformycin, a specific inhibitor of ADA, exhibited a similar defect (Fig. 6b), although LPS-stimulated proliferation was not affected in these conditions.

Furthermore, activation of cultured B lymphocytes in response to stimulation by anti-IgM was also impaired in ADA-deficient mice (Fig. 7, a and b), as shown by the reduced expression of the B and T cell activation marker CD69 and the costimulatory molecule CD86. It is noteworthy that the basal levels of both CD69 and CD86 were lower in ADA-deficient spleen B cells.

Effective and robust amplification of Ag-dependent humoral responses is a physiological landmark of the GC reaction, which includes the intramolecular switch from the IgM to the IgG isotype. The poor or nearly absent GC formation would therefore predict an accumulation of IgM and no changes in the levels of IgG, which may have resulted from extracellular Ig isotype switching. In line with this rationale, Fig. 8a shows that the production of IgG by cultured B cells was low and unremarkable. On the other hand, IgM production was 2-fold higher in ADA-deficient mice upon polyclonal expansion of IgM-producing cells by LPS. Treatment of normal B cells with dCF, an inhibitor of ADA activity, showed no effect on IgM or IgG production (Fig. 8b), further supporting the idea that while ADA deficiency affects the formation of GCs, it does not affect extracellular polyclonal cell proliferation.

**B-1 cells are present in normal numbers in ADA-deficient peritoneal fluid**

In contrast to the B-2 cells described in the preceding sections, B-1 cells are a self-renewing population derived from fetal liver. Normally, an enriched population of B-1 cells can be found in peritoneal fluid. Because proliferative and activation signaling through the B cell receptor (BCR) was defective in ADA-deficient splenic B cells, and since the development locales and signaling through the BCR differ in B-2 and B-1 cells (22, 23), we determined whether B-1 cells are present in ADA-deficient mice. Isolated B-1 cells from the peritoneal fluid, namely, IgM⁺CD5⁺, were present in similar proportions and numbers in ADA-deficient and wild-type mice (Fig. 9). This finding supports the idea that ADA deficiency has a major impact on Ag-dependent responses resulting from impaired GC formation.

**ADA-deficient B lymphocytes readily undergo apoptosis when stimulated through the BCR**

The fact that ADA-deficient B cells did not proliferate in response to Ag receptor-mediated signaling suggested a higher propensity to undergo programmed cell death or apoptosis. To investigate this possibility, we stimulated cultured B lymphocytes with anti-IgM and then analyzed annexin V and propidium iodide staining by
Flow cytometry. Although the viabilities of unstimulated B lymphocytes from ADA-deficient and wild-type mice were comparable, anti-IgM-stimulated ADA-deficient B cells showed a higher degree of apoptosis than control B cells (Fig. 10). Two-fold more ADA-deficient B cells were apoptotic, as identified by annexin V staining, than in the wild-type littersmates. Thus, an increased extrafollicular apoptosis rate could contribute to the impaired GC formation.

Elevated dATP levels in spleen may account for the increased propensity of ADA-deficient B cells to undergo apoptosis

Although primary B cell development in ADA-deficient bone marrow appeared unaltered in ADA-deficient mice, Ag-dependent B cell development in spleen was significantly affected. To further substantiate the differences between the two lymphoid compartments, we compared parameters that have long been suspected to be causes of lymphocyte demise. Elevated levels of ADA substrates or dATP are known to result in increased apoptosis in lymphocytes. Inhibition of SAH hydrolase could also cause B cell death in conditions of ADA deficiency. ADA substrate and dATP levels were measured using HPLC, and SAH hydrolase inhibition was measured with a TLC assay for radiolabeled reaction product. When we measured these parameters in bone marrow and spleen, we found increased levels of adenosine (Fig. 11a) and 2’-deoxyadenosine (Fig. 11b) in both ADA-deficient bone marrow and spleen. We also found SAH hydrolase inhibition in both locales (Fig. 11c). As shown in Fig. 11d, dATP was unaltered in the ADA-deficient bone marrow, a fact consistent with the unaltered B cell ontogeny. To our knowledge, we are the first to report metabolic results for bone marrow in ADA-deficient mice or humans. In contrast to bone marrow values and consistent with the defective GC immune responses, dATP was extremely elevated in the spleens of ADA-deficient mice. These data suggest that dATP accumulation plays a direct role in the death of mature B lymphocytes seen in ADA deficiency.

**FIGURE 8.** Ig production by ADA-deficient and control B cells. a, IgM production (top) and IgG production (bottom) by cultured splenic B cells from ADA-deficient and control littermate mice. b, IgM production (top) and IgG production (bottom) of cultured splenic B cells from control mice with or without 5 μM dCF. n = 4. *p < 0.05.

**FIGURE 9.** Similar amounts of B-1 cells are found in the peritoneal cavity fluid of ADA-deficient and control littermate mice at 14 days of age. Percentages are shown on histograms. Each histogram shows representative results of three trials.

**FIGURE 10.** Percentage of apoptotic spleen B220+ cells in control and ADA-deficient mice after stimulation with anti-IgM, as measured by the expression of annexin V (and exclusion of PI). SEs are shown as thin black bars. n = 4. *p < 0.05.

**FIGURE 11.** ADA substrate and dATP levels, and SAH hydrolase activity in bone marrow and spleen. Adenosine (a) and 2’-deoxyadenosine (b) levels in bone marrow and spleen. n = 3. c, SAH hydrolase activity in bone marrow and spleen. n = 3. d, dATP levels. n = 3. *p < 0.05.
Our findings collectively reveal B cell abnormalities in ADA-deficient mice that selectively perturb Ag-dependent GC responses. Such abnormalities are probably due to the toxic metabolic microenvironment of the spleen and a higher resulting B cell propensity to apoptosis.

**Discussion**

Both ADA-deficient T and B cells suffer developmental blocks in toxic environments with high dATP levels. For thymocytes, this block occurs early in ontogeny in the thymus. Developing B cells are spared the dATP toxicity in early ontogeny, because macrophages or other scavenger cells are likely to dispose of the deoxynucleotide in the bone marrow. Low nucleotide kinase activity or a high nucleotide phosphorylase to kinase activity ratio could also account for the low dATP levels seen in bone marrow. A low dATP level found in human ADA-deficient bone marrow (24) suggests that our findings in the mouse model are consistent with the human disease. However, and analogous to thymocytes in the thymus, B cells encounter toxic dATP levels in the spleen environment. Thus, the occurrence of a B cell developmental block at a later stage could explain why lymphoid normalization by polyethylene-glycolated ADA therapy occurs faster in B cells than in T cells (8).

Nucleoside levels do not normally exceed the levels of their phosphorylated nucleotides. This is the case in our normal mouse bone marrow and spleen samples. ADA-deficient mouse tissues, however, exhibit 2′-deoxyadenosine to dATP level ratios of 5–30. The high levels of 2′-deoxyadenosine compared with dATP seen in ADA-deficient spleens and bone marrow could be due to degradation of dATP to 2′-deoxyadenosine. Control experiments, however, adding internal dATP standards to tissues, showed that such dATP was not degraded to 2′-deoxyadenosine (data not shown).

GC B cell defects in ADA-deficient mice cannot be associated with limited CD4+ T cell help for the following reasons: 1) TCR-deficient mice that are completely depleted of T cells effectively have GC-like clusters that show the early phases of germinal center reaction (25); 2) knockout mice missing an adaptor protein crucial to TCR signaling (SLP-76) have impaired thymocyte maturation and no peripheral T cells, but normal B cell development and function (26); and 3) ADA-deficient spleens have some CD4+ cells, yet there is no GC formation.

Because low numbers of CD4+ T cells in ADA-deficient mice cannot completely explain the defects in GC formation, a defect in B cells themselves appears likely. B cells from ADA-deficient mouse spleens showed a greatly diminished ability to proliferate in response to LPS and anti-IgM. Indeed, splenic B lymphocytes from control mice cultured with dCF showed that ADA is necessary for anti-IgM-stimulated proliferation, but not for proliferation signaling through Toll-like receptor 4 by LPS. Activation signaling also, was impaired in ADA-deficient splenic B cells. Accumulation of immature B cells in ADA-deficient mouse spleens could explain this failure, since immature B cells undergo apoptosis when BCR-ligated, while mature B cells proliferate and become activated (27). Along the same line, accumulating extrafollicular plasmablasts were probably the source of the high levels of IgM produced by ADA-deficient splenic B lymphocytes. Faithful to their immature phenotype, ADA-deficient B cells underwent apoptosis more readily than their mature B counterparts and did not proliferate in response to Ag receptor signaling as well as mature B cells. If the basal level of apoptosis in control B cells is considered, the ADA-deficient B lymphocytes actually exhibited a 50% increase (from 10 to 15%) in apoptosis. Only when B lymphocytes from spleen were stimulated with anti-IgM did the increased apoptosis become apparent. Scavenging macrophages in spleen may efficiently remove apoptosed B cells, making it difficult to witness a large increase in the number of cells undergoing apoptosis.

In their in vitro study Fujita et al. (28) showed that ADA-positive fibroblasts could protect ADA-deficient B cells from the metabolic toxicity of high levels of deoxyadenosine. These results illustrate the widely accepted view that ADA-positive cells can provide metabolic protection for neighboring ADA-deficient cells. The successful use of ADA enzyme therapy is another example showing that exogenous ADA can provide metabolic protection for ADA-deficient B cells. Proper maturation would probably occur if metabolic protection was provided by ADA enzyme therapy or by neighboring cells with ADA activity. Our results clearly show that proper maturation of B cells does not occur within the spleens of ADA-deficient mice. The resulting B cells are impaired, as judged by a reduced mitogenic response to LPS or BCR activation. Our results indicate that B cells are especially sensitive to the metabolic consequences of ADA deficiency.

B and T cells may need ADA for proliferation and activation signaling. In line with our B cell findings, other studies have shown proliferation and activation signaling defects in T cells under conditions of ADA deficiency. For instance, ADA-deficient peripheral T cells have shown a reduction of tyrosine phosphorylation of TCR-associated signaling molecules and a block of TCR-mediated calcium flux (17). Also, extracellular adenine nucleotides, which accumulate in ADA deficiency, inhibit CD4+ T cell activation via an increase in cAMP induced by an unidentified purinergic receptor (29).

BCR signaling differs between B-2 and B-1 cells (30). Ligation of the BCR of B-2 cells leads to mobilization of intracellular calcium and proliferation of mature cells and apoptosis of immature cells. BCR ligation of B-1 cells, however, results in failure to enter the cell cycle, proliferate, and undergo apoptosis. B-1 cell numbers were normal in the peritoneal fluid of ADA-deficient mice. Their survival could be due to BCR signaling or signaling via Toll-like receptor 4, which is associated with the LPS receptor complex and is not dependent on ADA. The survival of B-1 cells in the peritoneum may be due to the fact that B-1 cells are fetal liver-derived and are a self-renewing population. These cells may survive because they were protected during development by placental ADA activity. If the peritoneal fluid does not accumulate dATP and does not represent a toxic environment, B-1 cells would be spared the fate of B-2 cells in spleen. It is also plausible that the peritoneal fluid is cleared of dATP and may not represent a toxic environment.

Several theories exist to explain the selective effects of ADA deficiency on lymphoid cells. Accumulation of the ADA substrates, adenosine and 2′-deoxyadenosine, could affect development of T and B cells in a number of ways. Elevated adenosine levels could trigger aberrant adenosine receptor signaling. Adenosine transduces extracellular signals by binding to G protein-coupled adenosine receptors (A1, A2a, A2b, and A3) that can mediate intracellular cAMP levels (31). Adenosine receptor engagement can lead to elevation of cAMP levels, which can lead to thymocyte apoptosis and developmental arrest (32–34). On the other hand, 2′-deoxyadenosine functions as a suicide inhibitor of the enzyme SAH hydrolase, an enzyme critical to cellular transmethylation metabolism (35). In this context, the resulting accumulation of SAH could modulate APO-1/Fas-mediated cell death (36). Accumulated deoxyadenosine can be converted to dATP, and elevated dATP levels could cause B cell apoptosis or the proliferative defects seen in ADA-deficient spleens. High levels of dATP can cause DNA nicking and apoptosis (37). The resulting unrepaired DNA strand breaks can induce poly-ADP ribose polymerase,
which, in turn, may induce the cells to undergo apoptosis by NAD depletion (38, 39). Moreover, dATP could directly initiate apoptosis by binding to apoptotic protease-activating factor-1, triggering the caspase-dependent apoptotic cascade (40). Alternatively, high dATP levels can also cause ribonucleotide reductase inhibition, leading to inhibition of deoxyribonucleotide synthesis and the reduced ability to repair DNA. Inhibition of ribonucleotide reductase can lead to nucleotide pool depletion, which can then interfere with the formation of N nucleotides in coding joints and impair T and B cell gene rearrangement. In line with these conclusions, alterations in N region insertions have been reported in human ADA-deficient patients (41). Therefore, while B cell maturation in the bone marrow appeared to be normal in ADA-deficient mice, the Ag binding diversity of the B cell repertoire may remain limited.

Although elevated adenosine and deoxyadenosine levels along with the inhibition of SAH hydrolyase have long been suspected of contributing to the lymphopenia seen in ADA deficiency, these mechanisms are less likely to play a definitive role in the fate of B cells. Nevertheless, B cell development is altered in the spleen, and higher levels of deoxyadenosine and dATP were observed, which, in turn, could exacerbate the propensity of B cells to undergo apoptosis. This evidence pointed to dATP as the cause of B cell development problems in ADA-deficient spleens. Consistent with this hypothesis, previous studies showed that dATP inhibits growth or induces apoptosis in T cells. T lymphoblastoid cells showed growth inhibition, which was correlated to the accumulation of dATP (42). Recently, dATP was implicated as a cause of thymocyte apoptosis in ADA-deficient FTOC (43) when a kinase inhibitor blocked the inhibitory effects of dCF. These findings together with the data reported in the present study indicate that dATP accumulation is the decisive defect leading to lymphocyte growth inhibition and apoptosis in ADA deficiency.

Autoimmunity, which appears to be linked to limited B and T cell diversity and defective intrathymic and GC repertoire selection, is often seen in ADA-deficient patients. Recently, in the MRL.Fas<sup>−/−</sup> mouse strain, which develops systemic lupus erythematosus, autoreactive B cells were found to accumulate not in GCs but at the T cell zone–red pulp border (44). Attheros, autoreactive B cells were found to accumulate not in GCs but at the T cell zone–red pulp border (44).

References


