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Article

Adenosine deaminase (ADA) deficiency in humans results in a severe combined immunodeficiency (SCID). This immunodeficiency is associated with severe disturbances in purine metabolism that are thought to mediate lymphotoxicity. The recent generation of ADA-deficient ($ADA^{-/-}$) mice has enabled the in vivo examination of mechanisms that may underlie the SCID resulting from ADA deficiency. We demonstrate severe depletion of T and B lymphocytes and defects in T and B cell development in $ADA^{-/-}$ mice. T cell apoptosis was abundant in thymi of$ADA^{-/-}$ mice, but no increase in apoptosis was detected in the spleen and lymph nodes of these animals, suggesting that the defect is specific to developing thymocytes. Studies of mature T cells recovered from spleens of $ADA^{-/-}$ mice revealed that ADA deficiency is accompanied by TCR activation defects of T cells in vivo. Furthermore, ex vivo experiments on $ADA^{-/-}$ T cells demonstrated that elevated adenosine is responsible for this abnormal TCR signaling. These findings suggest that the metabolic disturbances seen in $ADA^{-/-}$ mice affect various signaling pathways that regulate thymocyte survival and function. Experiments with thymocytes ex vivo confirmed that ADA deficiency reduces tyrosine phosphorylation of TCR-associated signaling molecules and blocks TCR-triggered calcium increases.

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Adenosine deaminase deficiency increases thymic apoptosis and causes defective T cell receptor signaling

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Adenosine deaminase (ADA) deficiency in humans results in a severe combined immunodeficiency (SCID). This immunodeficiency is associated with severe disturbances in purine metabolism that are thought to mediate lymphotoxicity. The recent generation of ADA-deficient (*ADA*$^{-/-}$) mice has enabled the in vivo examination of mechanisms that may underlie the SCID resulting from ADA deficiency. We demonstrate severe depletion of T and B lymphocytes and defects in T and B cell development in *ADA*$^{-/-}$ mice. T cell apoptosis was abundant in thymi of *ADA*$^{-/-}$ mice, but no increase in apoptosis was detected in the spleen and lymph nodes of these animals, suggesting that the defect is specific to developing thymocytes. Studies of mature T cells recovered from spleens of *ADA*$^{-/-}$ mice revealed that ADA deficiency is accompanied by TCR activation defects of T cells in vivo. Furthermore, ex vivo experiments on *ADA*$^{-/-}$ T cells demonstrated that elevated adenosine is responsible for this abnormal TCR signaling. These findings suggest that the metabolic disturbances seen in *ADA*$^{-/-}$ mice affect various signaling pathways that regulate thymocyte survival and function. Experiments with thymocytes ex vivo confirmed that ADA deficiency reduces tyrosine phosphorylation of TCR-associated signaling molecules and blocks TCR-triggered calcium increases.

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Introduction

Inherited immunodeficiencies were recognized as distinct human disease entities several decades ago. In subsequent years, the analysis of naturally occurring immunodeficiencies yielded considerable insight regarding the composition and function of the immune system. In 1972, adenosine deaminase (ADA) deficiency became the first of the immunodeficiency diseases for which a specific molecular defect was identified (1). The importance of ADA for immune function was revealed unexpectedly when it was discovered that the enzyme was absent in several immunodeficient patients being considered for bone marrow transplantation therapy. Within a few years of this discovery, it was determined that a deficiency in another purine catabolic enzyme, purine nucleoside phosphorylase (PNP), also results in a serious immunodeficiency disease in humans, although in this case the lymphotoxic impact is largely restricted to T cells (2). Together, these findings underscore the importance of these two metabolically linked purine catabolic enzymes to proper immune development and/or function. Because of its higher prevalence and more profound immunological impact, more attention has been focused on ADA deficiency.

ADA deficiency is one of the most severe of the immunodeficiencies in humans and is associated with a severe depletion of all three major categories of lymphocytes, T cells, B cells, and NK cells (3). ADA deficiency is the second-most prevalent form of severe combined immunodeficiency disease (SCID), accounting for approximately 20% of the group (3, 4). Without intervention, affected individuals die from overwhelming opportunistic infections within the first few months of life. Available evidence suggests that the metabolic basis for ADA-deficient immunodeficiency is related to the impact of its substrates, adenosine and 2′-deoxyadenosine (reviewed in ref. 4). However, the exact molecular mechanisms of lymphotoxicity remain obscure, and the relative contribution of intracellular lymphotoxic versus signaling properties of extracellular adenosine on ADA SCID have not been sufficiently explored. Adenosine may function as an extracellular signal transducer that mediates a variety of physiological effects by binding to G protein–coupled receptors present on the surface of target cells (5). In this regard, the accumulation of adenosine and the activation of adenosine receptors known to be present on T cells (6) may lead to impaired T cell development or function (7, 8). Alternatively, 2′-deoxyadenosine may act as a cytotoxic metabolite that can mediate its effects directly at the nucleoside level or after conversion to dATP. Potential downstream consequences of elevated 2′-deoxyadenosine and/or dATP include: (a) interference with cellular transmethylation reactions (9), (b) inter-

ruption of deoxynucleotide synthesis (10, 11), and (c) activation of apoptosis (12). Thus, ADA deficiency may provoke a variety of consequences either through metabolic disturbances caused by elevated 2′-deoxyadenosine or cell signaling disturbances caused by elevated adenosine. Efforts to identify the metabolic and molecular basis for the immune dysfunction associated with ADA deficiency are motivated in part by the desire to identify pharmacological targets that could be used to control the immune system. This could be of considerable benefit in treating conditions where lymphocytes proliferate or function abnormally, such as lymphocytic leukemias and autoimmune disease. To facilitate the analysis of the SCID phenotype associated with ADA deficiency, we have recently created a mouse model of ADA-deficient SCID (13).

A two-stage genetic engineering strategy was used to produce ADA-deficient mice that retain many of the features associated with ADA deficiency in humans (13, 14). These mice develop a combined immunodeficiency that is associated with profound disturbances in purine metabolism (13). The ability to analyze tissues from ADA-deficient mice, an experimental approach not possible with ADA-deficient humans, enabled us to make novel immunological and metabolic observations. Analysis of tissue-associated metabolic disturbances in these mice revealed that absolute increases in ADA substrates are greatest in the thymus and spleen (15), suggesting that metabolic disturbances in these major immune organs play a major role in the ensuing immunodeficiency (13). Here we report a detailed analysis of the impact of ADA deficiency on intrathymic T cell development and on T cell receptor–mediated (TCR-mediated) signaling in the periphery. Our results show that ADA deficiency is associated with enhanced thymocyte apoptosis and impaired TCR signaling.

Methods

Animals. ADA-deficient mice ($ADA^{-/-}$) were developed using a two-stage genetic engineering strategy (13). The development of this mouse model was based on previous studies that suggested that ADA expression in trophoblast cells of the placenta is critical for fetal development in the mouse (14). Thus, to generate completely ADA-deficient postnatal mice, an ADA minigene that targeted expression specifically to the trophoblast lineage, was introduced onto the ADA-deficient background (13). This was accomplished by intercrossing mice carrying the trophoblast-specific ADA minigene with mice heterozygous for the null *ada* allele. Subsequent intercrosses yielded litters that contained mice harboring the ADA minigene that were also homozygous for the null *ada* allele. Given that the regulatory elements used targeted ADA expression only to trophoblasts (14), once born, and with the loss of the placenta, ADA enzymatic activity was not observed in any of the tissues examined in $ADA^{-/-}$ mice (13).

Mice were bred and maintained in the NIH pathogen-free animal facilities. $ADA^{-/-}$ mice were first identified using ADA zymogram analysis of blood samples, and then genotypes were conclusively determined by Southern blot analysis of tail DNA (Figure 1a). Measurements of ADA activity in blood samples from littermates born to heterozygous ($ADA^{+/-}$) intercrosses were performed by zymogram analysis (13) using a modified protocol of Knudsen et al. (16). Zymogram analysis was conducted with G493 agarose gels in a temperature-controlled electrophoresis chamber (Innovative Chemistry, Marshfield, Massachusetts, USA). Heat inactivation and ADA inhibitor treatment (coformycin; Calbiochem-Novabiochem International, San Diego, California, USA) prevented ADA band visualization in control samples, thereby verifying the ADA enzymatic activity assays in zymogram gels. ADA wild-type ($ADA^{+/+}$) and heterozygous ($ADA^{+/-}$) littermates were used as controls for most studies, as no differences between heterozygous and wild-type mice were found in preliminary experiments (data not shown).

Cells and medium. Thymocytes or spleen cells were isolated from 3-week-old mice and either directly labeled with mAb's or incubated in RPMI-1640 (Biofluids Inc., Rockville, Maryland, USA) supplemented with 5% dialyzed FCS (heat inactivated) and 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 1 mM HEPES, nonessential amino acids, and 5×10^{-5} M 2β-mercaptoethanol. Where indicated, cells were incubated in ADA-free serum-free media (Life Technologies Inc., Gaithersburg, Maryland, USA).

Reagents. R-phycoerythrin-conjugated (R-PE-conjugated) rat anti-mouse anti-CD25 mAb, anti-mouse CD69, and FITC-conjugated rat anti-mouse CD8a mAb's, as well as Cy-Chrome–conjugated CD4, were purchased from PharMingen (San Diego, California, USA). Rat anti-mouse CD4 mAb's conjugated with RED-613 fluorochrome were purchased from Life Technologies Inc. Adenosine was prepared freshly as 20 mM stock solution. Adenosine and the ADA inhibitor EHNA were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). 2′-Deoxyadenosine was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Coformycin was purchased from Calbiochem-Novabiochem International. Annexin V-FITC was purchased from BioWhittaker Inc. (Walkersville, Maryland, USA).

Flow cytometry. Single-cell suspensions of murine thymocytes were generated by standard procedures, and cells were either directly analyzed or cultured in 96-well plates (0.5×10^6 – 1×10^6 cells per well) as described elsewhere (17). After incubation for 16–18 hours or as indicated, cells were harvested and analyzed by flow cytometry. Flow cytometric quantitation of live, apoptotic, and dead cells was done according to a modified flow cytometry procedure (18) as described previously (17). The effects of adenosine on thymocytes were studied after incubating thymocytes ex vivo in short-term culture. Determining the status of cells (live, dead, or apoptotic) was based on gating of cells by their size (side scatter and forward scatter),

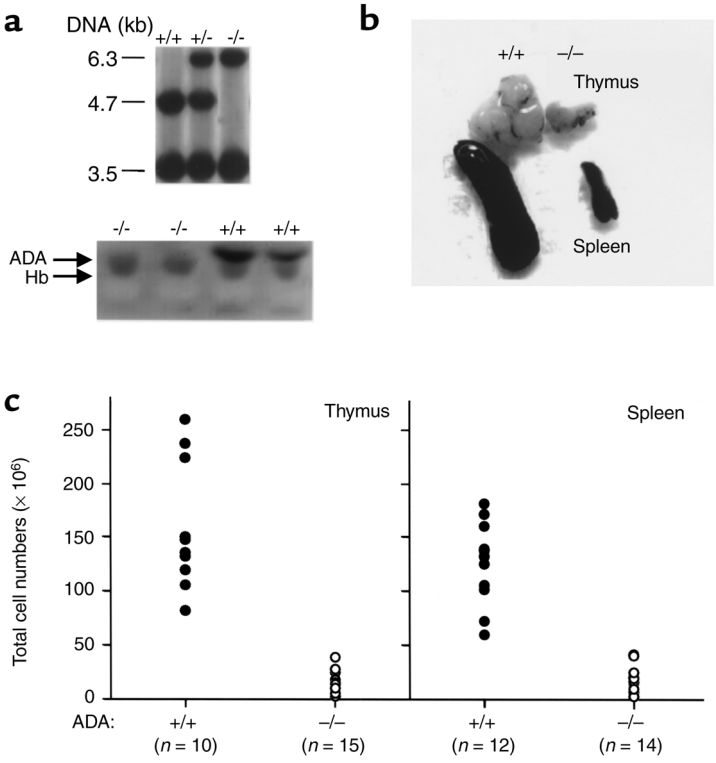

Figure 1
Demonstration of extensive cell death in thymus but not in peripheral lymphoid organs of *ADA*$^{-/-}$ mice. (**a**) Genetic and biochemical evidence of ADA deficiency in screening for *ADA*$^{+/+}$, *ADA*$^{+/-}$, and *ADA*$^{-/-}$ mice. Littermates of ADA heterozygous mice were first analyzed by zymogram assay to identify *ADA*$^{-/-}$ mice, and then tail DNA samples were analyzed by Southern blot as described in ref. 11. Arrowheads on the zymogram indicate position of ADA and hemoglobin (internal control). (**b**) Comparison of spleens and thymi from *ADA*$^{+/+}$ and *ADA*$^{-/-}$ littermates. ×3. (**c**) Decreased cellularity of lymphoid organs of *ADA*$^{-/-}$ mice. *n*, number of animals analyzed.

plasma membrane integrity (PI staining), and redistribution of plasma membrane phosphatidylserine (Annexin V–binding). The Annexin V binding assay was done as described previously (19). Briefly, 0.6×10^6 – 1×10^6 cells were resuspended in 100 μl of buffer containing 10 mM HEPES (pH 7.3), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$ and incubated with 0.3 μg/ml of FITC-conjugated Annexin V and 5 μg/ml propidium iodide for 15 minutes. After incubation, samples were diluted four times with buffer containing 1.8 mM $CaCl_2$ and were analyzed by FACScan (Becton Dickinson Immunocytometry Systems, San Jose, California, USA).

Statistical analysis of triplicate sample measurements was performed using the StatView statistic program (Abacus-Concepts Inc., Berkeley, California, USA). Standard deviations of triplicate measurements within the same experiment were lower than 1%. Flow cytometry data acquisition and analysis were done on FACScan using FACScan research software and CellQuest programs (both, Becton Dickinson Immunocytometry Systems).

Ca^{2+} measurements. For measurements of Ca^{2+} flux, freshly isolated thymocytes were preloaded with indo-1 (final 3 μM) in Ca^{2+} buffer (1% FCS, 10 mM HEPES, HBSS) for 30 minutes at 37°C and were washed and analyzed on a FACSVantage flow cytometer (Becton Dickinson Immunocytometry Systems) equipped with an argon laser tuned to 488 nm and a krypton laser tuned to 360 nm.

Indo-1 fluorescence was analyzed at 390/20 and 530/20 nm for bound and free probe as was described elsewhere (20, 21). The percentage of cells that responded by an increase in intracellular Ca^{2+} after stimulation with Concanavalin A (2.5 μg/ml) (Vector Laboratories, Burlingame, California, USA) was determined using CellQuest software program.

In situ analysis of apoptosis in thymocytes. Frozen tissue preparations and apoptosis analysis in the spleen, thymus, and lymph nodes of *ADA*$^{+/+}$ and *ADA*$^{-/-}$ mice were performed according to procedures used by Molecular Histology Inc. (Gaithersburg, Maryland, USA). The detection of apoptotic cells by in situ staining of single-strand breaks in nuclear DNA was preformed according to protocols described previously (22, 23).

Immunoblotting with anti-phosphotyrosine mAb's. After isolation, cells were incubated for 10 minutes at 37°C, washed once with PBS, and placed in lysis buffer containing 1% NP 40, 10 mM Tris-HCl (pH 7.2), 140 mM NaCl, 2 mM EDTA, 5 mM iodoacetamide, 1 mM Na_3VO_4 (Sigma Chemical Co.), and complete protease inhibitor cocktail (Boehringer Mannheim GmbH (Mannheim, Germany) for 25 minutes on ice. After removal of nuclear debris by centrifugation, the resultant supernatants were subjected to immunoprecipitation and analyzed by immunoblotting. ZAP-70 immunoprecipitation SDS-PAGE and immunoblotting were performed as described previously (24). The following antibodies were used in these experiments: rabbit antiserum to ZAP-70 (31); 4G10, a mouse mAb to phosphotyrosine (Upstate Biotechnology Inc., Lake Placid, New York, USA) and peroxidase-linked goat antibodies to mouse and rabbit Ig (Bio-Rad Laboratories Inc., Richmond, California, USA).

Results

Extensive apoptosis occurs in the thymus but not in lymph nodes or spleens of ADA-deficient mice. ADA deficiency in humans and mice results in lymphopenia (3, 13). Previously, the status of the immune system of *ADA*$^{-/-}$ mice was analyzed at 2 weeks of age, and, although a lymphopenia was seen, it was not severe (13). We therefore analyzed the immune status of *ADA*$^{-/-}$ mice at 3 weeks of age to determine whether the immune status was more severe at these later stages. Figure 1 demonstrates that there was a pronounced decrease in the size of immune organs in *ADA*$^{-/-}$ mice (Figure 1a). Furthermore, analysis of cells isolated from *ADA*$^{-/-}$ thymi, spleens, and lymph nodes demonstrated a large reduction in the absolute numbers of cells found in *ADA*$^{-/-}$ immune organs (Figures 1, b and c). These data demonstrated that a severe lymphopenia was present in *ADA*$^{-/-}$ mice at 3 weeks of age.

The metabolic consequences associated with ADA deficiency have been demonstrated to induce apoptosis in lymphocytes in vitro (25, 26) suggesting that apoptosis may play an important role in the lymphopenia associated with ADA deficiency. To determine whether apoptosis was increased in lymphoid tissues in association with ADA deficiency in vivo, we analyzed lymphocytes in immune organs of *ADA*$^{-/-}$ mice for the presence of live, apoptotic, and dead cells (Figure 2). Analysis of side scatter versus forward scatter in thymi from *ADA*$^{-/-}$ and *ADA*$^{+/+}$ littermates revealed an increase in the number of dead cells among immature T cells isolated from *ADA*$^{-/-}$ thymi (Figure 2a, top), whereas the proportions of dead cells among mature T cells found in the peripheral lymphoid organs (spleen and lymph nodes) were similar between *ADA*$^{+/+}$ and *ADA*$^{-/-}$ mice (data not shown). The double staining of cells with PI and Annexin V reveals an increase in apoptosis in the thymus of *ADA*$^{-/-}$ mice (Figure 2a, bottom), but not in peripheral lymph organs (data not shown). These data suggest that there was an increase in apoptosis in thymi of *ADA*$^{-/-}$ mice,

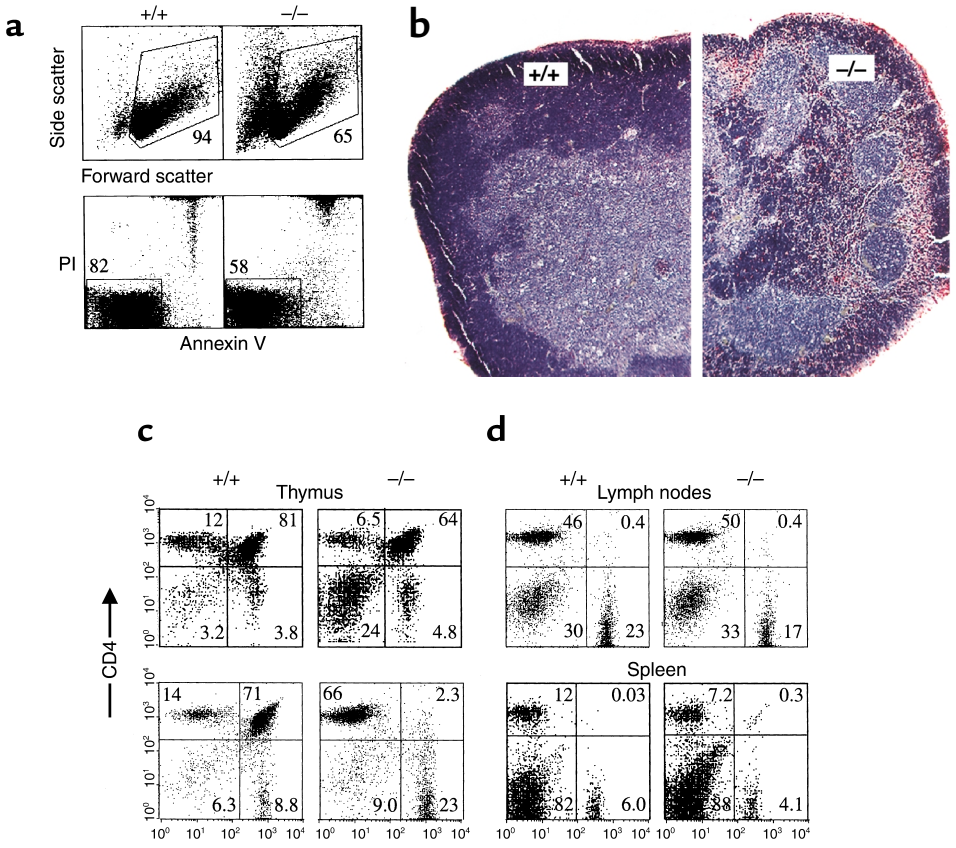

Figure 2

Increased apoptosis in the thymus but not in the lymph nodes or spleens of *ADA*$^{-/-}$ mice. (**a**) Top: Side scatter versus forward scatter evaluation of proportion of dead cells in thymus of *ADA*$^{+/+}$ and *ADA*$^{-/-}$ littermates. Numbers indicate percentage of live cells in the lymphoid organ. Bottom: Propidium iodide and Annexin V–aided evaluation of proportion of dead cells in thymus of *ADA*$^{+/+}$ and *ADA*$^{-/-}$ littermates. Numbers indicate the percentage of live cells in the lower left gate estimated using both Annexin V and PI cell death flow cytometry assay as described in Methods. (**b**) Cytochemical demonstration of extensive clusters of apoptotic cells in thymus of *ADA*$^{-/-}$ but not of *ADA*$^{+/+}$ littermates. Apoptotic cells are shown in red. The frozen tissue preparations and apoptosis detection were performed as described in Methods. (**c**) Flow cytometry demonstration of thymocyte distribution in thymi of *ADA*$^{-/-}$ and *ADA*$^{+/+}$ littermates. (**d**) Flow cytometry demonstration of normal subset distributions in lymph nodes and spleens of *ADA*$^{-/-}$ mice compared with organs of *ADA*$^{+/+}$ littermates. Numbers indicate the percentage of live cells in different subsets estimated as described in Methods.

but not in secondary immune organs such as the spleen and lymph nodes.

To determine which cells were undergoing apoptosis in *ADA*$^{-/-}$ thymi, the localization of DNA strand breaks in thymus compartments was analyzed by histochemistry in situ. As expected, there was an abundance of apoptosis in *ADA*$^{-/-}$ thymi (Figure 2b, red). Increased clusters of apoptotic cells were located predominantly in the cortical-medullary boundary (Figure 2b). These findings suggest that immature T cells that accumulate in this area of the thymus are susceptible to the consequences of ADA deficiency.

ADA-deficient mice show abnormal distribution of thymocytes but a relatively normal peripheral T cell distribution. The increased apoptosis at the cortical-medullary boundary of *ADA*$^{-/-}$ thymi prompted us to examine the distribution of lymphocyte populations in this organ. As expected, the majority (70–80%) of cells recovered from *ADA*$^{+/+}$ thymi were CD4$^+$CD8$^+$ double-positive cells (Figure 2c). In contrast, the reduction in the double-positive subset was observed in *ADA*$^{-/-}$ littermates. Although total cell numbers in *ADA*$^{-/-}$ thymi were always dramatically reduced, the decrease in the proportion of DP cell ranged from moderate (Figure 2c, top) to very severe in extreme cases (Figure 2c, bottom). There was a significant increase in the proportion of DN cells (24% vs. 3.2% in normal thymus), whereas the proportion of DP thymocytes was only moderately decreased (Figure 2c). Analysis of DN thymocytes for the expression of CD44 and CD25 demonstrated no significant differences in *ADA*$^{+/+}$ and *ADA*$^{-/-}$ mice (data not shown). Interestingly, both CD4$^+$ and CD8$^+$ single-positive cells were found in *ADA*$^{-/-}$ thymi, suggesting that a fraction of *ADA*$^{-/-}$ lymphocytes manage to proceed through intrathymic T cell development (Figure 2c). Consistent with this observation, relatively normal distributions of CD4$^+$ and CD8$^+$ lymphocytes were seen in *ADA*$^{-/-}$ lymph nodes and spleens (Figure 2d).

ADA deficiency is accompanied by the presence of T cells with abnormal expression of cell surface markers. Phenotypic changes in peripheral CD4$^+$ and CD8$^+$ T cells from spleens of *ADA*$^{-/-}$ mice were revealed when ex vivo splenocytes or lymph nodes were analyzed for the expression of different cell surface markers (Figure 3). Surface expression of CD69 and CD44 was increased and CD62L expression was decreased in both CD4$^+$ and CD8$^+$ peripheral *ADA*$^{-/-}$ T cells. These data suggest that *ADA*$^{-/-}$ T cells that manage to escape apoptosis in the thymus are abnormal. Interestingly, expression of CD43 was increased in *ADA*$^{-/-}$ CD4$^+$ T cells, but was somewhat decreased in CD8$^+$ cells.

ADA deficiency is accompanied by changes in the distribution of peripheral B cells among marginal, follicular, and newly formed zones in the spleen. To determine the status of B cells in 3-week-old *ADA*$^{-/-}$ mice, splenic B cells were analyzed using triple staining with mAb's to B220, CD21 and CD23 (27) and to B220, IgM and IgD (data not shown). In these experiments, we found fewer mature B cells among splenocytes mice than in marginal and follicular zones of spleen of *ADA*$^{-/-}$ mice, as was indicated by CD21 versus CD23 analysis (Figure 4b) of B220$^+$ splenocytes (Figure 4a).

ADA deficiency is accompanied by TCR activation defects of T cells in vivo. Previously, we demonstrated that adenosine can inhibit T cell activation and expansion in vitro (6, 7, 28), suggesting that elevated extracellular adenosine levels observed in conditions of ADA deficiency may contribute to the mechanisms of the depletion and function of peripheral lymphocytes by interfering with TCR signaling. To test this hypothesis in vivo, T cell activation was analyzed in *ADA*$^{-/-}$ mice that exhibited marked increases in circulating adenosine (13). T cell activation in vivo was accomplished by the injection of anti-TCR/CD3 mAb that is known to activate T cells both in vitro (29) and in vivo (30). *ADA*$^{+/+}$ and *ADA*$^{-/-}$ littermates were injected with anti-TCR/CD3 mAb, and 16 hours later, T cells were harvested from spleens and T cell activation was monitored by examining the upregulation of CD25 and

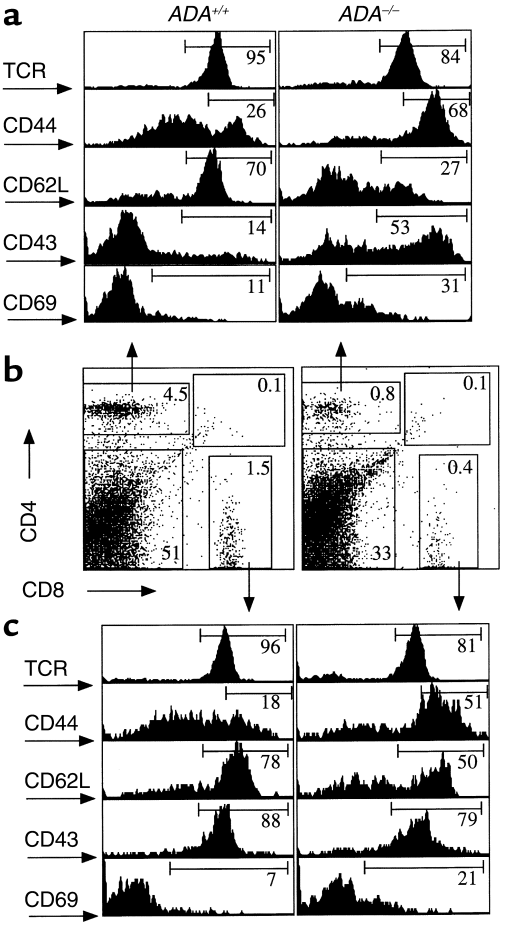

Figure 3
Changes in phenotype of peripheral CD4$^+$ and CD8$^+$ T cells from spleens of *ADA*$^{-/-}$ mice. Splenocytes of *ADA*$^{-/-}$ and *ADA*$^{+/+}$ littermates were analyzed by three-color flow cytometry. Expression of different cell surface markers on CD4$^+$ cells (**a**) and CD8$^+$ cells (**c**) were analyzed by using CD4/CD8 gates as indicated by arrows in **b**. Numbers indicate proportions of cell from total cell population (**b**) or among gated cells (**a** and **c**).

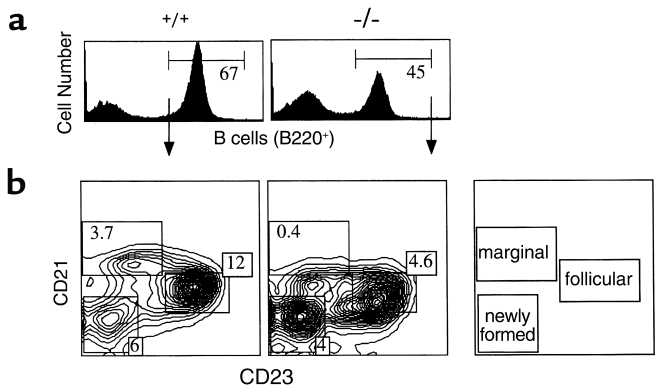

Figure 4
Changes in phenotype of peripheral B cells from spleens of *ADA*$^{-/-}$ mice. Splenocytes of *ADA*$^{-/-}$ and *ADA*$^{+/+}$ littermates were analyzed by three-color flow cytometry as described in Methods and indicated by arrows. (**a**) Evaluation of B220^{+} B cells in *ADA*$^{-/-}$ and *ADA*$^{+/+}$ littermates. Numbers represent percentage of B cells from total splenocytes. (**b**) Distribution of B cells among marginal, follicular, and newly formed zones of spleen in *ADA*$^{-/-}$ versus *ADA*$^{+/+}$ mice. Numbers indicate percentage of cells in different zones. Cartoon illustrates location of different B cell subsets according to staining with mAb to CD21 and CD23.

CD69 by flow cytometry. Initial dose-response experiments in control animals suggested that a dose of 5 μg of anti-TCR/CD3 mAb was optimal for stimulation of CD25 and CD69 upregulation on T cells (data not shown). This dose was therefore used to assess T cell activation in *ADA*$^{-/-}$ mice (Figure 5). Pronounced differences in T cell activation were seen in both CD8^{+} (Figure 5a) and CD4^{+} (Figure 5b) T cells from *ADA*$^{-/-}$ mice. CD25 activation in CD8^{+} T cells was up to 17 times stronger in *ADA*$^{+/+}$ mice than in *ADA*$^{-/-}$ littermates, and TCR-triggered upregulation of CD69 on CD8^{+} T cells was approximately two times stronger in *ADA*$^{+/+}$ mice than in *ADA*$^{-/-}$ mice (Figure 5a). Similar differences were seen with CD4^{+} T cells (Figure 5b). These results demonstrated that the activation of both CD8^{+} and CD4^{+} T cells was strongly inhibited in *ADA*$^{-/-}$ mice compared with control *ADA*$^{+/+}$ littermates.

Inhibition of TCR-triggered activation of *ADA*$^{+/+}$ T cells in vitro requires the addition of ADA inhibitors, suggesting the need to prevent the rapid degradation of adenosine by ADA (7). To test this interpretation, we incubated ex vivo T cells from *ADA*$^{+/+}$ an *ADA*$^{-/-}$ mice with anti-CD3 mAb in vitro in the absence of an ADA inhibitor (Figure 6). Results demonstrated that adenosine markedly inhibited TCR-triggered activation in *ADA*$^{-/-}$ but not *ADA*$^{+/+}$ T cells. It is shown that control incubations (0 μg of anti-CD3 mAb; Figure 6, top) had no effect on CD25 and CD69 expression in *ADA*$^{+/+}$ or *ADA*$^{-/-}$ T cells. In contrast, activation of T cells with anti-CD3 mAb (5 μg; Figure 6, bottom) resulted in dramatic upregulation of both CD25 and CD69 activation markers. This activation was observed in both *ADA*$^{+/+}$ and *ADA*$^{-/-}$ T cells, although strong activation of *ADA*$^{-/-}$ T cells (0.2– 64% of CD25-expressing cells) was somewhat lower than activation of *ADA*$^{+/+}$ cells (2.6–92% of CD25-expressing cells).

Importantly, no ADA inhibitors were required to observe the block of TCR-triggered CD25 and CD69 upregulation by adenosine in *ADA*$^{-/-}$ T cells, as the proportion of CD25^{+} cells decreased from 64% to 2.1% in the presence of adenosine alone. In contrast, *ADA*$^{+/+}$ T cells were affected very little (92% vs. 88% of CD25^{+} cells in the presence of adenosine). Similar observations of the effects of ADA deficiency and extracellular adenosine were made in evaluations of the CD69 activation marker. These findings demonstrated that activation of *ADA*$^{-/-}$ T cells are indeed susceptible to inhibition by elevated adenosine that occurs in ADA-deficient environments, and that effects of ADA inhibitors were indeed due to their ADA-inhibiting

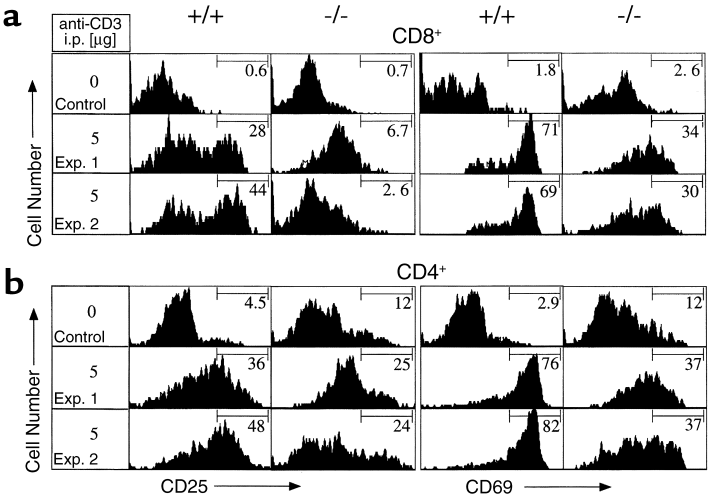

Figure 5
Inhibited TCR-triggered activation of T cells in an ADA-deficient environment in vivo. *ADA*$^{+/+}$ and *ADA*$^{-/-}$ littermates were injected intraperitoneally (i.p.) with PBS (control) or anti-CD3 mAb; 16 hours later, spleens were harvested and analyzed by flow cytometry for expression of T cell activation markers as described in Methods. Representative results of two (Exp. 1 and Exp. 2) of more than ten similar experiments using more than 20 pairs of littermates of *ADA*$^{-/-}$ and *ADA*$^{+/+}$ mice are presented. (**a**) Comparison of TCR-triggered upregulation of CD25 and CD69 surface antigens on CD8^{+} T cells in an *ADA*$^{-/-}$ or *ADA*$^{+/+}$ in vivo environment after injection of 5 μg of anti-CD3 mAb. (**b**) Comparison of TCR-triggered upregulation of CD25 and CD69 surface antigens on CD4^{+} T cells in an ADA-deficient or ADA-containing in vivo environment after injection of 5 μg of anti-CD3 mAb.

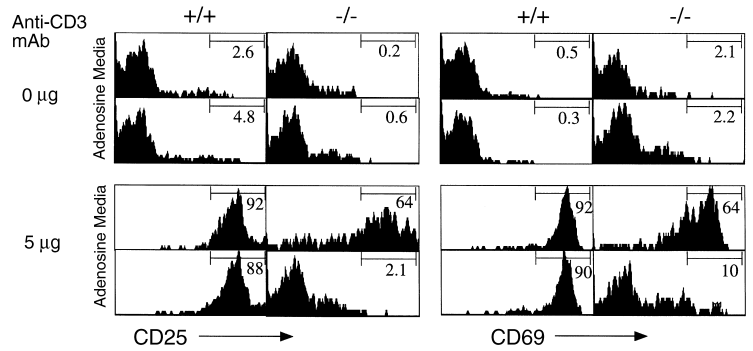

Figure 6
Effect of adenosine on TCR-triggered upregulation of CD25 and CD69 activation markers in vitro. Ex vivo spleen cells from *ADA*$^{-/-}$ and *ADA*$^{+/+}$ littermates were incubated in 96-well plates with immobilized anti-CD3 mAb (5 µg/ml mAb) or with serum-free, ADA-free media alone (0 µg/ml mAb) in the presence or absence of adenosine (100 µM); 16 hours later, TCR-triggered upregulation of the T cell activation markers CD69 and CD25 was evaluated by flow cytometry.

activities. Another notable observation made in these studies was that a much higher proportion of *ADA*$^{-/-}$ T cells were activated in vitro by anti-CD3 mAb than after anti-CD3 mAb incubation in vivo (cf. Figures 5 and 6). For example, about 64% of CD4$^+$ T cells from *ADA*$^{-/-}$ mice were activated by anti-CD3 mAb in vitro, whereas only 25–36% of *ADA*$^{-/-}$ CD4$^+$ T cells were activated by anti-CD3 mAb in vivo. These findings suggested that the degree of inhibition of TCR-triggered activation of T cells in vivo was likely related to the severity of the metabolic disturbances seen. Collectively, these studies present the first demonstration that TCR-triggered activation of T cells was inhibited in an ADA-deficient environment in vivo.

Adenosine, but not 2′-deoxyadenosine, inhibits TCR-triggered signaling as reflected in the rescue of thymocytes from TCR-induced apoptosis. Adenosine and 2′-deoxyadenosine levels are elevated in ADA-deficient humans and mice (4, 13). We proposed recently that TCR-inhibiting properties of extracellular adenosine signaling might contribute to the mechanisms of pathogenesis in ADA SCID (7). The availability of *ADA*$^{-/-}$ mice enabled us to directly test one of the predictions of that model without using ADA inhibitors. Thus, to determine the relative impact of adenosine and 2′-deoxyadenosine on TCR-triggered signaling we selected an assay in which the TCR-inhibiting effects of adenosine would be reflected in protection of thymocytes from cell death. Such a "cell survival" read-out of the experiment on the effects of adenosine was expected to simplify the interpretation of our observations, in that it would allow us to discount possible toxic effects of intracellular adenosine and of its metabolites. Figure 7 demonstrates the effects of exogenous adenosine or 2′-deoxyadenosine on T cells during their incubation with anti-CD3 mAb in vitro. Incubation of *ADA*$^{+/+}$ thymocytes in vitro resulted in some "spontaneous" cell death (Figure 7a), and the addition of anti-CD3 mAb increased the amount of cell death seen. Addition of adenosine did not affect the outcome of CD3-induced apoptosis in *ADA*$^{+/+}$ thymocytes, most likely because the added adenosine was degraded by ADA. In contrast, the same concentration of exogenously added adenosine was very efficient in blocking TCR-signaling, and protected *ADA*$^{-/-}$ thymocytes from both spontaneous and TCR-triggered apoptosis (Figure 7a, –/– graph).

In contrast to adenosine, we found that 2′-deoxyadenosine (Figure 7b) not only did not inhibit TCR-induced apoptosis of thymocytes, but it strongly

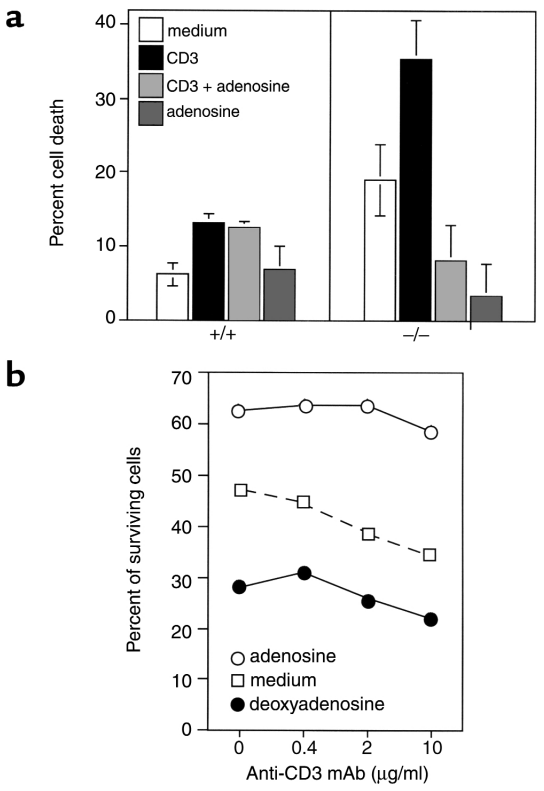

Figure 7
Extracellular adenosine inhibits TCR-induced signaling in thymocytes. (**a**) Adenosine alone inhibits TCR-triggered apoptosis in *ADA*$^{-/-}$ but not in normal *ADA*$^{+/+}$ thymocytes. (**b**) Demonstration of opposite effects of adenosine and 2′-deoxyadenosine on spontaneous and TCR-triggered apoptosis in *ADA*$^{-/-}$ thymocytes. Thymocytes from wild-type or *ADA*$^{-/-}$ mice were incubated for 16 hours in 96-well plates with apoptosis-inducing immobilized anti-CD3 mAb in the presence or absence of added adenosine (100 µM) or 2′-deoxyadenosine (100 µM). The effect of adenosine and 2′-deoxyadenosine on thymocyte survival (proportion of live cells) was evaluated using Annexin V assay as described in the Methods.

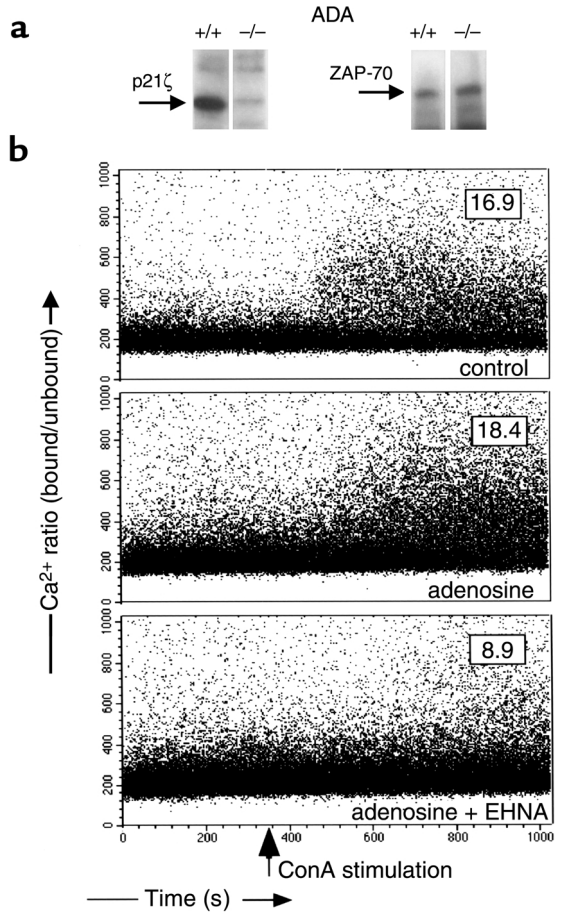

Figure 8
ADA deficiency is accompanied with defects in TCR signaling pathways in vivo and in vitro. (**a**) Partial phosphorylation of CD3 ζ chain is notably reduced in nonactivated ex vivo thymocytes from *ADA*$^{-/-}$ mice, whereas ZAP-70 levels are shown to be the same in parallel samples of immunoprecipitates from ex vivo thymocytes harvested from *ADA*$^{-/-}$ and *ADA*$^{+/+}$ littermates. (**b**) Intracellular Ca^{2+} mobilization upon ConA stimulation is inhibited by adenosine in normal thymocytes in the presence of the ADA inhibitor EHNA. Thymocytes from *ADA*$^{+/+}$ or *ADA*$^{-/-}$ mice were used for isolation of ZAP-70 immunocomplexes followed by immunoblotting analysis of TCR ζ chain phosphorylation with an anti phosphotyrosine mAb as described in Methods. Anti ZAP-70 mAb's were used in control immunoblotting. For measurements of calcium flux *ADA*$^{+/+}$ thymocytes were preloaded with indo-1 and analyzed on a FACSVantage flow cytometer as described in Methods. The percentages of cells that increase intracellular calcium after stimulation with Concanavalin A are shown on the graphs. Adenosine (100 μM) alone or in combination with EHNA (10 μM) was added a minute before the ConA stimulation. Arrow indicates time of injection of T cell–activating stimuli.

exacerbated both spontaneous and TCR-triggered apoptosis. These data confirmed the well-documented observations of 2′-deoxyadenosine lymphotoxicity and suggested that 2′-deoxyadenosine was not involved in the inhibition of TCR-triggered events in surviving T cells in *ADA*$^{-/-}$ mice or in experiments in vitro.

ADA deficiency is accompanied by defects in TCR signaling pathways in vivo and in vitro. Proper TCR signaling plays a critical role in survival and development of thymocytes in vivo. It has been extensively shown that TCR molecules trigger a complex series of early and late biochemical events including tyrosine phosphorylation and Ca^{2+} mobilization (20, 21, 24, 31,). It was therefore important to determine whether the inhibitory function of adenosine in *ADA*$^{-/-}$ thymocytes was the result of inhibition in TCR-triggered biochemical events in vivo. A number of studies have demonstrated the importance of the TCR ζ chain phosphorylation for initiating of TCR cell signaling. In primary thymic and lymph node T cells, the TCR ζ chain appears to be preassociated with ZAP-70, which plays a central role in the T cell signal transduction (32, 33). Using approaches developed in studies of phosphotyrosine phosphorylation of TCR-associated signaling CD3 chains (24, 31), we compared the level of ζ p21 tyrosine phosphorylation in ex vivo thymocytes from *ADA*$^{-/-}$ and *ADA*$^{+/+}$ control littermates. Partial phosphorylation of CD3 ζ chain was notably reduced in nonactivated ex vivo thymocytes from *ADA*$^{-/-}$ mice (Figure 8a), suggesting that an ADA-deficient environment in vivo is not conducive to normal levels of TCR signaling. An internal biochemical control was provided by blotting of the same samples with anti–ZAP-70 mAb's to show similar amount of ZAP-70 in immunoprecipitated samples from both *ADA*$^{-/-}$ and *ADA*$^{+/+}$ littermates (Figure 8a).

Observations of inhibited TCR-CD3 chain tyrosine phosphorylation most likely reflects inhibited TCR signaling in the in vivo thymic environment of *ADA*$^{-/-}$ mice and predicts that other early events of TCR signaling (e.g., intracellular Ca^{2+} accumulation) may be inhibited by adenosine in conditions of ADA deficiency. This was examined by in vitro studies of intracellular Ca^{2+} accumulation in activated *ADA*$^{+/+}$ thymocytes in the presence of the ADA inhibitor EHNA (Figure 8b). The addition of adenosine or EHNA alone did not have an inhibitory effect on Ca^{2+} levels in this short term Ca^{2+} measurement assay (Figure 8b and data not shown). However, the addition of adenosine in the presence of EHNA inhibited Ca^{2+} accumulation (Figure 8b, bottom). Adenosine alone was able to inhibit Ca^{2+} mobilization in a similar experiment with *ADA*$^{-/-}$ thymocytes, thereby confirming that effects of EHNA were due to its interactions with ADA (data not shown). Thus, early events of TCR-mediated signaling in thymocytes are inhibited in an ADA-deficient environment.

Discussion

ADA deficiency in humans results in an immunodeficiency characterized by a severe reduction in T, B, and NK cells (3). The generation of *ADA*$^{-/-}$ mice (13) has allowed the examination of molecular mechanisms that underlie the immunodeficiency seen in ADA-deficient humans. In this study, we demonstrated that there was a severe lymphopenia in *ADA*$^{-/-}$ mice at 3 weeks of age. Furthermore, there was an abundance of apoptosis occurring in thymi of *ADA*$^{-/-}$ mice, but not in the spleen and lymph nodes of these animals. These findings provided evidence that the metabolic disturbances associat-

ed with ADA deficiency induced apoptosis in developing thymocytes in vivo. Peripheral T and B cells were abnormal in *ADA*$^{-/-}$ mice as reflected in the expression of cell surface markers and localization in different zones of lymphoid organs. In addition, mature T cells recovered from spleens of *ADA*$^{-/-}$ mice were defective with regard to their ability to functionally signal through the TCR. Finally, ex vivo experiments on *ADA*$^{-/-}$ T cells demonstrated that elevated adenosine was responsible for inhibited and abnormal TCR signaling.

The immunodeficiency seen in ADA-deficient patients can be severe (3, 4), suggesting mature lymphocytes and/or their precursors are sensitive to the metabolic disturbances associated with ADA deficiency. Correspondingly, studies have demonstrated that apoptosis can be induced in lymphocytes after direct exposure to adenosine or 2′-deoxyadenosine in vitro (25, 26). Our ability to analyze apoptosis in the immune system of *ADA*$^{-/-}$ mice has provided the first in vivo evidence that apoptosis is abundant in *ADA*$^{-/-}$ thymi. Most of the apoptosis seen in *ADA*$^{-/-}$ thymi was localized to the cortical-medullary boundary, a location where CD4$^+$CD8$^+$ double-positive thymocytes should accumulate. Consistent with these findings, analysis of thymocyte populations in these and previous studies (13) show a reduction of in the number of CD4$^+$CD8$^+$ thymocytes in *ADA*$^{-/-}$ mice. Similar findings have been described in mice treated with the ADA inhibitor deoxycoformycin (26). Interestingly, increased apoptosis was not noticed in histological studies and lymphocytes collected from *ADA*$^{-/-}$ spleens or lymph nodes (data not shown), suggesting that mature lymphocytes are less sensitive to the cytotoxic properties of adenosine or 2′-deoxyadenosine. Indeed, in vitro observations suggest that mature CD4$^+$ T cells do not undergo apoptosis in response to 2′-deoxyadenosine exposure (26). These data suggest that CD4$^+$CD8$^+$ double-positive thymocytes, or their immediate precursors, are sensitive to the metabolic disturbances seen in ADA deficiency, and the depletion of this population is likely to account for the reduction of mature T cells found in the periphery of ADA-deficient mice (13) and humans (3, 4). Individual variations in ADA levels during early *ADA*$^{-/-}$ mice development may account for the severity of apoptotic and phenotypic changes in thymus. Drastic reduction with almost disappearance of double-positive thymocytes in some litters (Figure 2c) after 3 weeks of postnatal development could be the result of low level expression of ADA minigene in the progeny from particular breeders, and the exact mechanism of it is under study (S.G. Apasov, work in progress).

Both adenosine and 2′-deoxyadenosine exhibit potent biologic activities that may impact T cell development and function. Adenosine serves as a extracellular signaling molecule by engaging G protein–coupled receptors on the surface of target cells (5), whereas 2′-deoxyadenosine is cytotoxic to cells through mechanisms that include the inhibition of cellular transmethylation reactions (34), the disruption of cellular growth (10, 11) or differentiation (35) and the activation of apoptosis (12). The large amount of apoptosis detected in the thymi of *ADA*$^{-/-}$ mice, recent studies in fetal thymic organ cultures (36) and the observation of 2′-deoxyadenosine accumulation in the thymus of *ADA*$^{-/-}$ mice (13, 15) support the view that ADA substrates generated from apoptotic thymocytes contribute to the cytotoxicity and phenotypic outcomes of ADA deficiency.

Additionally, our findings suggest that the metabolic disturbances associated with ADA deficiency impact both intrathymic T cell development and mature T cell functions. We had shown previously that T cells treated with a combination of ADA inhibitors and adenosine in vitro exhibit defects in TCR induced activation (6, 7, 8, 28). In this study, we extended these observations to show similar effect on *ADA*$^{-/-}$ T cells, which challenged with anti-CD3 mAb in vivo. Both CD4$^+$ and CD8$^+$ cells in *ADA*$^{-/-}$ mice had a decreased capacity to upregulate CD25 and CD69 expression in response to anti-CD3 mAb in vivo. In important control experiments, these cells were activated by anti-CD3 mAb in vitro similarly to wild type, whereas added adenosine was inhibitory to the activation of *ADA*$^{-/-}$ but not *ADA*$^{+/+}$ T cells. These findings suggest that the increased levels of adenosine found in peripheral lymphoid organs such as the spleen (13) can affect normal T cell signaling. Disruptions in normal T cell signaling could in turn affect the function of existing peripheral T cells in *ADA*$^{-/-}$ mice, by preventing T cell activation during an immune response.

These observations also suggest that the T cell depletion in ADA SCID may be at least partially due to blocks in TCR-driven thymocyte maturation by adenosine, as well as to direct apoptotic effects of intracellular adenosine, 2′-deoxyadenosine, and dATP. Thus, we propose that there may be at least two alternative or simultaneously operating mechanisms of T cell depletion: (a) intracellular lymphotoxicity of intracellularly accumulated adenosine, 2′-deoxyadenosine, and dATP (4, 9, 10) and (b) inhibition of TCR signaling and, hence, the inhibition/block of TCR-driven processes of T cell selection (7, 8). Both of these mechanisms have potential to cause T cell depletion in our studies in *ADA*$^{-/-}$ mice and in patients with ADA SCID.

The challenging task for testing the signaling model of ADA SCID was in demonstrating that the inhibition of TCR signaling by extracellular adenosine was not due to translocation of exogenously added adenosine into the cytoplasm followed by intracellular lymphotoxicity of adenosine catabolites. One way to exclude considerations of lymphotoxicity of exogenously added adenosine was to use an assay system in which adenosine was rescuing T cells from death by virtue of acting as a TCR-antagonizing ligand. Accordingly, we used an assay in which incubation of TCR-transgenic thymocytes with antigenic peptides and antigen-presenting cells resulted in their activation followed by their apoptosis (7). The demonstrated rescue of thymocytes from

TCR-induced death (7) by adenosine was evidence of the ability of extracellular adenosine to antagonize the TCR-signaling and TCR-driven processes in conditions of ADA deficiency without being toxic in vitro. It was, however, still possible, that there was not enough extracellular adenosine to block T cell activation in an ADA-deficient environment in vivo. This issue could not be addressed without *ADA*$^{-/-}$ mice, and in this report, evidence in support of this was provided by showing that there was a strong inhibition of a T cell response in *ADA*$^{-/-}$ mice after injection with activating anti-TCR-CD3 mAb (Figure 5). Taken together, these findings suggest that the metabolic disturbances seen in ADA-deficient mice are capable of affecting various signaling pathways and intracellular processes that regulate thymocyte survival and function.

In conclusion, we have demonstrated that ADA deficiency in mice severely effects intrathymic T cell development and TCR-mediated signaling in the periphery. Our results show that ADA deficiency is associated with impaired TCR signaling and enhanced thymocyte apoptosis. This observations suggest a model that inhibition of TCR signaling in developing thymocytes may block positive selection resulting in the increase of cell death among immature thymocytes. This, in turn, will increase the accumulation of extracellular adenosine and 2′-deoxyadenosine facilitating apoptotic process in the thymus. Although the exact mechanisms by which the ADA substrates adenosine and 2′-deoxyadenosine promote immunodeficiency as a whole are still not clear, the data presented in this in vivo model have provided insight that may not have been previously appreciated. The observation that 2′-deoxyadenosine accumulation is greatest in the thymus (15), and the visualization of massive apoptosis in this organ, provide strong correlative evidence that this metabolite mediates apoptosis in this organ. The widespread accumulation of adenosine, together with the observation that adenosine inhibits TCR-triggered activation events in immature and mature T cells, suggest that systemic accumulations of adenosine may prevent the TCR signaling–dependent thymocyte differentiation and normal function of peripheral T cells. This model lends itself well to additional genetic manipulations that will help to determine the specific mechanisms involved in these processes. In addition, the ability to perform ADA enzyme therapy and gene therapy in this model (37) will provide a means to further assess the lymphoid specificity of this disease and the efficacy of these novel therapies.

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