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Restoration of Adenosine Deaminase-Deficient Human Thymocyte Development In Vitro by Inhibition of Deoxynucleoside Kinases$^{1,2}$

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Mutations in the gene encoding adenosine deaminase (ADA), a purine metabolic enzyme that catalyzes the deamination of both adenosine (Ado) and deoxyadenosine (dAdo), cause severe combined immunodeficiency in humans (1). ADA-deficient humans have a pronounced deficiency of T cells with variable numbers of B and NK cells, and they die early in life of infections unless treated with enzyme replacement therapy, bone marrow transplantation, or gene therapy (reviewed in Refs. 2, 3). The paucity of lymphoid cells in thymuses from ADA-deficient patients (5, 6), the ADA substrate dAdo became the prime candidate for causing lymphoid toxicity. However, little is known about the specific stage or stages of lymphocyte development that might be sensitive to a lack of ADA or of specific mechanism(s) responsible for the lack of lymphocyte differentiation and/or proliferation in ADA deficiency because of the difficulty in obtaining thymus samples from these rare patients and the lack of convenient culture systems for studying human thymocyte development.

The successive stages of human thymocyte maturation can be categorized as double negative (DN), double positive (DP), or single positive (SP) according to the presence or absence of the CD4 and CD8 coreceptors. Immature human DN thymocytes are categorized as double negative (DN), double positive (DP), or single positive (SP) (7). The transition from the DN to DP stage occurs via a CD4 immature single-positive (CD4 ISP) stage (8), accompanied by TCR gene rearrangements and the first developmental checkpoint, β-selection. β-selection involves the pairing of the TCRβ protein with the pre-TCR protein to produce a membrane-localized pre-TCR that signals survival, expansion, and allelic exclusion. β-selection occurs over a range of phenotypic stages from CD34+CD1a+ cells to DP cells (9, 10). Successful β-selection leads to the expression of the αβTCR, followed by positive and negative selection, and development into mature CD4 and CD8 SP T cells (7, 11). Thymuses from ADA-deficient patients have not been analyzed for their proportions of these normal human thymocyte subsets, nor has human thymocyte development been studied in vitro under ADA-deficient conditions.

Mouse models for ADA deficiency have, however, yielded important clues to the pathogenesis of the immunodeficiency. In an in vitro model for murine thymocyte development, fetal thymic organ culture (FTOC) (12–14), inhibition of ADA led to a marked reduction in lymphocyte development secondary to the accumulation
of intracellular dATP and mitochondria-dependent apoptosis. Few thymocytes survived past the point of β-selection, most likely because most thymocytes fail to pass this developmental checkpoint and die by apoptosis providing a source of ADA substrates. Thymocyte differentiation and proliferation could be rescued by the adenosine kinase (AK) inhibitor 5′-amino-5′-deoxyadenosine (5′A5′dAdo) (15), since the main route of dAdo phosphorylation in murine cells is via AK (16, 17).

It is well known that the pathway of dAdo phosphorylation in human thymocytes differs from that in the mouse (17, 18), making it essential that models of human thymocyte development be utilized to understand the pathogenesis of ADA deficiency. Despite many early studies, there is still contention regarding which deoxynucleoside kinase is primarily responsible for dAdo phosphorylation in human thymocytes. Deoxyctydine kinase (dCK) is usually thought to be the major cytoplasmic enzyme responsible for phosphorylation of deoxyctydine, deoxyguanosine, and deoxyadenosine (19). Human AK primarily utilizes Ado as a substrate, but it can also phosphorylate dAdo when it is present in high enough concentration (20, 21). Differential roles for these enzymes depend on experimental context. In cell extracts, dCK was the primary dAdo-phosphorylating enzyme (22), while in intact human T and B lymphoblastoid cells, AK activity was more important (21, 23). The relative roles of each of these nucleoside kinases in human ADA-deficient thymocyte development have yet to be investigated.

Herein we report the use of human/mouse chimeric fetal thymic organ culture (hu/moFTOC) to study the consequences of ADA deficiency upon human thymocyte development. In this model system, human CD34+ thymic precursor cells reconstitute murine thymocyte-depleted fetal thymic lobes and develop normally through positive selection (24, 25). We show that cell expansion and differentiation are severely impaired when ADA is inhibited. We also demonstrate the importance of both dCK and AK in the accumulation of dATP and induction of apoptosis in developing thymocytes, evidenced by the normalization of dATP levels and rescue of ADA-deficient thymocyte development in the presence of multiple deoxynucleoside kinase inhibitors. Our studies lend further support to the idea that dATP accumulation is the primary cause of toxicity to developing human thymocytes under conditions of ADA deficiency, and they underscore differences between murine and human development. Our findings also suggest that treatment of ADA-deficient patients with a combination of nucleoside kinase inhibitors may be an alternative therapeutic strategy to prevent metabolic toxicity to developing thymocytes.

Materials and Methods

Mice

C57BL/6 mice (The Jackson Laboratory) were bred in our animal facility under specific pathogen-free conditions and in compliance with the Oklahoma Medical Research Foundation Institutional Animal Care and Use Committee specifications.

Drugs and reagents

The specific ADA inhibitor 2′-deoxycoformycin (dCF) (26) was obtained from SuperGen. 5′A5′dAdo, dAdo, 2′-deoxyctydine (dCyd), and all other chemical reagents were obtained from Sigma-Aldrich.

Abs, thymocyte cell preparation, and immunofluorescent staining

Abs used were: FITC anti-CD3α, allopurinolcin anti-CD34, PE anti-γδTcR, FITC anti-CD8, and PE anti-αβTcR (BD Pharmingen); PE and PE-Texas Red anti-CD8α, PE-Cy5, PE-Cy5.5 and allopurinolcin anti-CD4, PE anti-CD34, FITC and PE anti-γδTcR, FITC and allopurinolcin anti-CD3, and FITC anti-γδTcR (Caltag Laboratories); PE anti-CD8β (Sero-otec); and PE anti-TCRβ (Anbell). Matched isotype control Abs were purchased from all of the above sources.

Human neonatal thymus was obtained from children (ages 1 day to 4 years) undergoing cardiac surgery at Children’s Hospital in Oklahoma City, OK, under protocols approved by the Institutional Review Boards of both the University of Oklahoma Health Sciences Center and the Oklahoma Medical Research Foundation. Thymocyte cell suspensions were made by forcing the thymic tissue pieces through a nylon filter. Human CD34+ thymocytes were enriched with anti-CD34 magnetic beads (Dy- nal Biotech) and sorted if needed to obtain ≥95% purity for use in hu/moFTOC. Staining for intracellular TCRβ (TcRβ) expression was performed by fixing cells with 1% formaldehyde and permeabilizing with 0.5% saponin (10). Data were collected using an LSR II or FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences). FITC annexin V (Caltag Laboratories) was used following the manufacturer’s instructions after surface staining with anti-CD4 and anti-CD8 Abs. Propidium iodide staining for cell cycle assessment was performed as described (10) and analyzed using the Dead-Jett-Fox cell cycle analysis algorithm in FlowJo analysis software (Tree Star).

Chimeric human/mouse fetal thymic organ culture

Hu/moFTOCs were performed as previously described (27). Briefly, fetal thymic lobes from timed pregnant C57BL/6 mice at day 15 of gestation (plag day = day 0) were placed in FTOC for 4–6 days with 1.35 mM 2′-deoxyguanosine to deplete endogenous murine thymocytes. The lobes were then washed in complete medium to remove deoxyguanosine and placed in Terasaki wells with 25 μl of Yssel’s medium (28) supplemented with 5% FCS/2% human serum (Yssel’s complete medium) containing 1–2 × 10⁶ human thymocyte precursors. After 2 days of hanging drop culture, the lobes were transferred to a standard FTOC format in Yssel’s complete medium and cultured with and without 5 μM dCF. 5′A5′dAdo was used at 5 μM, and dCyd was used at 50 μM with culture medium replenished daily. Cultures were incubated for 1–3 wk and then harvested by forcing the thymic lobes through a 70-μm nylon mesh. Thymocytes were counted in trypan blue to assess viability, stained with Abs, and analyzed by flow cytometry or processed for dATP analysis. Suspension cultures for dATP accumulation were performed in Yssel’s complete medium with 5 μM dCF and the indicated concentration of dAdo. Cultures were harvested at 20 h and 77 °C, and then cells were counted and processed for dATP analysis by HPLC.

TCR gene rearrangement analysis

Thymocyte genomic DNA from either control or dCF-treated hu/moFTOCs was prepared using the Puregene kit (Gentra Systems). TCR gene nomenclature is that of the IMGT (International ImMunoGeneTics database; imgt.cines.fr). Vβ20.1 rearrangements to either Jβ1.1–1.6 or Jβ2.1–2.3 were amplified from 200 ng of genomic DNA using JumpStart Taq Plus DNA polymerase (Sigma-Aldrich) (10). A portion of the RAG2 gene was amplified to normalize for input DNA in the PCR reactions. Primers used were: RAG2 forward, 5′-GGGTTTGTTGAGCTCAGTTGAATAG; Vβ20.1 reverse, 5′-GGTTTGTTGAGCTCAGTTGAATAG; Vβ20.1 forward, 5′-GATCGAATTCTCTCCCTTCGAATT; Jβ11.6 reverse, 5′-AC CCTGACCTCCTGCTTTCACTC; Jβ22.3 reverse, 5′-GGTGCTCCTGGCCCAAATACCTGGGT

HPLC analysis of dATP from thymocytes and S-adenosylhomocysteine (SAH) hydrolase activity assays

Single-cell suspensions were prepared at 4°C from 30 to 60 lobes of hu/moFTOC treated with dCF with and without deoxynucleoside kinase inhibitors for 8–14 days. Aliquots were removed for cell counts and immunophenotyping, and then the remainder of the cells were immediately pelleted and resuspended in 0.5 ml of ice-cold 6% MeOH. The cells were extracted at −80°C for ≥18 h, then analyzed for dATP content by HPLC as previously described (29). For SAH hydrolase activity measurements, thymocytes were washed with cold PBS and resuspended in 10 mM Tris-HCl (pH 7.5) plus protease inhibitors. Cell extracts were prepared by three rounds of freeze-thaw lysis, followed by clarification by centrifugation. SAH hydrolase enzyme activity was determined by measuring the formation of SAH from homocysteine and radiolabeled adenosine as described previously (29).

Results

Inhibition of human thymocyte development under ADA-deficient conditions in hu/moFTOC

To assess the impact of ADA deficiency on developing human thymocytes, hu/moFTOCs were seeded with CD34+ DN thymocytes and allowed to develop for various time periods up to 3 wk.
in the presence and absence of the specific tight-binding ADA inhibitor dCF. DP thymocyte development was efficient in control cultures (Fig. 1A, left panels) but was dramatically impaired by the addition of dCF (Fig. 1A, right panels). A broad range of developmental inhibition was observed (from <5% to >50% DP thymocytes) as illustrated by data from three independent experiments. The decreased percentage of DP thymocytes was always accompanied by increased percentages of CD4 ISP and DN thymocytes, indicating an inhibition of differentiation or a lack of survival and/or expansion of more mature thymocytes (or a combination of these). While cell yields progressively increased in control conditions, lack of ADA activity caused a cumulative decrease in cell yields, which were typically inhibited by ≥95% relative to controls at 3 wk (Fig. 1B). Note that while the percentages of DP thymocytes varied from culture to culture, the absolute numbers of DP thymocytes were consistently decreased by ≥90% in dCF-treated hu/moFTOCs. Similar results were obtained when starting the cultures with the earliest thymocyte progenitors, CD34+ CD1a- cells, or with more mature populations such as CD4 ISPs or early double positives (EDPs), although the kinetics of DP formation differed with the maturity of the starting population (data not shown). Similar results were also obtained when dCF was included in the hanging drop cultures.

Later stages of thymocyte development are most severely impacted by ADA deficiency

Percentages of $\alpha\beta^+$ thymocytes were always severely reduced in ADA-inhibited cultures, while percentages of $\gamma\delta^+$ thymocytes were increased after 3 wk of culture (Fig. 2A). An examination of the absolute numbers of thymocyte subpopulations revealed that both $\alpha\beta^+$ and $\gamma\delta^+$ thymocytes were decreased (Fig. 2B). The increased percentages of $\gamma\delta^+$ thymocytes likely reflects their development in an environment with very little overall cell expansion. Fig. 2B also shows that the earliest stage of thymocyte development (DN) is least impacted by ADA deficiency.

**Human thymocyte differentiation is not blocked at a discrete stage in the absence of ADA**

Because the yield of more mature populations of thymocytes was so severely compromised under ADA-deficient conditions, we investigated whether this might be the result of a block at a particular differentiation step. Key early steps in the differentiation of $\alpha\beta$ lineage T cells involve the processes of TCR$\beta$ recombination and $\beta$-selection. We first examined whether TCR$\beta$ gene rearrangements might be inhibited in thymocytes developing in ADA-deficient conditions. $V_{\beta}20.1$, known as $V\beta2$ in previous nomenclature, was chosen for this analysis because it is a commonly used $V\beta$ gene segment in the human T cell repertoire (30). Fig. 3A shows that complete $V \rightarrow D1$ TCR$\beta$ gene rearrangements to both D-J gene clusters, as analyzed by PCR, were readily detectable in both control and dCF-treated hu/moFTOCs and were comparable in amounts to the rearrangements detected in total human thymocyte DNA. Therefore, the absence of ADA activity did not lead to an inability to rearrange TCR$\beta$ genes.

Next, we assessed the expression of both intracellular TCR$\beta$ and CD8$\beta$ in ADA-inhibited hu/moFTOC as a way to gauge the efficiency of the $\beta$-selection process and later differentiation events. Staining thymocytes for TCR$\beta^{inc}$ expression is one way to evaluate whether an in-frame TCR$\beta$ gene rearrangement has occurred. DP cells from control and dCF-treated cultures both showed similar high levels of TCR$\beta^{inc}$ expression (Fig. 3B), indicating that this requirement for $\beta$-selection was met in ADA-deficient cultures. Secondary to $\beta$-selection, EDP CD8$\alpha$-expressing thymocytes up-regulate CD8$\beta$ expression (31). To determine whether this transition occurred normally in ADA-inhibited hu/moFTOC, DP cells...
were stained for the expression of cell surface CD8α and CD8β. Even though percentages of DP cells were greatly inhibited relative to controls, approximately the same percentages of cells that were CD4+/CD8α+ were also CD8α+/CD8β+, indicating that development of EDP to true DP cells was not blocked in dCF-treated cultures (Fig. 3C). These results demonstrate that the differentiation of cells in ADA-inhibited dCF was not blocked at a discrete stage of development, as assessed starting from the CD34+ thymocyte stage. Therefore, the selective loss of more mature thymocyte populations during ADA-inhibited development is likely due to increased cell death and/or lack of cell expansion.

ADA inhibition results in induction of apoptosis, inhibition of cellular proliferation, and accumulation of intracellular dATP

ADA-deficient patients have elevated levels of the ADA substrates dAdo and Ado in the plasma, and dATP in RBCs (32). dATP can induce mitochondrial cytochrome c release (33) and is a part of the apoptosome (34, 35). Therefore, we examined the induction of apoptosis in thymocytes from ADA-deficient hu/moFTOC by annexin V staining. The CD4/CD8 phenotype at 11 days showed the typical reduction in DP percentages (Fig. 4A), and annexin V staining showed that ADA-inhibited cultures had nearly 3-fold higher percentages of cells dying compared to controls (Fig. 4B). These results demonstrate that the differentiation of cells in ADA-inhibited dCF was not blocked at a discrete stage of development, as assessed starting from the CD34+ thymocyte stage. Therefore, the selective loss of more mature thymocyte populations during ADA-inhibited development is likely due to increased cell death and/or lack of cell expansion.

FIGURE 3. Human thymocyte development in the absence of ADA is not blocked at a particular stage. A, Cells from hu/moFTOC with and without 5 μM dCF were harvested at 3 wk and genomic DNA was prepared. TCR gene rearrangements from human Vβ20.1 to either the Jβ1 or the Jβ2 region were analyzed by PCR and agarose gel electrophoresis. Genomic DNA from total human thymocytes (Thymus) or human fibroblast cells (A549) were used as positive and negative controls, respectively. B and C, Cells from cultures were harvested as in A, stained with Abs to CD4, CD8α, and CD8β (C), and analyzed using flow cytometry. Following the surface stains, the cells were stained for TCRβ+ (B) as described in Materials and Methods. The dot plots shown are gated on all live cells, and the histograms for TCRβ+ expression are shown gated on the DP cells from each condition. Isotype control staining is indicated by the black line histograms.

FIGURE 4. Induction of apoptosis, inhibition of cell proliferation, and accumulation of dATP in cells from ADA-inhibited hu/moFTOC. A–D, Hu/moFTOCs were incubated for 11 days with and without 5 μM dCF; then cells were harvested and stained. One representative experiment out of three is shown. A, Surface staining with anti-CD4 and anti-CD8 of total thymocytes. B, Annexin V staining on total thymocytes from A. C, CD4/CD8 phenotype of annexin V+ cells in A. D, Cell cycle analysis of thymocytes from 11-day culture stained with propidium iodide as described in Materials and Methods. E, Cells from 11-day hu/moFTOCs were processed for dATP analysis as described in Materials and Methods. dATP is expressed in pmol/million cells (mean ± SD, n = 4). F, Total and CD34+ thymocytes were incubated with 5 μM dCF with and without dAdo for 20 h in suspension culture; then cells were harvested and analyzed for dATP as in E (mean ± SD, n = 4).
percentages of apoptotic cells compared with medium controls (Fig. 4B). Caspase activation assessed with a fluorogenic caspase substrate that can bind to all activated caspases (FAM-VAD-fmk) showed 4-fold elevated percentages of activated caspases in ADA-inhibited cells (data not shown). In both dCF-treated and control cultures, DP cells were the primary population undergoing apoptosis (Fig. 4C), although higher fractions of less mature cells (DNs and CD4 ISPs) were also seen in the dCF-treated cultures.

Because cell yields were so dramatically inhibited in the absence of ADA activity, we next analyzed the proliferative capacity of the developing thymocytes. Propidium iodide staining at 11 days of culture showed that nearly two-thirds fewer cells were in cycle in dCF-treated cultures compared with medium controls (Fig. 4D). Ki-67 staining supported this result, as fewer cells were in cycle in all phenotypic compartments in ADA-inhibited cultures (data not shown).

We next asked whether the induction of apoptosis in ADA-deficient hu/moFTOC might be caused by the accumulation of dATP as was found in ADA-deficient murine FTOCs (12). HPLC analyses revealed that dATP accumulated to high levels (>15-fold over control) in 11-day cultures of ADA-inhibited hu/moFTOC (p < 0.0001; Fig. 4E). dATP could be detected as early as 8 days of culture, peaked at days 10–12, and declined thereafter to ~2-fold over control at 3 wk of culture (data not shown).

Hu/moFTOCs were initiated with immature CD34+ DN thymocytes whose deoxynucleoside metabolism is known to change with differentiation (36). Because DN cells were least affected by ADA inhibition (Fig. 2B), we compared the ability of these cells to accumulate dATP to that of total thymocytes (~80% DP cells). CD34+ thymocytes and total thymocytes were incubated in suspension cultures with dCF and varying concentrations of dAdo for 20 h, followed by harvest of the cells for dATP analysis. On average, CD34+ thymocytes accumulated 10-fold as much dATP per cell as total thymocytes, even at relatively low dAdo concentrations (<10 μM) (Fig. 4F). Therefore, the relative survival advantage of immature thymocytes cannot be explained by an inability to accumulate dATP. In fact, these high levels of dATP did not induce any more caspase activation or apoptosis than was observed in cultures of total thymocytes at comparable concentrations of dAdo (data not shown).

Rescue of human thymocyte development in ADA-deficient hu/moFTOC by inhibition of deoxynucleoside kinases

The intracellular dATP in ADA-deficient thymocytes is derived from the ADA substrate dAdo by the action of cellular deoxynucleoside kinases. To attempt to prevent the accumulation of dATP and rescue thymocyte development in ADA-deficient hu/moFTOC, we inhibited the activity of dCK and AK, either separately or in combination (Fig. 5). AK activity can be efficiently inhibited by a structural analog of dAdo, 5′A5′dAdo, which has nanomolar affinity for AK (15), while micromolar concentrations of dCyd competitively inhibit the phosphorylation of dAdo by dCK and promote feedback inhibition of dCK by the phosphorylated product, dCTP (37). We first assessed the impact on development at an early stage (10–12 days) when robust development and expansion of DP thymocytes occur in control cultures. The addition of the inhibitors alone, either singly or in combination, did not significantly perturb the expansion and development of thymocytes in hu/moFTOC (Fig. 5A and data not shown). The addition of either dCyd or 5′A5′dAdo to the dCK-treated cultures did not significantly restore the development of DP thymocytes (Fig. 5A). Although the cell yields increased slightly with the addition of either dCyd or 5′A5′dAdo during dCF treatment (Fig. 5B), these increases were not statistically significant. However, when both dCyd and 5′A5′dAdo were used, a complete rescue of DP thymocyte development and cell expansion was observed (Fig. 5, A and B). Cells from dCF- and dCF+5′A5′dAdo-treated cultures had similar amounts of accumulated dATP, at levels ~15-fold over control cultures (Fig. 5C). Interestingly, cells from dCF+ dCyd-treated cultures produced significantly higher levels of accumulated dATP, consistently ~2.5-fold higher than cells from cultures treated with dCF alone (p = 0.003). However, there was no further decrease in cell yield (Fig. 5B) or increase in apoptosis (data not shown). This is consistent with the dATP concentration having peaked over a “threshold” value needed to induce the effects observed with inhibition of ADA, above which there is little further effect. The use of both kinase inhibitors (dCyd + 5′A5′dAdo) in dCF-treated cultures corrected both the phenotype and cell yields of the cultures, as well as normalized dATP levels to nearly those of the control.

We next asked whether dCyd + 5′A5′dAdo could also rescue longer (3 wk) ADA-inhibited cultures. Fig. 5D shows an assessment of αβTCR and γδTCR thymocyte development by flow cytometry. Low percentages of αβ+ thymocytes and higher percentages of γδ+ thymocytes were observed in dCF-treated and dCF +
of SAH hydrolase plays a significant role in the toxicity to development. It is therefore unlikely that inhibition as expected and remained inhibited in cultures that had been rescued. SAH hydrolase activity was inhibited under ADA-deficient conditions in cells from ADA-inhibited and rescued cultures (Fig. 6). SAH hydrolase activity is inhibited by dAdo in an active-site dependent manner. Inhibition of both deoxynucleoside kinases in long-term hu/moFTOCs could effectively restore the development of αβ and γδ thymocytes. Although the cell yields were not completely rescued (~40% of controls at 3 wk), the absolute numbers of both DP and αβ thymocytes showed substantial (50-fold) improvements. In summary, our data suggest that both dCK and AK play a role in the phosphorylation of the ADA substrate dAdo, leading to dATP accumulation in hu/moFTOC. Further, our data show that inhibition of both dCK and AK normalized dATP levels in hu/moFTOC and overcame the effect of ADA deficiency, allowing mature T cell development.

**Rescue of ADA-deficient hu/moFTOC by deoxynucleoside kinase inhibition does not prevent inhibition of SAH hydrolase**

SAH hydrolase is a high-affinity adenosine-binding protein that catalyzes the reversible reaction, Ado + 1-homocysteine ⇄ SAH. In the absence of ADA, two factors contribute to dysregulation of SAH metabolism. First, the equilibrium constant of this reaction favors SAH formation, and increased Ado levels potentiate the accumulation of SAH, a product and potent inhibitor of numerous S-adenosyl methionine-dependent methyl transferase reactions. This inhibition of transmethylation reactions likely accounts for nucleotide-independent adenosine toxicity to lymphoblasts (38) and has been considered as a potential contributing mechanism to the immunodeficiency seen in ADA-deficient patients. Second, SAH hydrolase activity is inhibited by dAdo in an active-site directed, “suicide-like” process (39). To assess the role of SAH hydrolase inhibition in the development of human thymocytes in ADA-deficient hu/moFTOC, we measured SAH hydrolase activity in cells from ADA-inhibited and rescued cultures (Fig. 6). SAH hydrolase activity was inhibited under ADA-deficient conditions as expected and remained inhibited in cultures that had been rescued with kinase inhibitors. It is therefore unlikely that inhibition of SAH hydrolase plays a significant role in the toxicity to developing thymocytes in ADA-deficient hu/moFTOC.

**Discussion**

In this study, we provide the first description of the impact of ADA deficiency on human thymocyte development using an in vivo-like model for ADA deficiency, dCF-treated hu/moFTOC. Hu/moFTOC is an ideal model system for investigating human thymocyte development because the early events in T cell differentiation take place spontaneously without the need for exogenous cytokines or growth factors. Furthermore, the early murine fetal thymic lobe lacks a thick capsule and is small enough to permit optimal exchange of gases and nutrients when cultured at the air/medium interface (40). Relatively little has been known about how human thymocytes develop in an ADA-deficient environment. Studies on patient RBCs or measurements of metabolites from patient fluids constituted the bulk of information known about purine metabolism in ADA-deficient patients. ADA-inhibited hu/moFTOC approximates the metabolic and developmental environment of an ADA-deficient thymus, since toxic metabolites are generated in situ, rather than being provided exogenously, as in previous models (41). Although we cannot be certain that the concentrations of toxic metabolites are exactly the same as in the thymuses of ADA-deficient patients, we show that the addition of the ADA inhibitor dCF to hu/moFTOC causes a cumulative loss of mature thymocytes, accompanied by induction of apoptosis, accumulation of intracellular dATP, and inhibition of proliferation, but not a block in differentiation. Importantly, we show that normal thymocyte development, including the generation of TCR+ cells, is restored when the activities of AK and dCK are inhibited during culture.

The effects of ADA inhibition in hu/moFTOC were cumulative, with the loss of mature thymocytes more pronounced with longer cultures (Figs. 1B, 2B, and data not shown). Differentiation was not blocked at a discrete developmental stage, evidenced by detection of complete TCR rearrangements and expression of DP differentiation markers (TCRβ+ and CD8β; Fig. 3). It is unknown whether the earliest multipotent thymic progenitors or stem cells are affected by ADA deficiency. Cultures initiated with pure sorted CD34+CD1a+ thymocytes (the earliest thymocyte progenitors) readily generated CD4 ISPs in the presence of dCF and gave results that were indistinguishable from those initiated with CD34+ DN cells (primarily CD1a+, data not shown). Although these data do not address this issue directly, we think it is unlikely that stem cell differentiation is impaired by ADA deficiency since enzyme replacement therapy provides an effective treatment for ADA-deficient patients. It may be that reduced cell division (Fig. 4D) is the more significant factor in the lack of thymocyte development in ADA-deficient thymus. Histological studies of ADA-deficient thymic tissue from fetuses revealed a small thymic tissue rudiment devoid of lymphoid cells or normal thymic architecture (4). Lack of thymocyte expansion would lead to a thymus devoid of lymphocytes, without which a normal thymic architecture fails to develop.

Although the absolute numbers of DP thymocytes were always inhibited by >90% relative to controls, the proportions of DP thymocytes in ADA-deficient hu/moFTOC varied from culture to culture (Fig. 1A). While some of this is likely due to human sample heterogeneity, most could be explained by the nonsynchronous occurrence of β-selection in human thymocyte development. Thymocytes that fail β-selection provide the first major source of ADA substrate to other thymocytes through degradation of apoptotic cell DNA. Cells that fail β-selection at an early stage (i.e., at the CD34+CD1a+ or CD4 ISP stage) generate substrate (dAdo) that can be taken up by adjacent cells and converted to dATP, inducing additional apoptosis before significant numbers of thymocytes reach the DP stage. Cultures where β-selection occurs earlier would yield low percentages of DP thymocytes (Fig. 1A, top panel). However, thymocytes with a delayed β-selection process, occurring mainly in the DP stage, would not generate toxic levels.
of ADA substrates until many DP thymocytes had already been produced, leading to higher percentages of surviving DP thymocytes (Fig. 1A, bottom panel). Our studies of TCRβ expression in isolated ex vivo thymocytes suggest that the point of β-selection varies substantially from thymus to thymus (10) and provide an explanation for the variable proportions of surviving DP thymocytes in ADA-deficient hu/moFTOC.

Cells from ADA-inhibited cultures had high levels of induced apoptosis accompanied by accumulation of intracellular dATP (Fig. 4). A consistent finding was that immature thymocytes were relatively resistant to ADA inhibition (Fig. 2B). Our initial hypothesis was that they had less ability to accumulate dATP. However, data in Fig. 4F show that CD34+ thymocytes accumulate ~10-fold higher levels of dATP per cell than do total thymocytes (which are ~80% DP thymocytes), likely a consequence of their relatively high deoxynucleoside kinase activities, coupled with low activity of enzymes (nucleotidases) that can carry out the reverse reaction (36, 42). Furthermore, CD34+ thymocytes do not undergo dATP-induced apoptosis in suspension cultures to a greater degree than more mature cells (43), perhaps because their greater size decreases the actual intracellular concentration of dATP. On average, CD34+ thymocytes are 8.7 μm in diameter, while DP thymocytes are 6.0 μm (data not shown). However, the diameters of the nuclei of CD34+ and DP thymocytes are similar, so the cytoplasmic volume of CD34+ cells is actually more than three times that of DP thymocytes. It may also be that immature cells are inherently more resistant to apoptotic signals, as suggested by the fact that immature T cell leukemias are very difficult to treat (44).

We propose that as thymocytes mature, the marked decrease in cell size from the CD34+ to the DP stage increases the intracellular dATP concentration enough to induce cytochrome c release from mitochondria, triggering apoptosis in the vast majority of cells. This idea is consistent with the finding that dATP was greatly reduced in cells from 3 wk dCF-treated hu/moFTOC compared with earlier time points. We postulate that the only surviving cells in longer cultures were rare cells that failed to accumulate dATP or to undergo apoptosis as a part of normal development. In the rescued cultures (Fig. 5), the elimination of both kinase activities lowered dATP enough to allow survival when the cells transitioned to the DP stage.

Our observation of hyperelevated dATP in ADA-deficient hu/moFTOC treated with dCyd (Fig. 5C) provides a plausible explanation for why dCyd therapy provided no clinical benefit in ADA-deficient patients (45). Treatment of ADA-inhibited thymocytes with dCyd in the presence of exogenous dADP in suspension cultures prevented the accumulation of dATP (42, 43) and was no doubt the rationale for the use of dCyd therapy in several ADA-deficient patients. However, our data suggest that AK also plays an important role in dAdo phosphorylation in our model. Therefore, analogous to the differences in enzymes that phosphorylate dAdo in cell extracts compared with intact cells, differences clearly exist in the phosphorylation of dAdo by thymocytes in suspension vs in organ culture, a more in vivo-like environment. Because RBCs from ADA-deficient patients accumulate high levels of dATP even though erythrocytes have virtually no detectable dCK activity (2), there is in vivo precedence for the involvement of AK in the formation of dATP during ADA deficiency. The fact that AK is responsible for dATP accumulation in vivo leads us to hypothesize that the hyperelevation in dATP in hu/moFTOC treated with dCf + dCyd could be due to a shift to AK as the major dAdo-phosphorylating enzyme when dCK is inhibited, consistent with previous studies in kinase-deficient human T lymphoblastoid cell lines (21). Alternatively, the addition of dCyd may be stimulating the formation of excess dTTP, which will in turn increase deoxyribonucleoside diphosphate reduction by ribonucleotide reductase (42, 46) and allow more dADP and dATP accumulation. Clearly, some facets of these enzymes’ regulation are not well understood and will need to be further studied to provide an explanation for the increased dATP accumulation when ADA and dCK are both inhibited.

Because both Ado and dAdo are elevated in ADA-deficient patients, it has been difficult to discern the relative contribution of Ado and dAdo in the toxicity to developing lymphocytes. Although the primary focus in more recent years has been on the effects of aberrant dAdo metabolism, some studies have suggested a possible role for Ado in ADA deficiency through the action of Ado receptors and blocking of NF-κB activity (47, 48). Studies with murine FTOCs convincingly ruled out a major role for Ado in inhibition of thymocyte development under ADA-deficient conditions (13). Our own studies also do not support a role for Ado in the inhibition of human thymocyte development in dCF-treated hu/moFTOC, as adenosine receptor antagonists failed to protect from the consequences of ADA deficiency and adenosine receptor agonists had no appreciable effect on thymocyte development (data not shown). Furthermore, rescue of dCF-treated hu/mo FTOC with a combination of dCyd and an AK inhibitor should increase intracellular Ado concentrations. Thus, while it is possible that Ado signaling through AdoR could play a role in immunomodulation of the few surviving T and B cells in the periphery of ADA-deficient patients (49), it is less likely that Ado has a major impact on development of T cells in the thymus.

It is also highly unlikely that SAH hydrolase inhibition, as occurs in the RBCs of ADA-deficient patients (39), has a large impact on thymocyte development during ADA deficiency. SAH hydrolase remained inhibited under ADA-inhibited conditions where dATP accumulation was prevented and development was rescued by the use of deoxynucleoside kinase inhibitors (Fig. 6). Furthermore, when murine thymocyte development was assessed in FTOC under conditions of inhibited methylation, thymocyte development was perturbed not by apoptosis, but by inhibition of transcription of molecules needed for development, such as the TCR and CD4/CD8 (50). Inhibition of SAH hydrolase during ADA deficiency is therefore likely to be a consequence, but not a major cause, of the pathology induced by aberrant dAdo metabolism during thymocyte development.

Treatments for ADA deficiency usually entail a bone marrow transplant or, if a suitable donor is not available, enzyme replacement therapy using pegylated bovine ADA enzyme. While the best chance for a cure is provided by bone marrow transplant, this is often not a feasible option. Although initially efficacious, treatment of ADA-deficient patients using pegylated bovine ADA enzyme results in low long-term lymphocyte restoration, with chronic deterioration of T cell responses (51). Furthermore, a recent report has revealed that a high proportion of bone marrow transplant-treated ADA-deficient patients are now experiencing progressive neurological abnormalities, indicating that hematopoietic reconstruction is insufficient to completely correct the systemic metabolic abnormalities associated with lack of ADA enzyme activity in all other organ systems (52). Although it is unknown whether the mechanisms of neurotoxicity are similar to those of the dATP-induced apoptosis occurring in lymphocytes, autopsy material from an ADA-deficient patient clearly showed accumulation of dATP in nonlymphoid tissues such as brain (53).

Our studies provide rationale for consideration of an additional therapeutic alternative for ADA-deficient patients: treatment with...
deoxynucleoside kinase inhibitors. Deoxycytidine tested in ADA-deficient patients was systemically nontoxic (45). Adenosine kinase inhibitors are currently being developed for treatments of various neurological manifestations such as stroke, as AK is the main enzymatic activity responsible for the regulation of extracellular adenosine levels (54) and adenosine is known to be neuroprotective. Interestingly, the AK inhibitor used in our study, 5'AS dA do, lacks in vivo efficacy due to its deamination by ADA (15); clearly, this would not be an issue in an ADA-deficient patient. Thus, treatment with deoxynucleoside kinase inhibitors could be a useful adjunct to using pegylated bovine ADA enzyme therapy and/or bone marrow transplant and might ameliorate the neurotoxicity associated with lack of ADA activity in the CNS.

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Disclosures

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