Inhibition of Transient Receptor Potential Channel Mucolipin-1 (TRPML1) by Lysosomal Adenosine Involved in Severe Combined Immunodeficiency Diseases*

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Impaired adenosine homeostasis has been associated with numerous human diseases. Lysosomes are referred to as the cellular recycling centers that generate adenosine by breaking down nucleic acids or ATP. Recent studies have suggested that lysosomal adenosine overload causes lysosome defects that phenotype patients with mutations in transient receptor potential channel mucolipin-1 (TRPML1), a lysosomal Ca2+ channel, suggesting that lysosomal adenosine overload may impair TRPML1 and then lead to subsequent lysosomal dysfunction. In this study, we demonstrate that lysosomal adenosine is elevated by deleting adenosine deaminase (ADA), an enzyme responsible for adenosine degradation. We also show that lysosomal adenosine accumulation inhibits TRPML1, which is rescued by over-expressing ENT3, the adenosine transporter situated in the lysosome membrane. Moreover, ADA deficiency results in lysosome enlargement, alkalization, and dysfunction. These are rescued by activating TRPML1. Importantly, ADA-deficient B-lymphocytes are more vulnerable to oxidative stress, and this was rescued by TRPML1 activation. Our data suggest that lysosomal adenosine accumulation impairs lysosome function by inhibiting TRPML1 and subsequently leads to cell death in B-lymphocytes. Activating TRPML1 could be a new therapeutic strategy for those diseases.

Adenosine, a purine nucleoside, plays an important role in the human body. It is produced constitutively by cells under normal conditions, and its concentration increases under metabolic stress (e.g. ischemia) or at sites of tissue injury (1–3). In mammalian cells, adenosine can be carried across cell membranes by the concentrative nucleoside transporters (also known as SLC28 family proteins) and the equilibrative nucleoside transporters (ENTs; also known as SLC29 proteins) (4). There are four known ENTs, designated ENT1, ENT2, ENT3, and ENT4. All four isofoms are widely distributed in mammalian tissues. The best characterized members of the family, ENT1 and ENT2, appear to function primarily at the cell surface (5). In contrast, ENT3 (6–8) and ENT4 (9) are predominantly expressed in intracellular membranes (10). Uniquely, ENT3 is pH-sensitive and optimally active under acidic conditions (optimal pH value of 5.5), probably reflecting the subcellular location of the transporters in acidic compartments, like lysosomes (6, 7). In addition, adenosine can be broken down by adenosine deaminase (ADA).

Impaired adenosine homeostasis has been associated with numerous human diseases. For example, individuals with ADA deficiency display impaired lymphocyte function and mortality, resulting in lymphopenia, severe combined immunodeficiency disease (SCID), and neurological abnormalities (2, 11–15). However, the molecular mechanisms underlying the ADA−/− phenotypes remain unclear. Interestingly, loss of ENT3 in mice results in lysosomal adenosine overload (7), subsequently causing enlarged and impaired lysosomes and defects in the clearance of apoptotic cells (7). These phenocopy mice with deficiency in transient receptor potential channel mucolipin-1 (TRPML1), a lysosome Ca2+-permeable channel regulating lysosomal membrane trafficking, lysosome function, and apoptotic cell clearance (16–22). Interestingly, ADA activity has been detected in lysosomes isolated from human fibroblasts (23, 24).

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4 The abbreviations used are: ENT, equilibrative nucleoside transporter; TRPML1, transient receptor potential channel mucolipin-1; ADA, adenosine deaminase; SCID, severe combined immunodeficiency disease; ML-SA1, mucolipin synthetic agonist 1; GPN, glycyll-phenylalanine β-naphthylamide; GECO, genetically encoded calcium indicator for optical imaging; Lamp1, lysosomal associated membrane protein 1; LDH, lactate dehydrogenase; TEM, transmission electron microscopy.
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Therefore, we hypothesize that in ADA mutant cells TRPML1 activity may be compromised by accumulated adenosine in lysosomes, and this might be involved in ADA deficiency. In this study, we report that lysosomal TRPML1 is suppressed by a high concentration of adenosine from the lumen. ADA−/− cells display impaired TRPML1 activity, which is rescued by increasing ENT3. We have also shown that deleting ADA results in lysosomal adenosine accumulation, lysosome enlargement, alkalinization, and dysfunction. Additionally, ADA deficiency causes B-lymphocyte cell death in response to oxidative stress. Importantly, all these phenotypes in ADA-deficient cells are rescued by TRPML1 activation. Our data suggest that ADA deficiency impairs TRPML1 activity, which may cause lysosome dysfunction and subsequent cell death in lymphocytes. This study provides a molecular mechanism underlying diseases associated with ADA deficiency.

Results

ADA Deficiency Results in Adenosine Accumulation in Lysosomes—Because ADA activity has been detected in lysosomes (23, 24), loss of ADA presumably leads to adenosine accumulation in lysosomes. To explore the role of adenosine in lysosomes, we first measured the level of adenosine in lysosomes isolated from human skin fibroblasts using high-performance liquid chromatography (HPLC) (25). We detected significant amounts of adenosine and ATP in the lysosomes of human fibroblasts. With the calculation method we described before (26), the concentrations of lysosomal adenosine and ATP were estimated to be ~0.3–3 and ~0.2–2 mM, respectively. The concentration of lysosomal ATP obtained here using HPLC is in agreement with the value we reported previously using an ATP bioluminescence assay (26). Next, we compared the amount of lysosomal ATP and adenosine between wild-type (WT) human fibroblasts and ADA−/− human fibroblasts derived from an ADA-SCID patient that contain very low ADA activity (2, 11–15). Interestingly, although the ATP level is not different between WT and ADA−/− human fibroblasts (Fig. 1, A and B), the lysosomal adenosine level was significantly increased in ADA−/− cells (Fig. 1, A and C). These data suggest that ADA deficiency causes adenosine accumulation in lysosomes, which may further inhibit lysosome function, resulting in cell dysfunction.

Adenosine Inhibits TRPML1 Activity—The similar cellular phenotypes between TRPML1-deficient cells and cells with lysosomal adenosine accumulation (7) suggest that luminal adenosine may inhibit TRPML1. To test this, HEK293T cells were transfected with surface-expressed mutant TRPML1 channels (TRPML1-L15A/L16A/L577A/L578A; abbreviated as TRPML1-4A), which serve as a surrogate of lysosomal TRPML1 (27–29). Because lysosomal pH is elevated to around 5.5 by adenosine accumulation (7), to mimic the environments of lysosomes with adenosine accumulation, extracellular solutions (analogous to the intralyosomal luminal side) were adjusted to pH 5.5. Consistent with our previous study, TRPML1 currents were activated by mucolipin synthetic agonist 1 (ML-SA1; a TRPML1 agonist; 5 μM) (29, 30) in cells transfected with GFP-TRPML1-4A but not in untransfected cells or cells transfected with GFP alone (data not shown). As shown in Fig. 2, A and B, adenosine induced a dose-dependent decrease in TRPML1 currents.

Because TRPML1 acts as a Ca2+-permeable channel (31, 32), to further evaluate the inhibitory effect of adenosine, TRPML1-4A activity was measured by a Fura-2-based Ca2+ imaging assay (29, 32). Consistent with another report (29), activation of TRPML1-4A by ML-SA1 induced a rapid increase in Fura-2 signal followed by a quick rundown. The mechanism underlying the rundown remains unclear. To avoid the contamination of the rundown, we co-applied ML-SA1 and adenosine by 56.7 ± 2.2% (Fig. 2, C and D), the Fura-2 signal in cells expressing TRPML1-4A was increased by co-application of ML-SA1 (10 μM) and adenosine (10 mM), presumably due to incomplete inhibition of TRPML1 by adenosine. However, a much larger response was observed when ML-SA1 (10 μM) was applied alone. ML-SA1 did not reveal Fura-2 response in untransfected cells (Fig. 2, C and D) or cells transfected with GFP alone (data not shown). These data suggest that adenosine inhibits TRPML1 activity.

To verify the inhibitory effect of adenosine on TRPML1 in the native environment, COS1 cells expressing GFP-TRPML1 were treated with 1 μM vaculolin-1, a small chemical known to increase the size of lysosomes (32), for 10 h, and then the enlarged lysosomes were recorded under luminal-side-out mode with the pipette containing 10 μM ML-SA1. TRPML1 currents in the luminal-side-out patch were markedly inhibited by bath application of adenosine (10 mM, pH 5.5) by 56.7 ± 2.2% (Fig. 2, E and F). These data suggest that luminal adenosine inhibits lysosomal TRPML1 channel activity.
Reduced TRPML1-mediated Ca\(^{2+}\) Release in ADA-deficient Cells—Next, we aimed to test whether adenosine accumulation inhibits TRPML1 in intact cells. TRPML1 activity in intact cells can be assessed by monitoring the cytoplasmic Ca\(^{2+}\) change using a Fura-2-based Ca\(^{2+}\) measurement. We compared ML-SA1-induced Fura-2 signals between WT and ADA deficiency human fibroblasts. If adenosine accumulation inhibits TRPML1, we would expect to detect less Fura-2 signal in response to ML-SA1 in ADA\(^{-/-}\) cells. As shown in Fig. 3, A and C, ADA\(^{-/-}\) cells exhibited a significantly smaller Fura-2 signal in response to ML-SA1 (50 \(\mu M\)), decreasing from 0.0600 ± 0.0052 in control fibroblasts to 0.0050 ± 0.0008 in ADA\(^{-/-}\) fibroblasts. This reduction in Fura-2 signal in ADA\(^{-/-}\) cells was not caused by a decrease in lysosomal Ca\(^{2+}\) content because ADA\(^{-/-}\) cells showed a comparable Fura-2 signal in response to GPN (400 \(\mu M\)), a cathepsin C substrate that induces osmotic

**FIGURE 2. Adenosine inhibited TRPML1 activity.** A, HEK293T cells transfected with GFP-TRPML1-4A were recorded under whole-cell mode. ML-SA1 (5 \(\mu M\))-induced TRPML1 currents were suppressed by co-applying adenosine (Ado; 1, 3, and 10 mM) in a dose-dependent manner. Left, representative currents in response to voltage ramps from -140 to 60 mV before and after bath application of ML-SA1 ± adenosine. Right, representative trace of current development at -140 mV responding to the indicated treatments. B, statistic data showing the inhibitory effect of adenosine on ML-SA1-induced TRPML1 currents. Currents measured at -140 mV were normalized to the peak currents under ML-SA1. C, representative images showing the changes of Fura-2 signal upon bath application of adenosine (10 mM), adenosine + ML-SA1, and ML-SA1 (10 \(\mu M\)) in HEK293T cells expressing GFP-TRPML1-4A. D, the removal of adenosine (10 mM) significantly increased ML-SA1 (10 \(\mu M\))-induced lysosomal Ca\(^{2+}\) release (measured with Fura-2 ratios) in GFP-TRPML1-4A-transfected HEK293T cells but not non-transfected cells. E and F, the lysosome isolated from COS1 cells transfected with GFP-TRPML1 was recorded under luminal-side-out mode. The pipette solution contained 10 mM ML-SA1. TRPML1 currents were inhibited by bath application of adenosine (10 mM, pH 5.5). The numbers in parentheses in panels B and F indicate the n (the number of cells tested). Data are presented as the mean ± S.E. Error bars represent S.E. Statistical comparisons were made using analysis of variance: **, \(p < 0.01\).
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FIGURE 3. TRPML1-mediated lysosomal Ca\(^{2+}\) release in ADA\(^{-/-}\) cells was compromised. A, ML-SA1 (50 \(\mu\)M) induced a smaller increase in cytosolic Ca\(^{2+}\) as measured by the Fura-2 (\(F_{340}/F_{380}\)) in ADA\(^{-/-}\) human fibroblasts than in control human fibroblasts. Ionomycin (Iono; 1 \(\mu\)M) was applied to induce maximal responses in the end of experiments. B, overexpression of ENT3 but not the mutant ENT3-G437R in ADA\(^{-/-}\) cells rescued the Fura-2 response induced by ML-SA1. C, summary of ML-SA-induced Fura-2 responses in WT fibroblasts, ADA fibroblasts, and ADA fibroblasts expressing ENT3 or ENT3-G437R. D, comparable GPN (400 \(\mu\)M)-induced Fura-2 responses in WT, ADA, and ADA fibroblasts expressing either ENT3 or ENT3-G437R. E and F, cells were transfected with GECO-TRPML1. ML-SA1 (10 \(\mu\)M)-induced GECO-TRPML1 responses in ADA\(^{-/-}\) human fibroblasts were smaller than that in WT human fibroblasts. G, GPN (200 \(\mu\)M)-induced comparable GECO-TRPML1 responses in WT and ADA\(^{-/-}\) human fibroblasts. H, ionomycin (1 \(\mu\)M)-induced comparable GECO-TRPML1 responses in WT and ADA\(^{-/-}\) human fibroblasts, indicating a similar expression level of GECO-TRPML1. The numbers in parentheses in panels F–H indicate the n (the number of cells tested). Data are presented as the mean \(\pm\) S.E. Error bars represent S.E. Statistical comparisons were made using analysis of variance: NS, no significant difference; **, \(p < 0.01\).

lysis of lysosomes to release lysosomal Ca\(^{2+}\) (Fig. 3D). Interestingly, overexpression of ENT3 but not ENT3-G437R, a mutant ENT3 with compromised transport activity but normal subcellular localization (8, 33), rescued the compromised Fura-2 response induced by ML-SA1 (Fig. 3, B and C) in ADA\(^{-/-}\) fibroblasts without changing lysosomal Ca\(^{2+}\) content (Fig. 3D).

To further measure lysosomal TRPML1 activity and lysosomal Ca\(^{2+}\) release in intact cells, we have generated a lysosome-targeted genetically encoded Ca\(^{2+}\) indicator by fusing GECO, a single wavelength genetically encoded Ca\(^{2+}\) indicator (34), to the cytoplasmic amino terminus of TRPML1 (30). GECO-TRPML1 has been used as a probe to specifically measure TRPML1-mediated lysosomal Ca\(^{2+}\) release, representing TRPML1 activity (30). To exclude the possible contamination of external Ca\(^{2+}\), low external Ca\(^{2+}\) (nominally free Ca\(^{2+}\) with 1 mM EGTA; free Ca\(^{2+}\) estimated to be <10 nM) was used for the GECO-TRPML1 fluorescence measurement. As shown in Fig. 3, E and F, ML-SA1 (10 \(\mu\)M)-induced GECO fluorescence intensity in ADA\(^{-/-}\) cells was significantly smaller than that in WT cells, decreasing from 0.68 \(\pm\) 0.09 in control fibroblasts to 0.24 \(\pm\) 0.03 in ADA\(^{-/-}\) fibroblasts. In contrast, a comparable GPN-induced GECO signal (Fig. 3G) and ionomycin-induced GECO signal (Fig. 3H) were observed between WT and ADA\(^{-/-}\) cells. These data suggest that TRPML1 activity is reduced in lysosomes of human skin fibroblasts with ADA deficiency.

Enlarged Lysosome in ADA-deficient Cells—TRPML1-mediated lysosomal Ca\(^{2+}\) release is essential for lysosomal membrane trafficking, and defective TRPML1 results in enlarged lysosomes (35–39). The above results suggest that by affecting TRPML1-mediated Ca\(^{2+}\) release lysosomal adenosine accumulation in ADA\(^{-/-}\) fibroblasts may result in enlarged lysosomes. In agreement with this, ADA\(^{-/-}\) fibroblasts displayed enlarged lysosomes as revealed by electron microscopy, and this was rescued by ML-SA1 treatment (Fig. 4, A and B). Overall, these results confirm that lysosomal adenosine accumulation inhibits TRPML1 activity, subsequently resulting in defective membrane trafficking.

Lysosome Dysfunction Induced by Adenosine Accumulation in Lysosomes—Because TRPML1 is important for lysosome function (16), to test whether adenosine accumulation impairs
lyosomal function, we first monitored lysosomal pH using LysoTracker Red DND-99. Strong LysoTracker signals were detected in WT but not ADA\(^{-/-}\) fibroblasts (Fig. 5, A and B). Notably, TRPML1-GFP but not lysosomal associated membrane protein 1 (Lamp1)-GFP or TRPML1-DDKK (non-conducting pore mutation)-GFP rescued the loss of LysoTracker signal in ADA\(^{-/-}\) cells. These data suggest that ADA deletion results in elevated pH in lysosomes, which is likely due to a reduction in TRPML1 activity.

Because LysoTracker provides only a qualitative assessment of lysosomal pH, a ratiometric approach has been used to more accurately measure lysosomal pH (40). Unfortunately, we did not have access to a ratiometric imaging system to perform this type of experiment. Alternatively, to validate the LysoTracker data, we loaded lysosomes with the pH-sensitive fluorescent indicator Oregon Green 488-dextran (green) and pH-insensitive tetramethylrhodamine-conjugated dextran (red) through endocytosis (41, 42) and then measured the relative intensity of Oregon Green 488-dextran. The ratio between green fluorescence intensity and red fluorescence intensity is a reliable index of the luminal pH. As shown in Fig. 5, C and D, ADA\(^{-/-}\) fibroblasts displayed stronger green signals compared with WT cells, whereas the red signals were comparable. Furthermore, expression of TRPML1 but not Lamp1 caused a decrease in the Oregon Green signal in ADA\(^{-/-}\) cells (Fig. 5, E and G) without affecting the signals of pH-insensitive tetramethylrhodamine-conjugated dextran (Fig. 5, F and G). This further suggests that ADA deletion results in an elevated pH in lysosomes, which is likely due to a reduction in TRPML1 activity.

We next assessed lysosomal function by applying DQ-BSA, a dye that indicates active proteolysis in lysosomes (43). Strong DQ-BSA signals were observed in WT but not ADA\(^{-/-}\) fibroblasts, although similar dextran signals were present (Fig. 6, A and B). Interestingly, ENT3 but not ENT3-G437R overexpression rescued attenuated DQ-BSA signals, suggesting that lysosome dysfunction in ADA\(^{-/-}\) fibroblasts results from adenosine accumulation. The loss of DQ-BSA signals in ADA\(^{-/-}\) fibroblasts was also rescued by expressing TRPML1-GFP but not its mutant TRPML1-DDKK-GFP, suggesting that lysosomal dysfunction in ADA\(^{-/-}\) fibroblasts is attributed to an impaired TRPML1 (Fig. 6, C and D).

Lymphocytes Death Induced by Adenosine Accumulation in Lysosomes—Dysfunction of lysosomes has been associated with cell death. Although ADA is ubiquitously expressed in most body cells, lymphocytes in the immune system contain the highest level of ADA, and lymphocytes are the most vulnerable to ADA deficiency (11, 13, 44, 45). To test whether ADA deficiency-mediated TRPML1 impairment is implicated in cell death, we monitored cell death by assaying levels of lactate dehydrogenase (LDH), a cytosolic enzyme that is released into cell culture medium once cells lose membrane integrity (43). An ADA-SCID patient-derived B-lymphocyte cell line, which displays a very low level of ADA activity (46), was used. ADA deficiency increased cell death (Fig. 6E, first two columns). Given that the lysosome is an important source of reactive oxidative species (47–49), we also tested our hypothesis under H\(_2\)O\(_2\) condition. H\(_2\)O\(_2\) (0.5 or 1 mM) induced a much higher level of cell death in ADA-deficient cells compared with WT cells, and this was rescued by activating TRPML1 using ML-SA1 (10 \(\mu\)M). Our data suggest that lysosomal adenosine accumulation in ADA-deficient cells may contribute to lymphocyte death by inhibiting TRPML1 activity.

Discussion

Adenosine, a ubiquitous signal molecule that is produced constitutively by the cell under both normal and pathological conditions, is engaged in regulation of numerous physiological systems, such as the cardiovascular, nervous, renal, and immune systems; lungs; and kidneys (1, 14). There are several pools of adenosine that are generated by intracellular and extracellular enzyme pathways. Although the function of extracellular adenosine is well characterized (1, 14), little is known about its roles in intracellular organelles, particularly in the lysosome. In this study, we have shown that lysosomes contain 0.3–3 mM adenosine, which can be further elevated by deleting ADA. Accumulation of adenosine in lysosomes impairs TRPML1 channel activity and subsequent lysosome function, leading to lymphocyte death. These defects in ADA-deficient cells are rescued by activating TRPML1.

One interesting question is how the lysosome maintains its adenosine homeostasis. Lysosomes contain a variety of degradative enzymes capable of breaking down all types of biological macromolecules, including proteins, nucleic acids, carbohydrates, and lipids. The degradation of nucleic acids in lysosomes is one of the major sources of nucleoside recycling (50). Meanwhile, lysosomes take up ATP from the cytosol via SLC17A9, an ATP transporter localized in the lysosomal membrane (43). Because 5'-nucleotidase, the enzyme that dephosphorylates AMP to generate adenosine, has been found in lysosomes (51, 52), it is likely that ATP breakdown in the lysosomal interior acts as an alternative pathway that generates adenosine.
addition, lysosomal adenosine homeostasis is maintained not only by the biogenesis pathway but also by the degradation pathway, which may be dominated by lysosomal ADA (23, 24), and the release pathway mediated by ENT3 (7). In agreement with this, the loss of either ADA (this study) or ENT3 (7) causes lysosomal adenosine accumulation. Therefore, lysosomal adenosine homeostasis is tightly controlled by generation, degradation, and transport pathways. Disruption of either pathway would result in lysosomal adenosine dyshomeostasis, subsequently resulting in human diseases.

It remains unclear how accumulative adenosine suppresses TRPML1 and subsequent lysosomal function. Previously, adenosine has been reported to directly interact with and inhibit the TRPV1 channel (53). Because adenosine inhibits TRPML1 currents under inside-out recoding mode (Fig. 2, E and F), it is possible that adenosine inhibits TRPML1 using the same mechanism, i.e. by directly binding to the channel. This awaits further investigation. Conversely, considering that TRPML1 activity decreases as lysosomal pH increases (32), adenosine accumulation could also compromise TRPML1 function by elevating lysosomal pH (7). Given that TRPML1 is required for maintaining lysosomal pH (54), compromised TRPML1 induced by adenosine accumulation may further alkalinize the lysosome, facilitating the impairment of lysosomal function (55) using a positive feedback mechanism.

Although adenosine accumulation impairs lysosomes, the physiological role of adenosine in lysosomes remains unclear. Because lysosomes keep recycling nucleic acids (50) and lysosomes contain a high level of ATP (43), the nucleoside concentration in lysosomes is expected to be higher (\(0.3–3\) mM as estimated) than that in the cytosol. It is very likely that ENT3-mediated adenosine release can replenish cytosolic adenosine, which is important for many intracellular processes under normal physiological condition. We can also speculate that lysosomal adenosine may be released into the cytosol to participate in ATP synthesis, especially during nutritional starvation (23). Lysosomes are dynamic organelles that constitutively fuse with the plasma membrane to release luminal substances, so-called lysosomal exocytosis. Lysosomes may release adenosine to the extracellular space to activate adenosine receptors under cer-

FIGURE 5. Lysosome alkalinization in ADA-deficient cells. A and B, ADA deficiency caused lysosome alkalinization that was indicated by a decrease in LysoTracker signal in ADA \(^{-/-}\) human fibroblasts. TRPML1-GFP but not Lamp1-GFP or TRPML1-DDKK-GFP rescued the loss of LysoTracker signal in ADA \(^{-/-}\) cells. C and D, ADA deletion caused enhanced Oregon Green 488 signals, whereas tetramethylrhodamine-conjugated dextran signals were comparable. These data indicate that ADA \(^{-/-}\) cells displayed an alkalized lysosomal pH. E, expression of TRPML1-mCherry but not Lamp1-mCherry rescued the enhanced Oregon Green 488 signals in ADA \(^{-/-}\) fibroblasts. F, expression of TRPML1-GFP and Lamp1-GFP resulted in similar signals of tetramethylrhodamine-conjugated dextran. G, mean intensity of the fluorescence of pH-sensitive Oregon Green 488-dextran and pH-insensitive tetramethylrhodamine-conjugated dextran. Data are presented as the mean ± S.E. Error bars represent S.E. Statistical comparisons were made using analysis of variance. NS, no significant difference; *, \(p < 0.05\); **, \(p < 0.01\).
tain conditions. In addition, because lysosomal exocytosis helps membrane repair during cell damage (56–58) and because elevated extracellular adenosine is commonly thought of as a beneficial signaling molecule during tissue damage (1–3, 14), it is possible that lysosomes replenish extracellular adenosine to protect tissues from damage (1–3, 59). Further investigation is needed to test this hypothesis.

Adenosine dyshomeostasis leads to a number of human diseases, including diseases associated with ADA deficiency (lymphopenia, SCID, and neurological abnormalities) (2, 11–15) and ENT3 deficits (familial Rosai-Dorfman disease, Faisalabad histiocytosis, and H syndrome) (7, 33). However, the mechanisms for these diseases are largely unknown. We have demonstrated that accumulation of adenosine in lysosomes inhibits TRPML1 channel function, and TRPML1 activation successfully corrects the abnormal phenotypes in ADA-deficient cells. Our findings provide a molecular mechanism underlying the pathogenesis of diseases associated with ADA or ENT3 deficiency. This may help develop a potential therapeutic strategy for these diseases, e.g. up-regulation of TRPML1 activity by the newly identified TRPML1 agonist ML-SA1.

Experimental Procedures

Cell Culture and Transfection—COS1 and HEK293T were obtained from ATCC (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 supplemented with 10% fetal bovine serum (Invitrogen). Wild-type human fibroblasts (GM00969) and ADA-deficient human fibroblasts (GM02605) were obtained from Coriell Institute (Camden, NJ) and cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum. Wild-type B-lymphocyte (AG14953) and ADA-deficient B-lymphocyte (GM11411) were obtained from Coriell Institute and cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum. Cells were cultured at 37 °C in a 5% CO2 atmosphere. For some experiments, cells were seeded on 0.01% polylysine-coated coverslips and cultured 24–48 h before further experiments. Cells from passage numbers 5–30 were used for subsequent assays. COS1 and HEK293T cells were transfected using Lipofectamine 2000. Human fibroblasts were transfected by electroporation with the Neon® Transfection System (Invitrogen) following the manufacturer’s optimized protocol (1525 V, 25 ms, one pulse). 70% transfection efficiency was regularly achieved.

Reagents—The following reagents were used: adenosine (Sigma-Aldrich), ML-SA1 (Princeton Biomolecular Research), GPN (Santa Cruz Biotechnology), ionomycin (Cayman Chemical), vaculin-1 (Santa Cruz Biotechnology), Fura-2 AM (Thermo Scientific), LysoTracker Red DND-99 (Thermo Scientific), DQ-BSA (Thermo Scientific), and hydrogen peroxide solution (H2O2; Sigma).

Plasmid Constructs—GFP-TRPML1 was made as described before (22, 32). TRPML1-L15A/L16A/L577A/L578A-GFP (abbreviated as TRPML1-4A-GFP) was constructed using a site-directed mutagenesis kit (Qiagen). The GECO-TRPML1

FIGURE 6. Lysosome dysfunction and cell death induced by adenosine accumulation in lysosomes. A and B, strong DQ-BSA signals were observed in WT but not ADA−/− fibroblasts. DQ-BSA is a dye that indicates active proteolysis in lysosomes. The dextran signal was examined at the same time, indicating a similar level of endocytosis to lysosomes between WT and ADA−/− cells. C and D, GFP-ENT3 and TRPML1-GFP but not their non-functional mutants GFP-ENT3-G437R and TRPML1-DDKK-GFP rescued attenuated DQ-BSA signals in ADA−/− fibroblasts. E, H2O2 significantly increased cell death in B-lymphocytes from patients with ADA deficiency, and this was corrected by activating TRPML1 with ML-SA1 (10 μM). Data are presented as the mean ± S.E. Error bars represent S.E. Statistical comparisons were made using analysis of variance: NS, no significant difference; **, p < 0.01.
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conduct was made by inserting the full-length GECO sequence (34) between the HindIII and BamH1 sites of a pCDNA6 plasmid that contains the mouse TRPML1 cDNA at the XhoI site. All constructs were confirmed by sequencing, and protein expression was verified by Western blotting. GFP-ENT3 and GFP-ENT3-G437R were kindly provided by Neil Morgan and Eamonn Maher. COS1, HEK293T, and human fibroblasts were transiently transfected with the plasmids for electrophysiology, Ca\(^{2+}\) imaging, and confocal imaging.

Whole-cell Electrophysiology—The pipette solution contained 140 mM potassium gluconate, 4 mM NaCl, 1 mM EGTA, 2 mM MgCl\(_2\), 0.39 mM CaCl\(_2\), 20 mM HEPES (pH 5.5; free [Ca\(^{2+}\)] = 100 nM). The bath solution (modified Tyrode’s solution) contained 145 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM HEPES, 10 mM glucose (pH 5.5). All bath solutions were applied via a perfusion system to achieve a complete solution exchange within a few seconds. Data were collected using an Axopatch 200B patch clamp amplifier, Digidata 1440, and pClamp 10.0 software (Axon Instruments). Whole-cell currents were digitized at 10 kHz and filtered at 2 kHz. All experiments were conducted at room temperature (21–23 °C), and all recordings were analyzed with pClamp 10.0 and Origin 8.0 (OriginLab, Northampton, MA).

Lysosomal Electrophysiology—Lysosomal electrophysiology was performed in isolated enlarged late endosome/lysosome vacuoles using a modified patch clamp method as described previously (32). Briefly, cells were treated for >2 h with 1 μM vacuolin-1, a lipophilic polycyclic triazine that can selectively increase the size of endosomes and lysosomes. Large vacuoles/lysosomes were observed in most vacuolin-1-treated cells. Whole-lysosome recordings were performed on manually isolated enlarged vacuoles. In brief, a patch pipette was pressed against a cell and quickly pulled away to slice the cell membrane. This allowed enlarged lysosomes to be released into the recording chamber and identified by monitoring GFP fluorescence. After formation of a gigaseal between the patch pipette and an enlarged lysosome, capacitance transients were compensated. Voltage steps of several hundred millivolts with millisecond duration(s) were then applied to break the patched membrane and establish the whole-lysosome configuration. Unless otherwise stated, bath (cytoplasmic) solution contained 140 mM potassium gluconate, 4 mM NaCl, 1 mM EGTA, 2 mM MgCl\(_2\), 0.39 mM CaCl\(_2\), 20 mM HEPES (pH was adjusted with KOH to 7.2; free [Ca\(^{2+}\)] was 100 nM). The pipette (luminal) solution was a standard extracellular solution (modified Tyrode’s: 145 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM HEPES, 10 mM glucose (pH was adjusted with HCl to 4.6)). Data were collected using an Axopatch 2A patch clamp amplifier, Digidata 1440, and pClamp 10.2 software (Axon Instruments). Whole-lysosome currents were digitized at 10 kHz and filtered at 2 kHz. All experiments were conducted at room temperature (21–23 °C), and all recordings were analyzed with pClamp 10.2 and Origin 8.0.

Fura-2 Ca\(^{2+}\) Imaging—Cells were loaded with 5 μM Fura-2 AM (Invitrogen). Fluorescence was recorded at different excitation wavelengths using an EasyRatioPro system (Photon Technology International). Fura-2 ratios (F\(_{340}\)/F\(_{380}\)) were used to monitor changes in intracellular [Ca\(^{2+}\)] upon stimulation. GPN (200 μM) was used to induce Ca\(^{2+}\) release from lysosomes. Ionomycin (1 μM) was added at the conclusion of all experiments to induce a maximal response for comparison. Ca\(^{2+}\) imaging was carried out within 0.5–3 h after plating and when cells still exhibited a round morphology.

GECO Ca\(^{2+}\) Imaging—Cells were trypsinized and plated onto glass coverslips after 18–24 h of transfection with GECO-TRPML1. Experiments were carried out within 0.5–2 h after plating when cells still exhibited a round morphology. The fluorescence intensity at 470 nm (F\(_{470}\)) was monitored using the EasyRatioPro system. Lysosomal Ca\(^{2+}\) release was measured under a “low external Ca\(^{2+}\)” solution, which contained 145 mM NaCl, 5 mM KCl, 3 mM MgCl\(_2\), 10 mM glucose, 1 mM EGTA, 20 mM HEPES (pH 7.4). Ca\(^{2+}\) concentration in the nominally free Ca\(^{2+}\) solution is estimated to be 1–10 μM. With 1 mM EGTA, the free Ca\(^{2+}\) concentration is estimated to be <10 nM based on the Maxchelator software.

HPLC—Lysosomes from human fibroblast (WT and ADA\(^{-/-}\)) were isolated as described previously (43). Briefly, cell lysates were obtained by Dounce homogenization in a homogenizing buffer (HM buffer; 0.25 M sucrose, 1 mM EDTA, 10 mM HEPES, pH 7.0) and then centrifuged at 1,500 × g for 20 min to remove the nuclei and intact cells. Postnuclear supernatants were then subjected to ultracentrifugation through a Percoll density gradient using a Beckman Optima L-90K ultracentrifuge. An ultracentrifuge tube was layered with 2.5 M sucrose, 18% Percoll in HM buffer and supernatant (top). The centrifugation was carried out at 90,000 × g (31,300 rpm) at 4 °C for 1 h using a Beckman Coulter 70.1 Ti rotor. Samples were fractionated into light, medium, and heavy membrane fractions. Heavy membrane fractions contained concentrated bands of cellular organelles and were further layered over a discontinuous iodixanol gradient generated by mixing iodixanol in HM buffer with 2.5 mM glucose (in v/v; 27, 22.5, 19, 16, 12, and 8%) and with osmolarity maintained at 300 mosm for all solutions. After centrifugation at 4 °C for 2.5 h at 180,000 × g (44,200 rpm), each sample was divided into 12 fractions (0.5 ml each) for further analyses. Note that the biological and ionic compositions of the lysosomes were largely maintained due to the low rate of transport across the lysosomal membrane at 4 °C.

Reversed phase HPLC analysis was carried out using a Prostar HPLC system equipped with a diode array ultraviolet UV-visible detector (Varian Canada Inc., Mississauga, Ontario, Canada). A Cosmosil-polymerylic octadecyl group C\(_{18}\) column (5-μm particle size, 250 mm × 46-mm inner diameter; Nacalai USA, Inc., San Diego, CA) was used. The column temperature was maintained at 30 °C. Standards and samples (100-μl injections) were separated using a binary gradient mobile phase of water:methanol (A:B); only A contains 0.4% phosphoric acid, and detection was at 257 nm. A linear gradient (0.9 ml/min flow rate) from 2 to 80% B in A was used: 2–10% in 25 min, 10–80% in 10 min, and 80–10% in 30 min. ATP and adenosine retention times were determined using co-injected standards.

Isolated lysosomes were resuspended with 1 ml of internal buffer and sonicated at 20% power input (6 × 20-s sonication on ice). After centrifuging at 12,500 rpm for 10 min at 4 °C, the supernatants were saved at −80 °C. For further HPLC analysis,
300 μl of supernatant was mixed well with 1 ml of cooled extraction medium of Tris-EDTA-statured phenol/chloroform/deionized water (6:2:2) and centrifuged at 10,000 × g for 5 min at 4 °C. The upper aqueous phase was carefully collected and filtered through a filtering cartridge containing a 0.45-μm nylon membrane using a disposable syringe set before HPLC analysis.

Quantification was based on the external standard method. Stock solutions of an ATP-Na, adenosine standard (1 mg/ml) were prepared by dissolving them in 80% ethanol. The working standard solutions for linear calibration were prepared by diluting the stock solution to produce a concentration sequence of 0.1, 1, 5, 10, 20, 50, 100, and 200 μg/ml.

Transmission Electron Microscopy (TEM)—Cell pellets of human fibroblasts and mouse embryo fibroblasts were used for these experiments. First, cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight. After fixation, cells were gently scraped and centrifuged to form cell pellets. Pellets were immersed in 1% osmium tetroxide in buffered saline. After a quick rinse with distilled water, pellets were stained with 0.25% uranyl acetate at 4 °C overnight. Further dehydration was done in a series of acetone dehydration steps (50% for 10 min, 70% for 10 min × 2, 95% for 10 min × 2, 100% for 10 min × 2) and dried with 100% acetone for 10 min. Infiltration was performed by a 3:1 mixture of dried 100% acetone and resin for 3 h and then a 1:3 mixture of dried 100% acetone and resin overnight. Pellets were embedded in 100% Epon Araldite resin for 3 h × 2 and then dried in an oven at 60 °C for 48 h. Thin sections were cut using a LKB Huxley Ultramicrotome with a diamond knife (100 nm) and placed on 300 mesh copper grids. Grids were stained with lead citrate. Samples were viewed with a JEOL JEM 1230 transmission electron microscope at 80 kV. Images were captured using a Hamamatsu ORCA-HR digital camera. Lysosome sizes were measured using an Advanced Microscopy Techniques image capture engine. Lysosomes displayed the typical morphology in the TEM images, i.e. round or ovoid in shape with dense bodies inside and a single membrane. For those lysosomes of ovoid shape, we averaged the diameter that runs through the shortest and longest part of the lysosome.

Confocal Microscopy—Confocal fluorescence images were taken using an inverted Zeiss LSM510 Axiovert 200M confocal microscope with a 63× oil immersion objective. Sequential excitation wavelengths at 488 and 543 nm were provided by argon and helium-neon gas lasers, respectively. Emission filters BP500–550 and LP560 were used for collecting green and red images in channels 1 and 2, respectively. After sequential excitation, green and red fluorescence images of the same cell were saved with ZEN2012 software. Images were analyzed by Zeiss software. Intensity of fluorescence was analyzed using ImageJ.

Lysotracker, Dextran, and DQ-BSA Staining—Cells were placed in the coverslip for 24 h. For LysoTracker loading, cells were incubated at 37 °C with LysoTracker Red DND-99 (L7528) (25 nM) for 30 min and chased for 30 min. For dextran loading, cells were incubated with Oregon Green 488-dextran (D22910) (250 μg/ml) and tetrarmethylrhodamine-conjugated dextran (D1868) (250 μg/ml) for 2 h and chased for 3 h at 37 °C. For DQ-BSA loading, cells were incubated at 37 °C with DQ-BSA (10 μg/ml) and dextran green (100 μg/ml) for 2 h and then chased for 3 h prior to taking the confocal images.

LDH Assay—Cells were cultured in 24-well plates. LDH activity was determined by measuring the NADH oxidation with pyruvate as substrate. For each assay, a 50-μl sample was incubated with 50 μl of reaction buffer (CytoTox-ONE™ Homogeneous Membrane Integrity Assay, Promega) for 10 min at 22 °C, and the fluorescence at 340 nm was measured using a spectrophotometer. Results were scaled to complete cell death induced by exposure to 1% Triton X-100 at room temperature for 20 min. Percentage of cell death = 100 × (Sample OD – Background medium OD)/(Triton X-100 OD – Medium background OD).

Data Analysis—Data are presented as mean ± S.E. Statistical comparisons were made using analysis of variance and Student’s t test. p values <0.05 were considered statistically significant. *, p < 0.05; **, p < 0.01.

Author Contributions—X. Z. Z. and Y. Z. performed electrophysiology recording and Ca2+ imaging experiments and data analysis. X. S., G. D., and A. P. performed HPLC experiments and data analysis. Q. C. performed EM and confocal microscopy experiments and data analysis. J. K. R. and X. Z. provided constructive comments on the manuscript. X.-P. D. designed the projects and wrote the manuscript with input from co-authors.

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