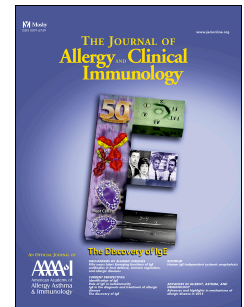


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Pui Y. Lee, MD, PhD, Yuelong Huang, PhD, Qing Zhou, PhD, Oskar Schnappauf, PhD, Michael S. Hershfield, MD, Ying Li, Nancy J. Ganson, Natalia Sampaio Moura, Ottavia M. Delmonte, MD, Scellig S. Stone, MD, PhD, Michael J. Rivkin, MD, Sung-Yun Pai, MD, Todd Lyons, MD, Robert P. Sundel, MD, Victor W. Hsu, MD, Luigi D. Notarangelo, MD, Ivona Aksentijevich, MD, Peter A. Nigrovic, MD



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## Disrupted N-linked glycosylation as a disease mechanism in deficiency of ADA2

Pui Y. Lee MD, PhD <sup>a,b,\*</sup>, Yuelong Huang PhD <sup>b</sup>, Qing Zhou PhD <sup>c</sup>, Oskar Schnappauf PhD<sup>c</sup>, Michael S. Hershtfield MD <sup>d</sup>, Ying Li <sup>b</sup>, Nancy J. Ganson <sup>d</sup>, Natalia Sampaio Moura <sup>c</sup>, Ottavia M. Delmonte MD <sup>e</sup>, Scellig S. Stone MD, PhD <sup>f</sup>, Michael J. Rivkin MD <sup>g</sup>, Sung-Yun Pai MD <sup>h,i</sup>, Todd Lyons MD <sup>j</sup>, Robert P. Sundel MD <sup>a</sup>, Victor W. Hsu MD <sup>b</sup>, Luigi D. Notarangelo MD <sup>e</sup>, Ivona Aksentijevich MD <sup>c</sup>, Peter A. Nigrovic MD <sup>a,b</sup>

- a. Division of Immunology, Boston Children's Hospital, Boston, MA 02115
- b. Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Boston, MA 02115
- c. Inflammatory Disease Section, National Human Genome Research Institute, Bethesda, MD 20892
- d. Department of Medicine and Biochemistry, Duke University School of Medicine, Durham, NC 27710
- e. Laboratory of Clinical Immunology and Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892
- f. Department of Neurosurgery, Boston Children's Hospital, Boston, MA 02115
- g. Departments of Neurology, Psychiatry and Radiology, Boston Children's Hospital, Boston, MA 02115
- h. Division of Hematology-Oncology, Boston Children's Hospital, Boston, MA 02115
- i. Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA 02115
- j. Division of Emergency Medicine, Boston Children's Hospital, Boston, MA 02115

**\* Correspondence to:** Pui Y. Lee, MD PhD,  
Boston Children's Hospital,  
300 Longwood Ave., Boston, MA 02115  
[pui.lee@childrens.harvard.edu](mailto:pui.lee@childrens.harvard.edu)

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**Summary:**

Deficiency of adenosine deaminase 2 is characterized by vasculitis, early-onset strokes, immunodeficiency, and bone marrow failure. We describe a novel pathogenic mutation affecting a consensus N-linked glycosylation sequence and illustrate the essential role of glycosylation in the biology of ADA2.

**Key words:** Adenosine deaminase 2, DADA2, vasculitis, hemorrhagic stroke, N-linked glycosylation

To the editors,

Deficiency of adenosine deaminase 2 (DADA2) causes systemic inflammation, immunodeficiency, vasculitis, and early-onset strokes (1, 2). This variable phenotype is caused by recessively inherited loss-of-function mutations of *CECR1* (Cat Eye Syndrome Chromosome Region 1), which encodes adenosine deaminase 2 (ADA2). At least 30 pathogenic mutations in the *CECR1* gene have been described in more than 125 patients (3, 4). While absent plasma ADA2 activity is a hallmark of the disease, how individual mutations affect ADA2 expression and function has been largely unexplored.

We studied a 2 year-old girl with erythematous papules, livedo reticularis, systemic inflammation and intracranial hemorrhage (Fig. 1A, B; see Methods section and Table E1 in Online Repository for clinical summary and laboratory data). The constellation of findings led to high clinical suspicion for DADA2, which was confirmed by near-absent plasma ADA2 activity (Fig. 1C). Maternal plasma ADA2 activity was within the range for DADA2 carriers. Genomic analysis of the patient revealed compound heterozygosity of two novel *CECR1* variants: a missense variant (c.385A>C; p.T129P) in exon 3 identified by Sanger sequencing, and a 13.5 kb deletion in the 3' region detected by multiplex ligation-dependent probe amplification (Fig. 1D). The large deletion was maternally-derived and encompassed exons 8, 9, and 10 (see Fig. E1 in Online Repository). The father was not available for analysis, therefore it remains unknown whether he carries the c.385A>C variant in heterozygous state, or whether this was a *de novo* mutation in the patient.

Interestingly, sequencing of complementary DNA (cDNA) from the patient revealed exclusive expression of the c.385A>C variant (Fig. 1E), suggesting that the large deletion in the maternally-derived allele impacted RNA expression and/or stability. RNA stability is likely

impaired as the deletion encompasses the normal stop codon and 3'untranslated region of the gene. We hypothesized that the T129P substitution must reduce ADA2 expression or function. The variant was not found in the Genome Aggregation Database (gnomAD) (5) and is predicted to be deleterious (SIFT score 0.003; PolyPhen2 score 0.99).

We expressed wild-type and mutant ADA2 in 293T cells using pcDNA3.1-myc/his plasmid (see Methods and Table E2 in Online Repository for experimental details and primer sequences). Wild-type ADA2 was abundantly secreted into the medium and also present intracellularly (Fig. 1F, G). Strikingly, ADA2 with T129P substitution displayed a lower molecular weight (MW) by 2 kD with increased intracellular retention and absent secretion. These characteristics were fully explained by the T129P substitution, as correction of this mutation by site-directed mutagenesis (P129T) reversed these findings and restored ADA2 secretion (Fig. 1H).

The MW reduction due to a missense mutation raised the possibility of disrupted post-translational modifications. Indeed, T129 and the preceding N127 together constitute a consensus N-linked glycosylation sequence (NXS/T) required for the attachment of oligosaccharides (6). N-glycosylation has well-recognized roles in protein folding, trafficking, and function (7). Confirming the presence of N-glycosylation at N127, substituting this residue with glutamine (N127Q) recapitulated the shift in MW and impaired secretion as seen with the T129P mutation (Fig. 1H). Correspondingly, ADA2 activity was significantly impaired by both T129P and N127Q substitutions. Sequence alignment showed that the NXT sequence at this site is highly conserved among eukaryotic species (See Fig. E2A in Online Repository).

Consistent with an important role for glycosylation in ADA2 function, addition of the N-glycosylation inhibitor tunicamycin profoundly inhibited the secretion of wild-type ADA2 (Fig.

11). The difference in MW between wild-type and mutant ADA2 was abolished by tunicamycin treatment, as unglycosylated protein displayed a lower MW near 52 kD, suggesting the presence of additional N-glycans (Fig. 1J).

To understand how N-linked glycosylation modulates ADA2 trafficking and secretion, we performed confocal microscopy on transfected cells. Wild-type ADA2 displayed a perinuclear Golgi staining pattern in addition to diffuse presence in the cytoplasm (Fig. 1K). In contrast, T129P mutant protein showed intense cytoplasmic foci with minimal Golgi colocalization. T129P and N127Q mutants both showed prominent co-localization with endoplasmic reticulum (ER) (Fig. 1L). These findings suggest that glycosylation at N127 is required for trafficking of ADA2 from the ER to the Golgi apparatus.

In defining the structure of ADA2, Zavialov and colleagues suggested asparagine 127, 185 and 378 as sites for N-glycosylation (8). Our *in silico* analysis further predicted N174 as a glycosylation target (see Fig. E2B in Online Repository). While the NXS/T consensus sequence is highly conserved for N127, N174 and N378, some species have lost N185 (see Fig. E2C in Online Repository). We searched gnomAD to identify variants that affect the predicted glycosylation sites. Out of 16 total variants, we found 5 missense mutations that disrupt the consensus sequence (see Table E1 in the Online Repository). These mutations are predicted to be deleterious although they have not yet been described in DADA2 patients.

Supporting the presence of glycosylation on asparagine 174, 185 and 378, we found that glutamine substitution of each site resulted in a 2kD reduction in MW similar to the N127Q and T129P mutants (Fig. 2A). Confocal microscopy demonstrated that disruption of N174 and N378 also result in ER retention and defective trafficking of ADA2 to the Golgi apparatus (Fig. 2B).

By contrast, substitution of the less evolutionarily-conserved N185 did not alter ADA2 localization.

In line with these findings, disruption of N127, N174 or N378 profoundly reduced ADA2 secretion while glycosylation at N185 was dispensable (Fig. 2C). Despite the low levels of secreted protein for three of the glycosylation mutants, ADA2 enzyme activity was detected from all samples (Fig. 2D). When normalized to protein concentration, the enzyme activity of mutant proteins was within 30% of wild-type ADA2 (Fig. 2E), illustrating that N-linked glycosylation is required for ADA2 secretion but less essential for its enzymatic function.

Gain-of-glycosylation mutations leading to attachment of oligosaccharides to unexpected regions of a protein have been implicated in immunodeficiency syndromes (9). We explored gnomAD and identified two predicted gain-of-glycosylation *CECR1* variants, I480T and K481N. Both variants are extremely rare in the general population and they create an additional NXS/T sequence at the C-terminus. Interestingly, only I480T was predicted to be glycosylated *in silico* (see Fig. E3A in Online Repository). This was verified experimentally as expression of I480T mutant resulted in an increased MW by 2 kD while K481N substitution displayed minimal impact (see Fig. E3B in Online Repository; note blurring of upper border of band). Functionally, the secretion of I480T mutant was severely compromised compared to wild-type ADA2, while the K481N mutant had moderate effects on ADA2 secretion (see Fig. E3C in Online Repository). ADA2 enzyme activity in the medium of cells expressing I480T and K481N mutants was directly proportional to the amount of secreted ADA2 protein. These data highlight that precise glycosylation is necessary for trafficking of ADA2 to the secretory pathway.

DADA2 is an intriguing disease with wide-spectrum of clinical findings spanning autoinflammation and immunodeficiency. Our work identifies not only a new set of causal

mutations but more generally demonstrates the importance of N-glycosylation for normal ADA2 function. To our knowledge this is the first report of an autoinflammatory disease caused by a defect in glycosylation. Interestingly, patients with congenital defects of glycosylation (CDG) can show features of skin inflammation, stroke-like episodes and immunodeficiency. The etiology of these manifestations is unclear, and whether ADA2 glycosylation and secretion is affected in CDG warrants investigation.

Pui Y. Lee MD, PhD <sup>a,b,\*</sup>  
Yuelong Huang PhD <sup>b</sup>  
Qing Zhou PhD <sup>c</sup>  
Oskar Schnappauf PhD <sup>c</sup>  
Michael S. Hershfield MD <sup>d</sup>  
Ying Li <sup>b</sup>  
Nancy J. Ganson <sup>d</sup>  
Natalia Sampaio Moura <sup>c</sup>  
Ottavia M. Delmonte MD <sup>e</sup>  
Scellig S. Stone MD, PhD <sup>f</sup>  
Michael J. Rivkin MD <sup>g</sup>  
Sung-Yun Pai MD <sup>h,i</sup>  
Todd Lyons MD <sup>j</sup>  
Robert P. Sundel MD <sup>a</sup>  
Victor W. Hsu MD <sup>b</sup>  
Luigi D. Notarangelo MD <sup>e</sup>  
Ivona Aksentijevich MD <sup>c</sup>  
Peter A. Nigrovic MD <sup>a,b</sup>

- a. Division of Immunology, Boston Children's Hospital, Boston, MA 02115  
b. Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Boston, MA 02115  
c. Inflammatory Disease Section, National Human Genome Research Institute, Bethesda, MD 20892  
d. Department of Medicine and Biochemistry, Duke University School of Medicine, Durham, NC 27710  
e. Laboratory of Clinical Immunology and Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892  
f. Department of Neurosurgery, Boston Children's Hospital, Boston, MA 02115  
g. Departments of Neurology, Psychiatry and Radiology, Boston Children's Hospital, Boston, MA 02115

- 180 h. Division of Hematology-Oncology, Boston Children's Hospital, Boston, MA 02115  
181 i. Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA 02115  
182 j. Division of Emergency Medicine, Boston Children's Hospital, Boston, MA 02115  
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**FIGURE LEGENDS**

**Figure 1.** Deficiency of ADA2 associated with a novel mutation that disrupts N-linked glycosylation. A) Photographs of erythematous papules and livedo reticularis. B) Non-contrast CT images of the brain demonstrating intracranial hemorrhage episodes occurring 9 days apart. C) Quantitation of plasma ADA2 activity using a spectrophotometric assay. D) Pedigree illustration of *CECR1* variants in the patient and parents. NM, no mutation. \*Paternal genotype is inferred. E) Chromatograms show patient's complementary DNA (top) and genomic DNA (middle) sequences for the c.385A>C variant. Deletion break-point sequence is shown in the bottom panel. F) ELISA of ADA2 in the medium and G) western blot of intracellular ADA2 expression in 293T cells. Wedge indicates a gradient of plasmid concentration. H) Western blot of wild-type and mutant ADA2. I) ELISA of secreted wild-type ADA2 with or without tunicamycin (TM; 1  $\mu$ M). J) western blot of intracellular ADA2 expression with or without TM (1  $\mu$ M). K) Confocal microscopy of ADA2 and giantin staining. Arrow indicates the Golgi apparatus. L) Confocal microscopy of ADA2 and ERp72 staining. Images are representative of 3 independent experiments. \*  $p < 0.05$ .

**Figure 2.** N-linked glycosylation at three sites is required for ADA2 secretion. A) Western blot of ADA2 mutants with disrupted N-glycosylation sites. B) Confocal microscopy of ADA2 and ERp72 staining. Arrow indicates perinuclear foci consistent with Golgi apparatus. C) ELISA of ADA2 mutants. D) Enzymatic activity of wild-type and mutant ADA2 quantified using a spectrophotometric assay. ADA activity is coupled to consumption of the substrate NADH measured at OD340. E) Enzymatic activity of ADA2 mutants in the medium of transfected cells normalized to the amount of secreted protein. Data are representative of 3 independent experiments. \*  $p < 0.05$ .

Figure 1.

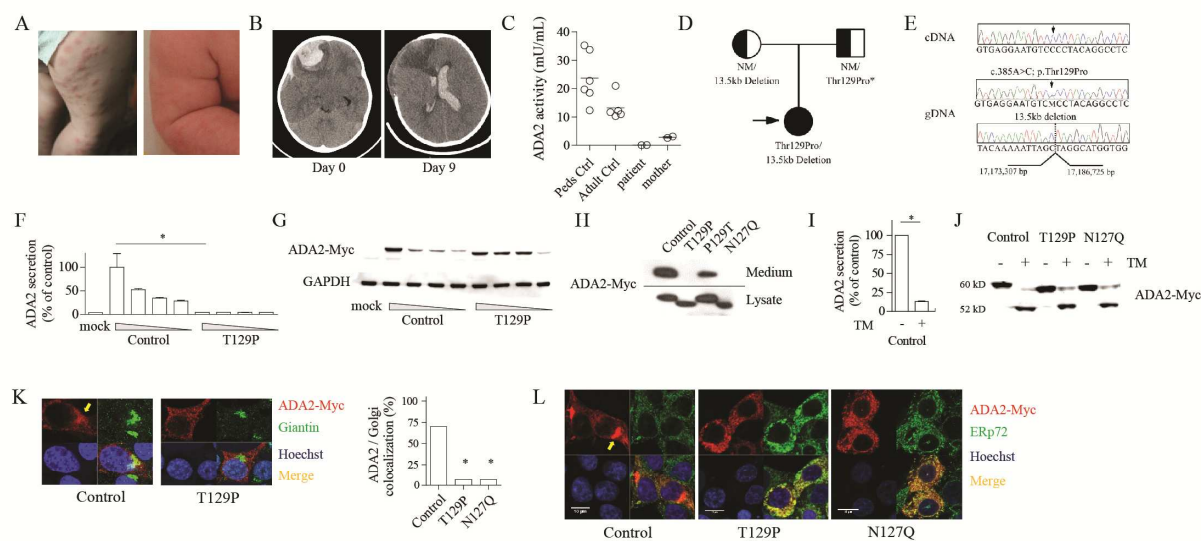
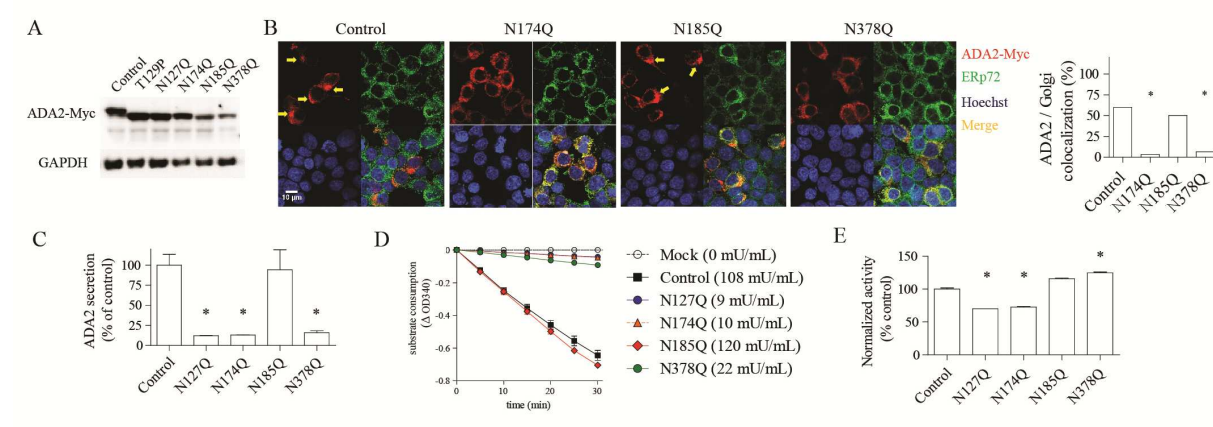


Figure 2.



**Disrupted N-linked glycosylation as a disease mechanism in deficiency of ADA2**

Online Repository

Methods

Figure Legends

Table E1. Laboratory parameters during the subject's clinical course.

Table E2. Primers used for site-directed mutagenesis

Table E3. Variants in gnomAD database affecting ADA2 glycosylation sites

## METHODS

**Human subjects:** All studies were approved by the Institutional Review Boards of Boston Children's Hospital (protocol 04-09-113R), the Brigham and Women's Hospital (protocol 2009P002379) and the National Institutes of Health (protocol 94-HG-0105). Informed consent was obtained from healthy controls and the patient's family.

**Clinical summary:** The patient is a 2 year-old female with a history of frequent viral infections and intermittent rash characterized as erythematous papules and livedo reticularis (Fig. 1A). She presented with fatigue, poor oral intake and failure to thrive with weight trending from the 25<sup>th</sup> to 7<sup>th</sup> percentile. Laboratory studies were notable for chronic anemia and elevated inflammatory markers (Table E1). Infectious disease work-up was unrevealing and neurology evaluation including brain MRI and lumbar puncture were within normal limits. Autoinflammatory syndromes were considered but commercial sequencing of *NLRP3*, *ELA2*, *LPIN2*, *MEFV*, *MVK*, *PSTPIP1* and *TNFRSF1A* did not identify any candidate variants (not shown).

As serum IL-6 levels were elevated at 24 pg/mL (normal <5 pg/mL) on a serum cytokine panel, tocilizumab was initiated, which led to a rapid clinical response and improvement of laboratory parameters. Her energy levels and appetite returned normal and the elevated inflammatory markers (erythrocyte sedimentation rate and C-reactive protein), thrombocytosis, and anemia improved within several weeks (Table E1). Two months later, however, she was brought to the emergency department for recurrent emesis and was found to have altered mental status, severe hypertension and bradycardia. CT scan showed a large right-sided acute-on-chronic subdural hemorrhage and right frontal intracerebral hemorrhage accompanied by mass effect and midline shift (Fig. 1B). The hematoma was promptly evacuated and the patient was

stabilized. Nine days later, she developed prolonged seizures and was found to have a large hematoma near the left basal ganglia with hemorrhage into the ventricles (Fig. 1B). Emergency surgery was again performed for clot evacuation and an external ventricular drain was placed.

The patient was started on etanercept after the second episode of catastrophic hemorrhagic stroke and tocilizumab was discontinued. Consistent with the reported benefit of anti-tumor necrosis factor therapy for DADA2 (1-3), she had no further stroke episodes and markers of systemic inflammation also remained normal (Table E1). She made a remarkable recovery and has minimal neurologic deficits at 18 month follow-up.

**Reagents:** All molecular biology reagents were purchased from New England Biolabs (Ipswich, MA) unless otherwise stated. Primers were produced by Integrated DNA Technologies (Coralville, IA). For immunoblotting, monoclonal antibody to Myc (clone 9E10) and GAPDH (mAbcam 9484), and secondary goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) and mouse anti-His tag conjugated to HRP were purchased from Abcam (Cambridge, MA) and Biolegend (San Diego, CA), respectively. For confocal microscopy, rabbit antibodies to giantin (ab80864) and ERp72 (clone D70D72) were purchased from Abcam and Cell Signaling Technology (Danvers, MA), respectively. Hoechst33342 was purchased from Thermo Fisher Scientific (Waltham, MA). All reagents for ADA2 activity assay were purchased from Sigma-Aldrich (St. Louis, MO).

**Plasmid construction:** *CECR1* was amplified from control and patient cDNA by PCR using the primers containing restriction digest sequences (Forward: CAACCGAATTCACCATGTTGGTGGATGGCCCAT; Reverse:

CAACCAAGCTTCTTTGTAGCCACATCTGCT). PCR products and pcDNA3.1(-)/Myc-His A vector (Thermo Fisher) were digested with HindIII and EcoRI. Products were gel purified and ligated using T4 ligase prior to transformation into competent DH5 $\alpha$ . Selected clones were verified DNA sequencing and plasmids for transfection were produced using Purelink HiPure MiniPrep Kit (Thermo Fisher).

**Site-directed mutagenesis:** Q5Site-Directed Mutagenesis Kit was used to introduce single base substitutions. Primers were designed using the NEBase Changer web tool (<http://nebasechanger.neb.com/>). Reversion of the patient's T129P mutation (P129T) was performed to eliminate potential confounding effects from common polymorphisms. The resulting plasmid encoding wild-type ADA2 was used as the template for other N-glycosylation mutants. Primer sequences used for site-directed mutagenesis are provided in Table E2 in the Online Repository. Sequencing of plasmids was performed by Eton Bioscience (Cambridge, MA) using provided universal T7-promoter and BGH-Reverse primers.

**Cell culture and transfection:** Human embryonic kidney 293T cells were cultured in DMEM with 10% FCS and penicillin-streptomycin (Thermo Fisher). Plasmid transfection was performed using Fugene 6 reagent (Promega, Madison, WI) following manufacturer's instructions. At 72 hours post-transfection, medium and cells were collected for analysis. In some experiments, a gradient of plasmid concentrations was used (1, 0.5, 0.25 and 0.1  $\mu$ g per  $2 \times 10^5$  cells).

**DNA sequencing and variant analysis:** Sanger sequencing of *CECR1* was as described previously. A custom-made Multiplex ligation-dependent probe amplification (MLPA) kit was used to screen for genomic deletions (MRC-Holland, Amsterdam, the Netherlands) (4). Pathogenicity of ADA2 variants were analyzed using SIFT and Polyphen2 (5, 6). N-glycosylation site analysis was performed using NetNGlyc 1.0 (<http://www.cbs.dtu.dk/services/NetNGlyc/>). Protein sequence alignment was performed using COBALT (Constraint-based Multiple Alignment Tool) (7).

**ADA2 activity assay:** The patient was initially shown to be deficient in plasma ADA2 enzymatic activity by testing performed at Duke University using two independent assays: a screening spectrophotometric assay that couples the release of ammonia from adenosine with the consumption of NADH catalyzed by glutamic dehydrogenase in the presence of  $\alpha$ -ketoglutarate (8, 9) (as modified by NJ Ganson, SJ Kelly, MS Hershfield, unpublished); and a somewhat more sensitive HPLC-based method to directly quantify the conversion of adenosine to inosine (as described (4), but performed in 100 mM Tris-acetate buffer, pH 7.0 instead of phosphate buffer). With both assays, the activities of ADA1 and ADA2 were distinguished using the selective ADA1 inhibitor EHNA (erythro-9-Amino- $\beta$ -hexyl- $\alpha$ -methyl-9H-purine-9-ethanol hydrochloride). Further studies of ADA2 activity in human plasma and culture medium of transfected cells in this study were performed using the spectrophotometric assay.

**ADA2 ELISA:** ADA2 protein in the medium of transfected cells were quantified using enzyme-linked immunosorbent assay (ELISA). 96-well plates were pre-coated with anti-Myc antibodies overnight (1  $\mu$ g/mL) and blocked with PBS with 5% bovine serum albumin. After incubation

with samples for 1 hour, wells were washed and secondary antibody (mouse anti-His tag conjugated to HRP; 1:1000 dilution) was added for 1 hour. After final washes, plates were developed with TMB substrate (Biolegend) and read by a Synergy Hybrid Microplate reader. Samples were quantified using a standard curve derived from known concentrations of ADA2.

**Western blot:** Transfected cells ( $5 \times 10^5$ ) were pelleted and directly resuspended in 100  $\mu$ l of Laemmli's buffer with 2-mercaptoethanol. SDS PAGE was performed using 10% polyacrylamide gel. After transfer to PVDF membrane and blocking with 5% dry milk, primary antibodies for Myc and GAPDH were applied for 1 hour. Membranes were washed in TBS with 0.1% Tween 20 and HRP-conjugated goat anti-mouse IgG (1:5000) was used for detection.

**Confocal microscopy:** 293T cells were plated on glass coverslips in 6-well plates ( $5 \times 10^5$  cells / well) and transfected as described above. After 48 hours, cells were fixed using 4% paraformaldehyde for 15 minutes followed by permeabilization using PBS with 0.2% saponin and 1% bovine serum albumin (BSA) for 30 minutes. After washing with PBS with 1% BSA and blocking in PBS with 5% goat serum, cells were incubated with primary antibodies overnight. Coverslips were washed and incubated with fluorophore-conjugated secondary antibodies and Hoechst 33342 for 2 hours before final washes and mounting onto slides using FluorMount. Images were acquired using a Zeiss Axio Observer Z1 Inverted Microscope with Zeiss LSM 800 with Airyscan confocal system (Zeiss, Oberkochen, Germany). For quantification of cells with ADA2 and Golgi staining co-localization (expressed as % of cells with ADA2 expression), thirty cells with ADA2 expression were analyzed per sample and bars represent pooled data from two independent experiments.

**Statistical Analysis:** Differences between groups were analyzed by the unpaired Student's t test.

All tests were two-sided, and  $P < 0.05$  was considered significant. Statistical analyses were performed using Prism 5.0 software (GraphPad Software, La Jolla, CA). For bar graphs, bars represent mean  $\pm$  SEM of duplicate samples. All data are representative of 2 or 3 independent experiments.

### Figure Legends

**Figure E1.** Identification of a large deletion in *CECR1* by multiplex ligation-dependent probe amplification. A) Schematic illustration of the identified 13.5 kb deletion found in the patient and her mother. B) MLPA analysis of reference probe to *CECR1* probe ratio in various regions of the gene. C) Enzymatic activity of wild-type and mutant ADA2 quantified using a spectrophotometric assay. \*  $p < 0.05$

**Figure E2.** Consensus N-glycosylation sites of ADA2 are highly conserved. A) Sequence alignment of N127 and T129 across various species. B) *in silico* prediction of native N-linked glycosylation sites on wild-type ADA2 using NetNGly1.0. A potential score above 0.5 is considered predictive of N-linked glycosylation. C) Sequence alignment of predicted glycosylation sites across various species.

**Figure E3.** Gain-of -glycosylation variants affect ADA2 secretion. A) *in silico* prediction of N-linked glycosylation sites on ADA2 variants using NetNGly1.0. B) Western blot of intracellular ADA2 protein in cells expressing wild-type or gain-of-glycosylation ADA2 mutants. C) ELISA quantification (left) and enzyme activity (right) of secreted ADA2 in the medium of cells expressing wild-type or gain-of-glycosylation ADA2 mutants. Bars represent mean  $\pm$  SEM of duplicate samples. Data are representative of 2 independent experiments. \*  $p < 0.05$ .

**Table E1.** Laboratory parameters during the subject's clinical course.

| Age of Patient                                |                 | 2 yr 1 mo     | 2 yr 5 mo   | 2 yr 8 mo  | 4 yr 3 mo  |
|-----------------------------------------------|-----------------|---------------|-------------|------------|------------|
| Treatment                                     |                 | Pre-treatment | Tocilizumab | Etanercept | Etanercept |
| Duration                                      |                 | -             | 2 months    | 2 months   | 18 months  |
| Weight percentile for age                     |                 | 7             | 12          | 34         | 66         |
|                                               | (Ref range)     |               |             |            |            |
| <b>WBC (k cells / <math>\mu</math>L)</b>      | (5.9 - 10.8)    | 10.6          | 6.9         | 6.8        | 5.9        |
| % Neutrophil                                  | (30 - 73)       | 70            | 59          | 55         | 45         |
| % Lymphocyte                                  | (11 - 59)       | 22            | 33          | 30         | 36         |
| % Monocyte                                    | (4 - 8)         | 4             | 4           | 14         | 18         |
| % Eosinophil                                  | (1 - 4)         | 1             | 1           | 0          | 1          |
| <b>Hemoglobin (g / dL)</b>                    | (11.1 - 12.9)   | 8.2           | 10.9        | 9.5        | 11.7       |
| <b>Platelet (k cells / <math>\mu</math>L)</b> | (208 - 410)     | 681           | 190         | 267        | 245        |
| <b>AST (U / L)</b>                            | (2 - 40)        | 31            | 61          | 46         | 40         |
| <b>ALT (U / L)</b>                            | (3 - 30)        | 10            | 22          | 13         | 18         |
| <b>ESR (mm / hr)</b>                          | (1 - 20)        | 69            | 2           | 7          | 4          |
| <b>CRP (mg / dL)</b>                          | (< 0.50)        | 16.8          | <0.03       | 0.22       | <.03       |
| <b>IgG (mg / dL)</b>                          | (400 - 1,300)   | 672           | 417         | 780        | 1,051      |
| <b>IgM (mg / dL)</b>                          | (30 - 120)      | 32            | 20          | 46         | 77         |
| <b>IgA (mg / dL)</b>                          | (20 - 230)      | 39            | 36          | 24         | 69         |
| <b>Anti-nuclear antibodies</b>                | (Negative)      | Negative      | Negative    |            |            |
| <b>Lymphocyte panel</b>                       |                 |               |             |            |            |
| CD3+ %                                        | (49 - 84)       | 60            |             |            |            |
| CD3+ (cells / $\mu$ L)                        | (1,900 - 6,200) | 2792          |             |            |            |
| CD3+ CD4+ %                                   | (28 - 52)       | 44            |             |            |            |
| CD3+ CD4+ (cells / $\mu$ L)                   | (1,300 - 3,400) | 2060          |             |            |            |
| CD3+ CD8+ %                                   | (13 - 30)       | 14            |             |            |            |
| CD3+ CD8+ (cells / $\mu$ L)                   | (620 - 2,000)   | 647           |             |            |            |
| CD19+ %                                       | (13 - 37)       | 35            |             |            |            |
| CD19+ (cells / $\mu$ L)                       | (620 - 2,600)   | 1618          |             |            |            |
| CD3- CD16+ / CD56+ %                          | (3 - 15)        | 5             |             |            |            |
| CD3- CD16+ / CD56+ (cells / $\mu$ L)          | (160 - 1,100)   | 221           |             |            |            |
| <b>Serum Cytokines (pg / mL)</b>              |                 |               |             |            |            |
| Interleukin 2                                 | (<=22)          | <5            |             |            |            |
| Interleukin 2 Receptor                        | (<=1033)        | 1383          |             |            |            |
| Interleukin 12                                | (<=5)           | <5            |             |            |            |
| Interferon gamma                              | (<=5)           | <5            |             |            |            |
| Interleukin 4                                 | (<=5)           | <5            |             |            |            |
| Interleukin 5                                 | (<=5)           | <5            |             |            |            |
| Interleukin 10                                | (<=18)          | 7             |             |            |            |
| Interleukin 13                                | (<=5)           | <5            |             |            |            |
| Interleukin 1 beta                            | (<=36)          | <5            |             |            |            |
| Interleukin 6                                 | (<=5)           | 24            |             |            |            |
| Interleukin 8                                 | (<=5)           | <5            |             |            |            |
| TNF alpha                                     | (<=5)           | <5            |             |            |            |

**Table E2.** Primers used for site-directed mutagenesis

| Substitution      | Forward                    | Reverse                   |
|-------------------|----------------------------|---------------------------|
| P129T (reversion) | TCACCTACAGGCCTCACTGCC      | CATTCCTCACCAGCCAGTCCATAG  |
| N127Q             | TCACCTACAGGCCTCACTGCC      | CTTGCCTCACCAGCCAGTCCATAG  |
| N174Q             | GCGGGTGCAGCAGGTCAGT        | TTCCGATAATCCTCCAGCAG      |
| N185Q             | AGCTTGCTGAGGCAGTTCAGTCTGG  | GTCATCAAACCTCAGTGACGTTT   |
| N378Q             | CTACCAGAATCGGCCATGGATTTG   | TCTGCAGCATCAGAGCATCCAGAAT |
| I480T             | CATGAACTCTACCAAGTACAGTACCC | GCCAGCTGTTTGAGGGTC        |
| K481N             | ACTCTATCAATTACAGTACCCTGTTG | TCATGGCCAGCTGTTTGA        |

**Table E3.** Variants in gnomAD database affecting ADA2 glycosylation sites.

| Variant in <i>CECR1</i>         | Type       | Consequence | Effect on NXS/T sequence | Allele count | Allele Frequency | PolyPhen2 Prediction | SIFT Prediction |
|---------------------------------|------------|-------------|--------------------------|--------------|------------------|----------------------|-----------------|
| 22:17688123 T / A (rs547716532) | missense   | p.Asn127Ile | Loss                     | 3            | 1.22E-05         | Possibly Damaging    | Deleterious     |
| 22:17688123 T / C (rs547716532) | missense   | p.Asn127Ser | Loss                     | 1            | 4.06E-06         | Probably Damaging    | Deleterious     |
| 22:17684647 T / G (rs752890414) | missense   | p.Thr187Pro | Loss                     | 1            | 4.07E-06         | Probably Damaging    | Deleterious     |
| 22:17663594 G / A (rs765154508) | missense   | p.Thr380Ile | Loss                     | 2            | 8.12E-06         | Probably Damaging    | Deleterious     |
| 22:17663595 T / C               | missense   | p.Thr380Ala | Loss                     | 1            | 3.23E-05         | Possibly Damaging    | Deleterious     |
| 22:17662713 A / G               | missense   | p.Ile480Thr | Gain                     | 1            | 3.22E-05         | Probably Damaging    | Deleterious     |
| 22:17662466 C / A (rs774795047) | missense   | p.Lys481Asn | Gain                     | 4            | 1.62E-05         | Benign               | Deleterious     |
| 22:17688116 G / A               | synonymous | p.Thr129Thr | None                     | 2            | 8.12E-06         | N/A                  | N/A             |
| 22:17687981 G / A (rs746651975) | synonymous | p.Asn174Asn | None                     | 4            | 1.63E-05         | N/A                  | N/A             |
| 22:17687980 C / T (rs757839578) | missense   | p.Val175Ile | None                     | 1            | 4.06E-06         | Benign               | Tolerated       |
| 22:17687980 C / A (rs757839578) | missense   | p.Val175Phe | None                     | 1            | 4.06E-06         | Benign               | Deleterious     |
| 22:17687975 A / T               | synonymous | p.Thr176Thr | None                     | 1            | 4.06E-06         | N/A                  | N/A             |
| 22:17687975 A / G               | synonymous | p.Thr176Thr | None                     | 1            | 4.06E-06         | N/A                  | N/A             |
| 22:17684651 A / G (rs758525993) | synonymous | p.Asn185Asn | None                     | 8            | 3.26E-05         | N/A                  | N/A             |
| 22:17663599 G / A (rs763728425) | synonymous | p.Asn378Asn | None                     | 1            | 4.06E-06         | N/A                  | N/A             |
| 22:17663598 T / C (rs561591791) | missense   | p.Thr379Ala | None                     | 10           | 4.06E-05         | Benign               | Tolerated       |
| 22:17663598 T / A (rs561591791) | missense   | p.Thr379Ser | None                     | 2            | 8.12E-06         | Benign               | Tolerated       |
| 22:17663596 A / T               | synonymous | p.Thr379Thr | None                     | 1            | 4.06E-06         | N/A                  | N/A             |

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