Human ADA2 deficiency is characterized by the absence of an intracellular hypoglycosylated form of adenosine deaminase 2

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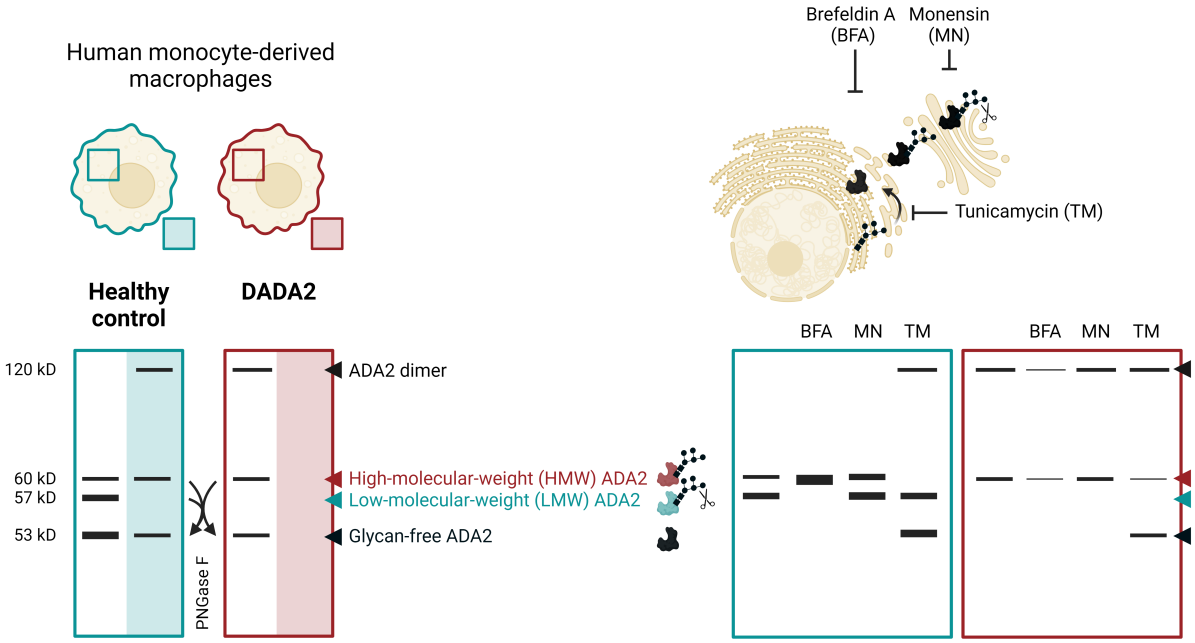
Abstract

Human deficiency of adenosine deaminase 2 (DADA2) is a rare autoinflammatory disease with a complex clinical phenotype of recurrent fever, vasculitis and stroke as well as immunodeficiency and bone marrow failure. It is caused by pathogenic variants in *ADA2* that lead to impaired ADA2 protein secretion and reduced deaminase activity. However, the mechanisms driving the disease on a cellular level remain elusive. Here, we analyze protein expression of mutant ADA2 in human monocyte-derived macrophages from 10 DADA2 patients. We identify a low-molecular-weight (LMW) form of ADA2 expressed exclusively intracellularly in healthy control macrophages. This LMW-ADA2 is hypoglycosylated compared with secreted high-molecular-weight (HMW) ADA2 and it is absent in DADA2 patients' monocyte-derived macrophages. We confirm the absence of LMW-ADA2 upon overexpression of 11 pathogenic *ADA2* variants in HEK293T cells. By analyzing protein trafficking and glycan processing, we show that LMW-ADA2 is generated from wild-type HMW-ADA2 after transfer to the Golgi apparatus. In conclusion, we describe a previously unreported intracellular hypoglycosylated form of ADA2 and establish the absence of this LMW-ADA2 as a cellular characteristic of DADA2.

Graphical abstract



Introduction

Human deficiency of adenosine deaminase 2 (DADA2) is an inborn error of immunity caused by biallelic deleterious mutations in the *ADA2* gene (1, 2). The disease manifests with a diverse phenotype displaying features of both autoinflammation and immunodeficiency (3). Almost 800 missense or predicted loss-of-function variants in *ADA2* are listed in the Genome Aggregation Database (gnomAD v4.0.0) (4). In the Infevers registry, 114 *ADA2* variants are classified as (likely) pathogenic (5). Complete loss-of-function variants are more likely to cause bone marrow failure. Missense variants which account for 80% of pathogenic variants are found in patients with a primarily vasculitis phenotype as well as in those presenting with cytopenias and hypogammaglobulinemia (6, 7).

How mutations in *ADA2* give rise to this diverse phenotype, even in patients with identical genotypes, is not understood. Inflammatory symptoms of the disease typically respond well to TNF inhibitors (TNFi) (8). Insufficient resolution of bone marrow failure in response to this treatment however often necessitates hematopoietic stem cell transplantation and disease lethality still remains 8% (9). To develop improved treatment options, we require a better understanding of the pathomechanisms underlying the immunological and clinical phenotype of DADA2.

ADA2 is a 59-kD glycoprotein with a signal peptide that mediates secretion of the protein via the endoplasmic reticulum (ER), and exhibits extracellular adenosine deaminase activity as a homodimer (10). The protein has four N-glycosylation sites and insufficient glycosylation leads to ER retention and intracellular aggregate formation of ADA2 transfected into HEK293T cells (11, 12). Impaired protein secretion and absent serum ADA2 enzyme activity have been established as characteristics shared by the majority of pathogenic ADA2 variants (13). Mechanistic approaches to the pathophysiology of ADA2-deficient cells have therefore been based on impaired extracellular adenosine deamination driving inflammation (14, 15). This assumption fails to acknowledge the weak affinity of ADA2 for adenosine and the presence of functional ADA1 in

DADA2 patients (16). Research into alternative functions of the ADA2 protein and DADA2-causing variants is therefore warranted. Biochemical characterization of pathogenic ADA2 variants has until now only been performed in a HEK293T overexpression system with supraphysiological intracellular levels of ADA2 while expression of the mutant protein has been shown to be minimal in DADA2 CD14+ monocytes (13, 17). Disease-associated ADA2 variants have to date hardly been studied in their physiological milieu.

In this study, we analyze trafficking and glycosylation profile of endogenous ADA2 expressed in primary human immune cells from healthy donors and DADA2 patients. We provide evidence of distinct glycosylation patterns of ADA2 expressed in human macrophages and show that DADA2 macrophages are characterized by the absence of intracellular LMW-ADA2.

Results

In vitro macrophage differentiation partially restores ADA2 protein expression in DADA2 monocytes

Of the circulating leukocytes, monocytes express ADA2 most abundantly. We analyzed ADA2 protein expression and *ADA2* mRNA levels in peripheral blood monocytes of 10 DADA2 patients and three heterozygous carriers (**Table 1 and Figure S1A+B**). CD14+ monocytes from DADA2 patients showed very low or absent ADA2 protein expression despite normal *ADA2* mRNA levels in patients with missense mutations which account for 80% of pathogenic variants found in DADA2 patients (**Figure 1A and Figure S2A**). Upon macrophage differentiation, we observed an increase in mutant ADA2 protein expression in the patient cells while ADA2 protein expression was reduced in healthy control (HC) human monocyte-derived macrophages (HMDM) (**Figure 1C**). Successful differentiation of DADA2 HMDM was achieved both with granulocyte–macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) (**Figure S2B**), which confirms the reported restoration of macrophage differentiation of

monocytes from DADA2 patients under treatment with TNFi (8). Interestingly, western blotting of pathogenic ADA2 protein variants expressed in DADA2 HMDM revealed the presence of only a high-molecular-weight (HMW) band of around 60 kD compared to HC HMDM displaying two bands at 60 and 57 kD, with the low-molecular-weight (LMW) band being more pronounced (**Figure 1B and Figure S2C**). A novel ADA2 variant serendipitously created in the process of generating an ADA2$^{-/-}$ Jurkat cell line showed the same phenotype (**Figure S2D**). The molecular weight of secreted wild-type (WT) ADA2 in the supernatant of HC HMDM corresponded to the intracellular HMW form – the only form expressed in DADA2 HMDM (**Figure 1B**). We confirmed the secretion of HMW-ADA2 in the monocytic cell lines U-937 and THP-1 (**Figure S2E**). HMDM from DADA2 patient P8 showed residual secretion of HMW-ADA2 with a molecular weight equivalent to secreted WT ADA2 (**Figure S2F**). This HMW-form of ADA2 also corresponded to the ADA2 form detected in human serum (**Figure 1C**). Importantly, overexpression of tagged WT ADA2 and several confirmed pathogenic variants in HEK293T cells uniformly led to predominant intracellular expression of HMW-ADA2 (63 kD) which was equal in size to the secreted protein (**Figure 1D**). In line with our findings in DADA2 HMDM, intracellular LMW-ADA2 was absent after overexpression of mutant ADA2 in HEK293T cells (**Figure 1D**). Interestingly, this band was still present – albeit at lower intensity – in the variants p.N127Q and p.W326R, two variants not found in DADA2 patients but created for the study of ADA2 glycosylation and dimerization, respectively (10, 11). Collectively, we show that HC HMDM intracellularly express a form of ADA2 that is lower in molecular weight than the secreted protein. This LMW-form is absent in DADA2 HMDM which only express the HMW-form intracellularly.

Human monocyte-derived macrophages intracellularly express a hypoglycosylated low-molecular-weight form of ADA2 that is absent in DADA2 macrophages

Differences in the molecular weight of a protein can be caused by posttranslational protein modifications and N-glycosylation of ADA2 has previously been confirmed (10, 12, 17). Therefore, we initially hypothesized that the LMW-form of ADA2 unique to cells expressing WT ADA2 corresponded to glycan-free ADA2. To test this, we performed glycan removal on lysates and supernatants containing ADA2 protein. Complete removal of N-glycans by Peptide-N-Glycosidase F (PNGase F) yielded a difference in molecular weight of 7 kD between HMW-ADA2 and glycan-free ADA2 both overexpressed in HEK293T cells and endogenously expressed in the monocytic cell lines U-937 and THP-1 as well as HMDM (**Figure 2A+B**), revealing that LMW-ADA2 was in fact slightly larger than glycan-free ADA2. Importantly, the anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam) was superior in detecting LMW-ADA2 but poorly detected glycan-free ADA2 (**Figure S3A**). Glycan removal by PNGase F eliminated the differences in molecular weight between the LMW-form and the HMW-form in HMDM, yielding a single band of glycan-free ADA2 at 53 kD (**Figure 2B+C**). Hence, the difference in molecular weight between intracellular WT ADA2 (LMW) and secreted WT ADA2 or intracellular pathogenic ADA2 (both HMW) was due to differential protein glycosylation. Of note, glycan removal yielded a smaller band of 53 kD in addition to the glycan-removed 56 kD protein for tagged WT ADA2 overexpressed in HEK293T cells that was absent for all tested pathogenic variants and did not appear after inhibition of N-glycosylation by tunicamycin (**Figure 2A**). As this band was absent after glycan removal of endogenous WT ADA2, its physiological relevance however is unclear.

Overall, we showed that the difference in size between HMW- and LMW-ADA2 is due to differential protein glycosylation.

Inhibition of N-glycosylation or ER glucosidases affects the glycosylation of high-molecular-weight ADA2

In order to better characterize the glycosylation of endogenous ADA2, we inhibited N-glycosylation in HMDM and the monocytic cell lines U-937 and THP-1 by 24-hour treatment with tunicamycin. We found that this treatment only acted on the fully glycosylated, fainter HMW-band while LMW-ADA2 remained unchanged in size (**Figure 2C+D**). In accordance with previous findings (12), we showed that adenosine deaminase activity of ADA2 was preserved after glycan removal while treatment with tunicamycin reduced enzymatic activity. This was true for ADA2 overexpressed in HEK293T cells as well as endogenous ADA2 from HC HMDM (**Figure 3A+B**). WT and mutant ADA2 were both sensitive to glycan removal by Endo H (**Figure 3C**), albeit incomplete compared to PNGase F (**Figure 3C**), hinting at the presence of high-mannose glycans in both LMW- and HMW-ADA2 expressed in HMDM. In contrast with PNGase F treatment, glycan removal by Endo H revealed differences between WT and mutant ADA2. Compared with mutant ADA2, Endo H-treated WT ADA2 visualised as a smear on western blot as opposed to the clean single band observed for mutant ADA2 in DADA2 HMDM (**Figure 3C**). This suggests that WT ADA2 underwent additional glycan processing as complex glycans are less sensitive to Endo H. Treatment with castanospermine, an inhibitor of the ER glucosidases I and II, caused an additional increase in the size of HMW-ADA2 in both HC and DADA2 HMDM (**Figure 3D**). As seen previously upon incubation with tunicamycin, the LMW-form of ADA2, which is absent in DADA2 HMDM, was unaffected by this treatment (**Figure 3D**). We confirmed this finding in the three ADA2-expressing cell lines U-937, THP-1 and Jurkat (**Figure S3B**). Since the glycan pattern of LMW-ADA2 was unaffected by treatment with tunicamycin – an inhibitor of the initial step of N-glycosylation – we concluded that glycosylation of this form must have occurred prior to the treatment incubation period, suggesting high stability of LMW-ADA2. Accordingly, LMW-ADA2 was responsive to prolonged incubation with tunicamycin at a lower concentration (**Figure S3C**). The same was true for inhibition of ER glucosidases: the more stable LMW-ADA2 was unaffected

by 24-hour incubation with castanospermine and the detected LMW-form had likely already matured before the beginning of the incubation period. Considering that HMW-ADA2 is responsive to both treatments, it is unlikely that LMW-ADA2 represents an intermediate – not yet fully glycosylated – form of yet unsecreted ADA2. We hypothesize that it might instead be generated by partial intracellular glycan trimming of the fully glycosylated form.

Since our data highlight the crucial role of N-glycosylation in ensuring successful folding and secretion of ADA2, we analyzed ADA2 expression in a patient with a mild form of *PMM2*-congenital disorder of glycosylation. ADA2 expression and secretion in CD14+ monocytes and HMDM were equal to HC and no differences in molecular weight were noted, indicating unaffected ADA2 glycosylation (**Figure 3E**). This might however be due to the patient's mild phenotype and residual PMM2 activity. The patient had normal serum ADA2 enzyme activity (**Figure 3E**).

Collectively, our findings suggest that the N-glycan structures of ADA2 contain high-mannose oligosaccharides and that the LMW-form is generated from HMW-ADA2 by intracellular glycan trimming.

The pathogenic ADA2 variant p.R169Q shows increased binding to proteins of the protein folding machinery

In order to elucidate whether the differential glycosylation of HMW- and LMW-ADA2 is associated with different protein functions, we performed immunoprecipitation followed by mass spectrometry (IP-MS) to analyze the interactome of WT ADA2 and the pathogenic variant p.R169Q transfected into HEK293T cells. p.R169Q displayed increased interaction with proteins involved in the ER quality control machinery for glycoproteins (**Figure 4A and Table S1**). Upon inhibition of N-glycosylation by tunicamycin, we observed a similar increase also for the WT protein (**Figure 4A and Table S1**), highlighting the importance of adequate N-glycosylation for

successful folding and secretion of ADA2. Additionally, we observed an increase in lysosomal proteins in the ADA2 interactome after treatment with tunicamycin, suggesting autophagic degradation of non-glycosylated ADA2. Among the induced interactors were several proteins of the chaperone-mediated autophagy pathway, e.g. LAMP2 and HSPA13, and we verified the presence of a KFERQ-related motif (QKFVE, amino acids 274-278) in the sequence of ADA2 (18). IP-MS of HC and DADA2 HMDM did not yield any specific binders, which was possibly due to low protein abundance in the pull down of endogenous ADA2 (**Figure S4A**). Notably, IP preferentially pulled down the HMW-form of ADA2 (**Figure S4B+C**). The experiment consequently did not allow us to draw conclusions about the interactome and potential distinctive function of LMW-ADA2.

Given the increased binding of mutant ADA2 to the ER folding machinery, we verified whether pathogenic ADA2 variants induced an increased ER stress response. In line with previous reports (13), we observed that some pathogenic ADA2 variants caused signs of activation of an ER stress response, measured by increased levels of CHOP (*DDIT3*) and BiP (*HSPA5*) (**Figure S5A**). These differences were however no longer present after down-titrating the amount of transfected DNA to achieve near-physiological protein levels (**Figure 4B and S5B+C**) and neither was there a difference in BiP expression in CD14+ monocytes from DADA2 patients compared to healthy controls (**Figure 4C**). Thus, our experiments revealed the interaction of the ADA2 variant p.R169Q overexpressed in HEK293T cells with mediators of the folding machinery and ER quality control despite lack of increased ER stress in the presence of pathogenic ADA2 variants. The interactome analyses were inconclusive regarding potential alternative functions of LMW-ADA2.

Impaired ADA2 secretion partially explains the intracellular predominance of fully glycosylated HMW-ADA2 in DADA2

We next sought to understand how protein glycosylation and trafficking differ between WT and mutant ADA2. We observed that fully glycosylated HMW-ADA2 persisted in cells transfected with the mutant p.R169Q after 24-hour incubation with tunicamycin (**Figure S4B**). Kinetics over 48 hours confirmed the increased presence of the glycosylated form of mutant ADA2 in comparison with WT ADA2 (**Figure S7A**). This trend was consistent in five additional pathogenic ADA2 variants (p.G47R, p.G47V, p.N127Q, p.T129P, p.P344L), albeit to different extents (**Figure 5A**). Of note, p.N127Q and p.T129P are glycosylation site variants and therefore show a reduced molecular weight of the glycosylated HMW-band. To understand whether the persistence of glycosylated ADA2 was due to increased protein stability of mutant ADA2, we performed a cycloheximide chase assay which revealed higher levels of intracellular ADA2 protein expression in HEK293T cells transfected with mutant ADA2 compared to WT 24 hours after inhibition of protein synthesis (**Figure 5B**). Additionally, the assay showed that LMW-ADA2, only present in cells transfected with WT ADA2, was more stable than the HMW-form (**Figure 5B**).

Since ADA2 is a secreted protein, protein secretion must be taken into account when assessing the intracellular signal reduction in this assay over time. Impaired secretion has previously been established as a characteristic of pathogenic ADA2 variants (2, 13). We confirmed the secretory defect in HMDM from our DADA2 cohort (**Figure 6A**). Overexpression in HEK293T cells recapitulated the endogenous behavior of the respective variants well (**Figure 6A**). While residual ADA2 enzyme activity largely correlated with preserved secretion, the catalytic domain variant p.N370K displayed absent deaminase activity despite comparatively high protein levels in the supernatant (**Figure 6A**). The partially preserved secretion of the variant p.G47R (2) was verified in HMDM from patient P8 who is homozygous for this variant (**Figure 6A**) and corresponded to higher serum ADA2 levels (**Figure 6A**) in the absence of serum deaminase activity (0.3 U/L).

Comparing the levels of increased ADA2 stability observed by cycloheximide chase assay with residual protein secretion, the variants p.T129P and p.R169Q that exhibited complete absence of secretion did indeed show the highest levels of intracellular ADA2 24 hours after inhibition of protein synthesis (**Figure 5B and 6A**). However, intracellular levels of the secreted mutant p.P344L were still higher compared to WT. By blocking the secretory pathway to impair secretion of WT ADA2, we confirmed that the secretory defect strongly contributed to the increased intracellular protein levels of mutant ADA2 (**Figure 6B and S7B**). Overall, we demonstrated the intracellular persistence of HMW-ADA2 in HEK293T cells transfected with pathogenic *ADA2* variants, which was largely due to impaired protein secretion.

Pathogenic ADA2 variants form intracellular dimers

Upon overexpression of mutant ADA2 in HEK293T cells, we observed intracellular ADA2 dimer formation by western blot of whole cell lysates (**Figure 7A**). This behavior had previously been reported only for the ADA2 variant p.L351Q (19). We observed that intracellular dimer formation largely correlated with impairment of ADA2 secretion although even variants with residual secretion, p.G47A, p.G47R, p.N127Q and p.P344L, showed increased dimer formation compared to WT. Inhibition of N-glycosylation – but not of the secretory pathway – led to aggregate formation of WT ADA2 (**Figure 7B and S8B**), confirming the role of N-glycosylation in ensuring the structural integrity of ADA2 (12). Geldanamycin has been described to induce refolding of misfolded proteins by inhibiting HSP90 (20). For ADA2, this treatment did not reduce intracellular aggregate formation or improve protein secretion (**Figure S8C**). Instead, we observed an increase in intracellular ADA2 dimers upon inhibition of the chaperone. Proteasome inhibition by delanzomib also increased intracellular ADA2 aggregates supporting the hypothesis that they are formed by misfolded ADA2 protein (**Figure S8C**). We also detected intracellular dimers in DADA2 HMDM, although to a lesser extent due to the strongly reduced protein expression of the

pathogenic variants (**Figure 7C**). These findings indicate that impaired folding due to mutations or defective N-glycosylation promotes intracellular aggregate formation of misfolded ADA2.

To elucidate whether these aggregates actively disturb ADA2 secretion or form as a result of impaired protein transport, we co-transfected HEK293T cells with varying amounts of WT and mutant ADA2. We found that the presence of the pathogenic variant p.R169Q caused dimer formation and impaired secretion of WT ADA2 overproportionally to the transfected ratios of WT and mutant (**Figure 7D**), indicating that aggregate formation may indeed actively disturb ADA2 secretion. In line with this finding, we also observed that ADA2 serum protein levels of heterozygous carriers were in fact only 25% of healthy control (**Figure S8D**), while intracellular protein levels represented 75% of HC in CD14+ monocytes (**Figure 7E**). Thus, intracellular aggregate formation of pathogenic ADA2 protein variants appears to contribute to impaired secretion of mutant ADA2, at least for the tested variant p.R169Q.

Inhibitors of the secretory pathway differentially affect ADA2 variants

We showed that the secretory defect of mutant ADA2 was phenocopied by WT ADA2 upon inhibition of the secretory pathway by brefeldin A (**Figure 6B**). However, this treatment also caused increased degradation of the mutants p.G47V, p.T129P and p.R169Q (**Figure 6B**). To determine whether this effect was specific to brefeldin A, we also inhibited protein trafficking with monensin. Brefeldin A blocks protein transport from the ER to the Golgi apparatus while monensin inhibits trans Golgi transport (21, 22). Treatment with monensin also caused retention of WT ADA2 but protein levels of mutant ADA2 were unaffected (**Figure 8A**). Moreover, incubation with monensin led to an increase in the LMW-band present in cells overexpressing WT ADA2. The differential behavior in response to the secretory pathway inhibitors brefeldin A and monensin was confirmed in DADA2 HMDM (**Figure 8B**). Since treatment with brefeldin A caused increased protein degradation in several pathogenic ADA2 variants while resulting in intracellular accumulation of WT ADA2, we hypothesized that trafficking of mutant ADA2 differs

from the WT protein. ER retention of mutant ADA2 has previously been shown in the HEK293T overexpression system (11, 13). Strikingly, protein expression of pathogenic ADA2 variants in CD14+ monocytes from DADA2 patients is almost absent (**Figure 1A**). Considering the normal *ADA2* mRNA expression, this suggests rapid protein degradation of mutant ADA2, at least for the studied variants. Classically, misfolded secretory proteins are identified in the ER to be transferred to the cytosol for proteasome-mediated degradation, a process referred to as ER-associated degradation (23). Indeed, inhibition of the proteasome by delanzomib rescued the increased protein degradation of mutant ADA2 induced by treatment with brefeldin A in HEK293T cells (**Figure 8C**). Importantly, inhibition of the proteasome did not rescue increased protein degradation of mutant ADA2 in DADA2 HMDM (**Figure 8D**) Interestingly, p.R169Q degradation was also reduced by inhibiting lysosomal degradation with chloroquine (**Figure 8C**). This was in line with the increased interaction of misfolded ADA2 with lysosomal proteins identified by IP-MS. Lysosomal degradation of mutant ADA2 by autophagy might account for the differential response to treatment with brefeldin A and treatment with monensin, a molecule that – besides its impact on Golgi transport – also inhibits autophagy (24). Monensin and chloroquine did however not have identical effects on the expression of LMW-ADA2 after transfection with WT ADA2. The absence of LMW-ADA2 upon inhibition of ER to Golgi trafficking and the increase in LMW-ADA2 upon inhibition of trans Golgi transport indicated that the hypoglycosylated LMW-form is generated from HMW-ADA2 after transport to the Golgi apparatus. In order to better understand the subcellular localization of LMW-ADA2, we performed subcellular fractionation experiments. We showed that HMW-ADA2 localized exclusively to the membranous fraction that also contains ER and Golgi membrane proteins (**Figure 8E**). LMW-ADA2 was also present in the membrane fraction. Moreover, this form was strongly detected in the cytosolic and lysosomal extracts. Overall, we showed that blocking ER to Golgi transport promotes protein degradation of mutant ADA2 and that LMW-ADA2 localizes to the cytosol and lysosomes.

Discussion

In this study, we showed that pathogenic protein variants of ADA2 expressed in HMDM from DADA2 patients exhibit a glycosylation pattern that is characteristic of secreted WT ADA2. DADA2 HMDM lack the LMW-form of ADA2 which is the predominant intracellular ADA2 protein in HC HMDM. We reported that LMW-form localized to the cytosol and lysosomes and that it is generated from HMW-ADA2 by glycan trimming after transport to the Golgi apparatus. The importance of N-glycosylation in trafficking and function of ADA2 has previously been established (11, 12). Yet, the behavior of mutant ADA2 on the cellular level has to date only been examined in overexpression systems of cells that do not express the protein endogenously (12, 13, 19). Importantly, expression of glycosyltransferases and glycosidases and therefore glycosylation patterns of individual proteins show striking variation even between different immune cell populations (25). It is therefore essential to understand the behavior of ADA2 in the patients' primary cells. In our cohort of 10 DADA2 patients, we identified the intracellular predominance of fully glycosylated HMW-ADA2 in the absence of LMW-ADA2 as a feature that was shared by all missense variants, independently of the patients' clinical phenotype. Differential patterns of glycosylation of the same protein being associated with distinctive protein functions have previously been described: IL-24 is a signal peptide-containing glycoprotein that is secreted via the ER. A partially glycosylated cytosolic form of IL-24 has been shown to mediate protein kinase R activation independently of its extracellular function (26). The predominance of the partially glycosylated LMW-form of ADA2 in HC HMDM raises the question of whether, in a similar way, this form differs in function from fully glycosylated secreted ADA2. In fact, the function of ADA2 has repeatedly been subject to discussion for several reasons: (i.) Pathogenic ADA2 variants display a wide spectrum of residual adenosine deaminase function which does not clearly correlate with disease severity (27). (ii.) ADA2 has evolved towards lower affinity to adenosine in higher species (10). (iii.) ADA1 exhibits superior adenosine deaminase activity (Km of 20-50 μM vs. 2-3 mM for

ADA2) and extracellular ADA1 bound to CD26 theoretically compensates for the absence of ADA2 at physiological levels of adenosine (28). Yet, the majority of studies proposing possible pathomechanisms underlying the immunological phenotype of DADA2 rely on the role of ADA2 as an extracellular adenosine deaminase (14, 15). In contrast, Greiner-Tollersrud and colleagues introduced the possibility of an intracellular function of ADA2 as a lysosomal DNase (17). The authors characterized the glycan structures of ADA2 isolated from porcine brain and identified a high mannose-6-phosphate content, indicating targeting of ADA2 to the lysosome. Indeed, they showed an overlap between the glycosylation pattern of ADA2 with several known lysosomal proteins. We showed that the glycan structures of LMW- and HMW-ADA2 expressed in HMDM were sensitive to glycan removal by Endo H, suggesting the presence of high-mannose oligosaccharides (29). This would be in line with a potential lysosomal localization of ADA2. Whether the predominant intracellular LMW-form of ADA2 that we detected in HMDM represents this lysosomal protein cannot be conclusively established from our data. Due to insufficient pull-down of LMW-ADA2 by immunoprecipitation, we could not elucidate its interactome and approach its potential function in this way. We did however detect LMW-ADA2 by western blot in the lysosomes of U-937 cells after lysosomal enrichment. The subcellular fractionation experiments provide only an approximation of the intracellular localization of LMW-ADA2 since the enriched fractions are not sufficiently pure to exclude interfractional contamination. They do however show a convincing signal for LMW-ADA2 in the cytosolic fraction indicating that LMW-ADA2 – like cytosolic IL-24 – might represent a hypoglycosylated cytosolic form of the ADA2 protein. Because of the increase in LMW-ADA2 after treatment with monensin, we hypothesize that HMW-ADA2 follows the classical secretory pathway after being directed to the ER via its signal peptide. After transport to the Golgi apparatus, it would then undergo glycan trimming before or after retro-translocation to the cytosol. Retro-translocation classically occurs in the context of ER-associated degradation to enable proteasomal degradation of misfolded proteins in the cytosol. LMW-ADA2 protein levels did however not increase upon

proteasome inhibition (**Figure S8C**). We therefore assume that glycan trimming and retro-translocation of LMW-ADA2 generate a functional cytosolic or lysosomal protein that might be different in function of secreted HMW-ADA2, as suggested by Greiner-Tollersrud and colleagues (17).

In the HEK293T model system, transfected ADA2 was predominantly expressed as the HMW-form corresponding to secreted ADA2 in these cells, highlighting the limitations of this system to understand the behavior of endogenous ADA2. Discrepancies between the two systems are also evident from the fact that ADA2 in whole cell lysates from transfected HEK293T cells was equally sensitive to glycan removal by PNGase F or Endo H (12), while we observed some residual glycan structures upon Endo H treatment of ADA2 in lysates from HMDM (**Figure 3C**). In HMDM, we did not observe the rescue of brefeldin A-induced degradation of mutant ADA2 by inhibition of proteasomal or lysosomal degradation shown in HEK293T cells. It is possible that in these cells the two protein degradation pathways mutually compensate for the inhibition of the other.

Another limitation of the relevance of macrophage LMW-ADA2 in the pathogenesis of DADA2 is that in primary CD14+ monocytes, the cells with the highest endogenous expression of ADA2, differentially glycosylated forms of ADA2 cannot be clearly distinguished (**Figure 1A+B**). Moreover, patient monocytes – both from patients with missense variants with normal *ADA2* mRNA expression and from patients with variants that exhibit nonsense-mediated mRNA decay – show complete absence of mutant ADA2 protein expression. This implies that mutations in the *ADA2* gene might manifest differently on a cellular level depending on the expressing cell.

This notion needs to be taken into account when examining the potential induction of ER stress by pathogenic variants of ADA2. Intracellular retention of ADA2 has only been reported after overexpression in HEK293T cells (2, 13). While primary monocytic cells also show impaired secretion of mutant ADA2, intracellular protein levels are very low in HMDM and close to absent in CD14+ monocytes. In our cohort of DADA2 patients, we did not observe an increased ER stress response in CD14+ monocytes. This was in line with single-cell RNA-sequencing data

reported by Watanabe et al. that showed downregulation of eIF2 signaling – a main pathway of the integrated stress response (30, 31).

Although we could not confirm that the presence of misfolded mutant ADA2 contributes to the pathophysiology of the disease by induction of an unfolded protein response, our data highlight the need for a better understanding of the folding and trafficking mechanisms of WT and mutant ADA2. We verified that the presence of pathogenic ADA2 protein variants as well as inhibition of N-glycosylation provoked the formation of intracellular protein aggregates, thereby broadening previous reports (12, 19). In addition, we showed that aggregate formation also occurs after co-transfection in the presence of the wild-type protein. These findings offer a possible explanation for heterozygous carriers presenting with clinical symptoms and underscore the importance of gaining in-depth understanding of the intracellular pathophysiology of DADA2. Next to Sanger sequencing of the *ADA2* gene, the measurement of serum ADA2 enzyme activity currently represents the diagnostic gold standard for DADA2. Assuming an intracellular function of the protein, it is possible that this strategy may overlook a proportion of patients. In this article, we introduced the absence of LMW-ADA2 as a new characteristic of pathogenic ADA2 variants that was verified for all pathogenic variants included in this study. Despite the focus on pathogenic missense variants in our cohort, we propose the absence of LMW-ADA2 as cellular feature of DADA2 since complete loss of ADA2 protein expression due nonsense-mediated mRNA decay also leads to absence of this form. Our findings suggest a possible role of LMW-ADA2 as a biomarker in DADA2, which would need further validation in a larger set of pathogenic and benign ADA2 variants.

Protein misfolding represents a major mechanism in the pathogenesis of mendelian diseases. The function of some proteins can be rescued by pharmaceutically modifying protein folding. Biallelic mutations in *CFTR* cause cystic fibrosis, one of the most common mendelian diseases. The most common pathogenic variant p.ΔF508 is almost entirely degraded via the ER-associated degradation pathway (32). CFTR correctors have been shown to promote folding of the mutant protein and

thereby partially restore the function of the channel to clinically relevant levels (33). In order to envision similar approaches to treat DADA2, a thorough understanding of ADA2 transport and function is crucial. The fact that ADA2 behaves differently depending on the expressing cell further complicates this approach. Until now, monocytes as the main producers of endogenous ADA2 have received the most attention in deciphering the pathophysiology of the disease. Considering the role of glycosylation in protein folding and the potential impact of this modification on protein function, we cannot exclude that the main ADA2 function might vary even between different immune cells. This might also account for the diverse clinical phenotype of the disease and the varying involvement of different immune cell populations. The large number of pathogenic variants and their diversity with respect to biochemical characteristics are additional factors contributing to the difficulties in delineating a single all-encompassing pathomechanism that would explain the complexity of the disease.

In conclusion, we demonstrate for the first time the differential N-glycosylation of pathogenic ADA2 in patient-derived macrophages. We establish LMW-ADA2 as a hypoglycosylated non-secreted form of ADA2 in healthy controls. This LMW-ADA2 is absent in DADA2. Further studies will be needed to confirm the functional relevance of these results in the pathogenesis of the disease.

Materials and methods

Human subjects

The patients and heterozygous carriers were treated at University Hospitals Leuven and their parents were included as heterozygous carriers. Healthy donors were recruited at University Hospitals Leuven and KU Leuven. Our study examined male and female patients, and similar findings are reported for both sexes.

Sanger sequencing

Genomic DNA samples were prepared from heparinized peripheral blood or $1x10^6$ Jurkat cells following the instructions of the QIAamp DNA Blood Mini kit (#51104; QIAGEN). Primers were designed with the help of Oligo Primer Analysis Software version 7 (Molecular Biology Insights). ADA2-specific gDNA amplification was performed using Platinum™ SuperFi™ PCR Master Mix (#12358010; Thermo Fisher Scientific). PCR products were purified using the QIAquick PCR purification kit (#28106; QIAGEN). Sanger sequencing was performed on an ABI 3730 XL Genetic Analyzer (Applied Biosystems) at LGC Genomics (Berlin, Germany). Sequencing data were analysed using Chromas 2.6.5 (http://www.technelysium.com.au).

Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep™ (#1114546; PROGEN) in SepMate™ isolation tubes (#85450; STEMCELL Technologies) according to the manufacturer's instructions. Prior to monocyte isolation, the final centrifugation step was performed at 300xg for 10 minutes at 4°C. CD14+ monocytes were isolated magnetically by positive selection using human CD14 MicroBeads (#130-050-201; Miltenyi Biotec) on LS columns (#130-042-401; Miltenyi Biotec) according to the manufacturer's protocol. The cells were eluted into complete medium (RPMI 1640 medium (#61870044; Gibco) supplemented with 10% fetal calf serum (FCS) (#S181BH-500, Biowest) and 1% penicillin-streptomycin (#15140122; Gibco). Purity > 97% of CD14+ monocytes after magnetic sorting was verified by flow cytometry (anti-CD14-FITC clone MφP9, 1:40, #345784; BD Biosciences). For macrophage differentiation, $3x10^5$ CD14+ monocytes were seeded in a 12-well plate in 1 mL complete medium containing 20 ng/mL GM-CSF (#300-03; Peprotech) or 50 ng/mL M-CSF (#300-25; Peprotech). The cells were differentiated for ten days, with medium changes every three days. U-937, THP-1 and Jurkat cells (all purchased from ATCC) were cultured in complete RPMI medium. HEK293T

cells (ATCC) were cultured in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin. For transfection, the cells were seeded in medium containing no antibiotics.

Inhibition of the secretory pathway was achieved by 24-hour incubation with 1 μg/mL brefeldin A (#00-4506-51; Thermo Fisher Scientific) or 2 μg/mL monensin (GolgiStop™, #554724, BD Biosciences). For analysis of N-glycosylation and glycan trimming, the cells were incubated with tunicamycin (#T7765; Sigma-Aldrich) at 2.5 μg/mL (HEK293T cells) or 5 μg/mL (HMDM) and castanospermine (#BML-S107-0100; Enzo Life Sciences) at 100 μg/mL for 24h. Monocytes were treated with 1 μg/mL tunicamycin for 24h for ER stress induction. Cells were treated with 50 μM chloroquine (#C6628; Sigma-Aldrich) for 24h to inhibit autophagy. Proteasome inhibition was achieved by 14 to 24-hour incubation with 200 nM delanzomib (#S1157; Selleck Chemicals GmbH). HSP90 was inhibited by 24-hour incubation with 360 nM geldanamycin (#G3381; Sigma-Aldrich).

Microscopy

HMDM were differentiated with GM-CSF or M-CSF for 10 days in a 12-well plate as described above. Cell morphology was evaluated in the culture plate by brightfield microscopy on an EVOS M7000 Microscope Imaging System (#AMF7000; Invitrogen) using the 20x objective.

Transfection

The plasmid expressing myc-DDK-tagged wild-type ADA2 (transcript variant 3, NM_001282225) was purchased from OriGene Technologies (#RC238645). Pathogenic ADA2 variants were created by site-directed mutagenesis using the Q5® Site-Directed Mutagenesis Kit (#E0554; New England Biolabs) according to the manufacturer's instructions. Stable competent E. coli (#C3040H, New England Biolabs) were transformed with the generated constructs. Plasmid DNA was purified with the help of the QIAprep Spin Miniprep Kit (#27104, QIAGEN). Successful

mutagenesis was verified by Sanger sequencing (LGC Genomics). HEK293T cells were seeded at $5x10^4$ cells/well in 1 mL in a 24-well plate or $2.5x10^5$ cells/well in 2 mL in a 6-well plate 24h prior to transfection. The cells were transfected with 5 ng and 25 ng plasmid DNA, respectively, using Lipofectamine™ 2000 Transfection Reagent (#11668019; Invitrogen) according to the manufacturer's instructions. The medium was changed after 24h. Cells and supernatants were collected 48h after transfection.

Generation of ADA2$^{-/-}$ cell lines by CRISPR/Cas9

Single-guide RNAs targeting ADA2 from the human CRISPR Brunello library (#73179; addgene) (34) were cloned into the lentiCRISPRv2 puro plasmid. lentiCRISPRv2 puro was a gift from Brett Stringer (plasmid #98290; addgene; http://n2t.net/addgene:98290; RRID: Addgene_98290). Jurkat cells were transfected by electroporation using the Neon™ Transfection System (#MPK5000; Thermo Fisher Scientific) according to the manufacturer's instructions: $2x10^5$ cells were resuspended in Resuspension Buffer R and mixed with 1 μg plasmid DNA. Electroporation was achieved with the following conditions: 1325V, 10ms, 3 pulses. After electroporation, the cells were directly transferred into 500 μL prewarmed complete RPMI 1640 medium. On day 1 after transfection, puromycin (#ant-pr-1; InvivoGen) was added at a final concentration of 1 μg/mL. After 36h, cells were seeded at 1 cell/well in 100 μL RPMI 1640 medium supplemented with 20% FCS in a round bottom 96-well plate. After 14 days, the clones were screened for knock out of ADA2 by western blot.

Subcellular fractionation

Membrane and cytosolic fractions were generated from $10x10^6$ U-937 cells using the Mem-PER™ Plus Membrane Protein Extraction Kit (#89842; Thermo Fisher Scientific). Incubation times were optimized to increase protein yield to 45 minutes for the permeabilization buffer and 90 minutes

for the solubilization buffer. The lysosomal fraction was produced with the help of the Lysosome Enrichment Kit for Tissues and Cultured Cells (#89839; Thermo Fisher Scientific) according to the manufacturer's protocol.

Immunoblotting

Adherent cells were detached with trypsin-EDTA (0.05%) (#25300062; Gibco) for 5 minutes at 37°C and the reaction was blocked by addition of complete medium. Cells were pelleted by centrifugation at 400xg for 5 minutes at 4°C. Whole cell lysates were obtained by lysing $1x10^6$ cells in 25 µL RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 8.0) or NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40, pH 7.4) containing protease inhibitor (#78429; Thermo Fisher Scientific) for 30 minutes on ice, followed by centrifugation at 13,500xg for 20 minutes at 4°C.

To collect supernatants, cells were cultured in serum-free medium for 24h. For immunoblotting, supernatants were concentrated by added 1200 µL ice-cold acetone to 300 µL supernatant and overnight storage at -20°C. Supernatant proteins were then precipitated by centrifugation at 15,000xg for 15 minutes at 4°C. The protein pellet was resuspended in 25 µL NP-40 buffer containing protease inhibitor. Bolt™ LDS sample buffer (#B0007; Thermo Fisher Scientific) mixed with Bolt™ Sample Reducing Agent (#B0009; Thermo Fisher Scientific) was added to the samples prior to gel electrophoresis. For analysis of intracellular dimer formation, the reducing agent was omitted. Samples were heated for 5 minutes at 70°C. Supernatants from HEK293T cells transfected with ADA2 were diluted 1:10 in 2X Bolt™ LDS sample buffer (#B0007; Thermo Fisher Scientific), serum was diluted 1:200. SeeBlue™ Plus2 Pre-stained Protein Standard (#LC5925; Thermo Fisher Scientific) was used as protein molecular weight marker.

Proteins were transferred onto PVDF transfer membranes. The membranes were probed with the following primary antibodies: anti-ADA2 (clone EPR25430-131, #ab288296, 1:1000; abcam), anti-ADA2 (#HPA007888, 1:500; Sigma-Aldrich; RRID: AB_1078495), Clone OTI4C5, anti-DDK

(FLAG) (clone OTI4C5, #TA50011, 1:1000; OriGene Technologies; RRID: AB_2622345), anti-LC3B (#ab51520, 1:1000; abcam; RRID: AB_881429), anti-BiP (#3183, 1:1000; Cell Signaling; RRID: AB_10695864), anti-LAMP-1 (clone H4A3, #sc-20011; 1:500; Santa Cruz Biotechnology; RRID: AB_626853), anti-CD98 (clone E-5, #sc-376815, 1:500; Santa Cruz Biotechnology; RRID: AB_2938854), anti-GAPDH (clone 7B, #sc-69778, 1:500; Santa Cruz Biotechnology; RRID: AB_1124759), anti-β-actin (clone: AC-15, #A5441, 1:9,000; Sigma-Aldrich; RRID: AB_476744) at 4°C overnight or at room temperature for two hours. The membranes were washed and incubated with the respective HRP-coupled secondary antibodies for one hour at room temperature: goat anti-rabbit IgG H&L (#ab205718, 1:5000; abcam; RRID: AB_2819160), Goat Anti-Mouse IgG (H + L) (#71045, 1:5000; Sigma-Aldrich; RRID: AB_11211441) or VeriBlot Detection Reagent (#ab131366, 1:1000 in tris saline/5% skim milk ; abcam). Protein expression was visualized by enzymatic chemiluminescence using Pierce™ ECL western blotting substrate (#32106; Thermo Fisher Scientific) or SuperSignal™ West Pico PLUS Chemiluminescent Substrate (#34580; Thermo Fisher Scientific) in a ChemiDoc XRS+ Imaging System (Bio-Rad).

Glycan removal

Glycan removal was performed on whole cell lysates generated with NP-40 lysis buffer or precipitated supernatant protein reconstituted in NP-40 lysis buffer. Glycan removal was performed on 20 µg or 25 µg total protein using PNGase F (#P0704; New England Biolabs), Endo H (#P0702; New England Biolabs) or PNGase F (#A39245; Gibco) according to the manufacturer's instructions. Glycan removal with PNGase F (#P0704; New England Biolabs) was completed under denaturing conditions for 1h at 37°C or under non-denaturing conditions for 4h at 37°C as indicated.

Immunoprecipitation

Whole cell lysates were prepared from HEK293T cells transfected with wild-type ADA2 or the variants p.R169Q or HMDM from HC or DADA2 patients in NP-40 lysis buffer at a protein concentration of 1 mg/mL. Primary antibodies were added to 1000 μg lysate or 500 μg lysate, respectively, and incubated rotating at 4°C overnight. Alternatively, 1 mL supernatant was directly incubated with the primary antibodies. After overexpression, tagged ADA2 was pulled down using 1 μg anti-DDK (FLAG) clone OTI4C5 (#TA50011, OriGene Technologies). An isotype control sample was incubated with 1 μg mouse IgG1 (#02-6100; Invitrogen) in parallel. Endogenous ADA2 was pulled down using 1 μg anti-ADA2 (clone EPR25430-131, #ab288296, abcam) with rabbit monoclonal IgG (clone EPR25A, #ab172730; abcam) used as isotype control. For immunoprecipitation, the samples were rotated with 100 μL SureBeads™ Protein G Magnetic Beads (#1614023, Bio-Rad) for 1h at room temperature. The beads were magnetized and washed four times with PBS/0.1% Tween 20. Samples for mass spectrometric analysis were washed twice with NP-40 lysis buffer and twice with PBS. For western blot, the samples were eluted from the beads in Bolt™ LDS sample buffer (#B0007; Thermo Fisher Scientific).

Protein digest

Beads from immunoprecipitation experiments were resuspended in 20 μL denaturation buffer (6 M Urea, 2 M Thiourea, 10 mM HEPES, pH 8.0), reduced for 30 min at 25°C in 12 mM dithiothreitol, followed by alkylation with 40 mM chloroacetamide for 20 min at 25°C. Samples were first digested with 0.5 μg endopeptidase LysC (Wako, Osaka, Japan) for 4h. After diluting the samples with 80 μL 50 mM ammonium bicarbonate (pH 8.5), 1 μg sequence grade trypsin (Promega) was added overnight at 25°C. The peptide-containing supernatant was collected and acidified with formic acid (1% final concentration) to stop the digestion. Peptides were desalted and cleaned up using Stage

Tip protocol (35). After elution with 80% acetonitrile/0.1% formic acid, samples were dried using speedvac, resolved in 3% acetonitrile/0.1% formic acid and analysed by LC-MS/MS.

LC-MS/MS analyses

Peptides were separated on a reversed-phase column (20 cm fritless silica microcolumns with an inner diameter of 75 μm, packed with ReproSil-Pur C18-AQ 3 μm resin (Dr. Maisch GmbH) using a 90 min gradient with a 250 nL/min flow rate of increasing Buffer B concentration (from 2% to 60%) on a High-Performance Liquid Chromatography (HPLC) system (Thermo Fisher Scientific) and ionized using an electrospray ionization (ESI) source (Thermo Fisher Scientific) and analyzed on a Thermo Q Exactive HF-X instrument (*HEK293T*) or on an Q Exactive Plus instrument (*HMDM*). The Q Exactive HF-X instrument was run in data dependent mode selecting the top 20 most intense ions in the MS full scans, selecting ions from 350 to 2000 m/z, using 60 K resolution with a 3×10^6 ion count target and 10 ms injection time. Tandem MS was performed at a resolution of 15 K. The MS2 ion count target was set to 1×10^5 with a maximum injection time of 100 ms. Only precursors with charge state 2–6 were selected for MS2. The dynamic exclusion duration was set to 30 s with a 10-ppm tolerance around the selected precursor and its isotopes. The Q Exactive Plus instrument was run in data dependent mode selecting the top 10 most intense ions in the MS full scans, selecting ions from 350 to 2000 m/z, using 70 K resolution with a 3×10^6 ion count target and 50 ms injection time. Tandem MS was performed at a resolution of 17.5 K. The MS2 ion count target was set to 5×10^4 with a maximum injection time of 250 ms. Only precursors with charge state 2–6 were selected for MS2. The dynamic exclusion duration was set to 30 s with a 10-ppm tolerance around the selected precursor and its isotopes.

Raw data were analyzed using MaxQuant software package (v1.6.3.4) (36). The internal Andromeda search engine was used to search MS2 spectra against a human UniProt database (HUMAN.2020-06), including ADA2 mutant sequence, containing forward and reverse sequences. The search included variable modifications of methionine oxidation, N-terminal acetylation and fixed

modification of carbamidomethyl cysteine. The FDR was set to 1% for peptide and protein identifications. Unique and razor peptides were considered for quantification. The LFQ (label-free quantitation) algorithm was activated. The resulting text file was filtered to exclude reverse database hits, potential contaminants, and proteins only identified by site.

SomaScan Assay

Serum samples (55 µL) from 12 healthy controls, 9 heterozygous ADA2 carriers and 12 DADA2 patients were analyzed by the 7,000-plex SomaScan® Assay (SomaLogic, Inc.). One carrier was identified as an outlier by principal component analysis and excluded from further analysis. For two carriers and 10 patients, samples from multiple time points were included. The cohort details are presented in **Table S2**. The assay readout was provided in relative fluorescent units (RFU) for all measured aptamers for each serum sample.

ADA2 enzyme activity

Adenosine deaminase 2 enzyme activity was determined in whole cell lysates from HEK293T cells overexpressing ADA2 or HMDM with and without glycan removal as well as in supernatants from transfected HEK293T cells and human serum. Deaminase activity was measured in a colorimetric assay adapted from Giusti and Galanti (37). Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) (#E114; Sigma-Aldrich) was used to inhibit ADA1 activity. Triplicate measurements were performed for all samples. Enzymatic activity of pathogenic ADA2 variants overexpressed HEK293T cells was normalized to the activity of wild-type ADA2.

qPCR

5-10x10^5 cells were lysed in TRIzol™ Reagent (#15596018; Thermo Fisher Scientific) for 3 minutes at room temperature and homogenized before storage at -80°C. To measure the ER stress

response, HEK293T cells were seeded at 5x104 cells/ml in a 24-well plate 24h prior to transfection. TRIzol™ samples were prepared 48h after transfection. RNA was extracted using the PureLink™ RNA Mini Kit (#12183018A; Thermo Fisher Scientific) according to the manufacturer's instruction. cDNA was generated from 20 ng RNA using the SuperScript™ VILO™ cDNA Synthesis Kit (#11754050; Thermo Fisher Scientific). Quantitative polymerase chain reaction (qPCR) analysis was performed with SsoAdvanced™ Universal SYBR® Green Supermix (#1725271; Bio-Rad Laboratories) and the following primers:
ADA2(929-1038)_F_GGCTCCGAATCAAGTTCCCC;
ADA2(929-1038)_R_TAAGGCAGCTTAACGCCATCC;
ADA2(1138-1257)_F_ACCAGAATCGGCCATGGA;
ADA2(1138-1257)_R_CTACAGGGTGGTTCCTCAAGTCA;
HSPA5_F_CAAGCAACCAAAGACGCTGGA; HSPA5_R_GCCACCCAGGTCAAACACCA; DDIT3_F_AGAGGAAGAATCAAAAATCT; DDIT3_R_AGCTCTGACTGGAATCTGGA; GAPDH_F_GTCTCCTCTGACTTCAACAGCG;
GAPDH_R_ACCACCCTGTTGCTGTAGCCAA. For all conditions, three technical replicates were measured. The experiment was run on a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific) and analyzed using the QuantStudio™ Design & Analysis Software v1.5.2. The relative abundance of *ADA2* was normalized to the expression level of *GAPDH*. and different conditions were compared using the $2^{-\Delta\Delta Ct}$ method (38).

Statistics

For the SomaScan assay, raw counts expressed in relative fluorescent units (RFU) were normalized across samples using a cyclic loess algorithm implemented by the package limma (39). Two outlier samples identified with principal component analysis were removed before downstream analysis. Differentially expressed genes were identified using a linear model based on subject condition (HC, Carrier, DADA2), subject age, and number of freeze-thaw-cycles of the sample. Pseudoreplicates

coming from subjects with multiple samples were handled using blocking. Statistics were moderated using the empirical Bayes method of the package limma (39).

Statistical analysis of the mass spectrometry data was performed using Perseus software (v1.6.2.1) (40). Log2 transformed LFQ intensity values were filtered for minimum of 3 valid values in at least one experimental group and missing values were imputed with random low intensity values taken from a normal distribution. Differences in protein abundance between groups were calculated using two-sample Student's t-test using an FDR-based significance cut-off of 5% (*HEK293T*) or 10% (*HMDM*) in order to define enriched proteins.

Study approval

This study was approved by the Ethics Committee for Research of Leuven University Hospitals (project numbers S63077, S63807). The patients provided written informed consent prior to participation. It was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.

Data availability

The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD046128. The authors provide a supplemental file containing the original blots of all the presented data. Values for all data points in graphs are reported in the Supporting Data Values file. Further information is available upon request.

Author contributions

LE, LM and IM were responsible for the study conception and design. Material preparation and data collection was performed by LE, AH, MW, BP, SD, MD, MK and LM. LE, AH, MB, MK and LM performed the data analysis. MJ and DD assisted in the generation of ADA2 knock-out cell lines. The proteomics analyses were performed in collaboration with MK, PM and TM. FE and PA supported the design of the ER stress and localization experiments. LE, SD, GB, LDS, RS, SV, DC and IM collected clinical data and biological materials from patients. The first draft of the manuscript was written by LE. All authors reviewed the manuscript and approved its submission. IM is responsible for the supervision of the study.

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Abbreviations

ADA2	Adenosine deaminase 2
DADA2	Deficiency of adenosine deaminase 2
Endo H	Endoglycosidase H
HC	Healthy control
HMDM	Human monocyte-derived macrophages
PNGase F	Peptide-N-Glycosidase F
TNFi	TNF inhibitors
WT	Wild type

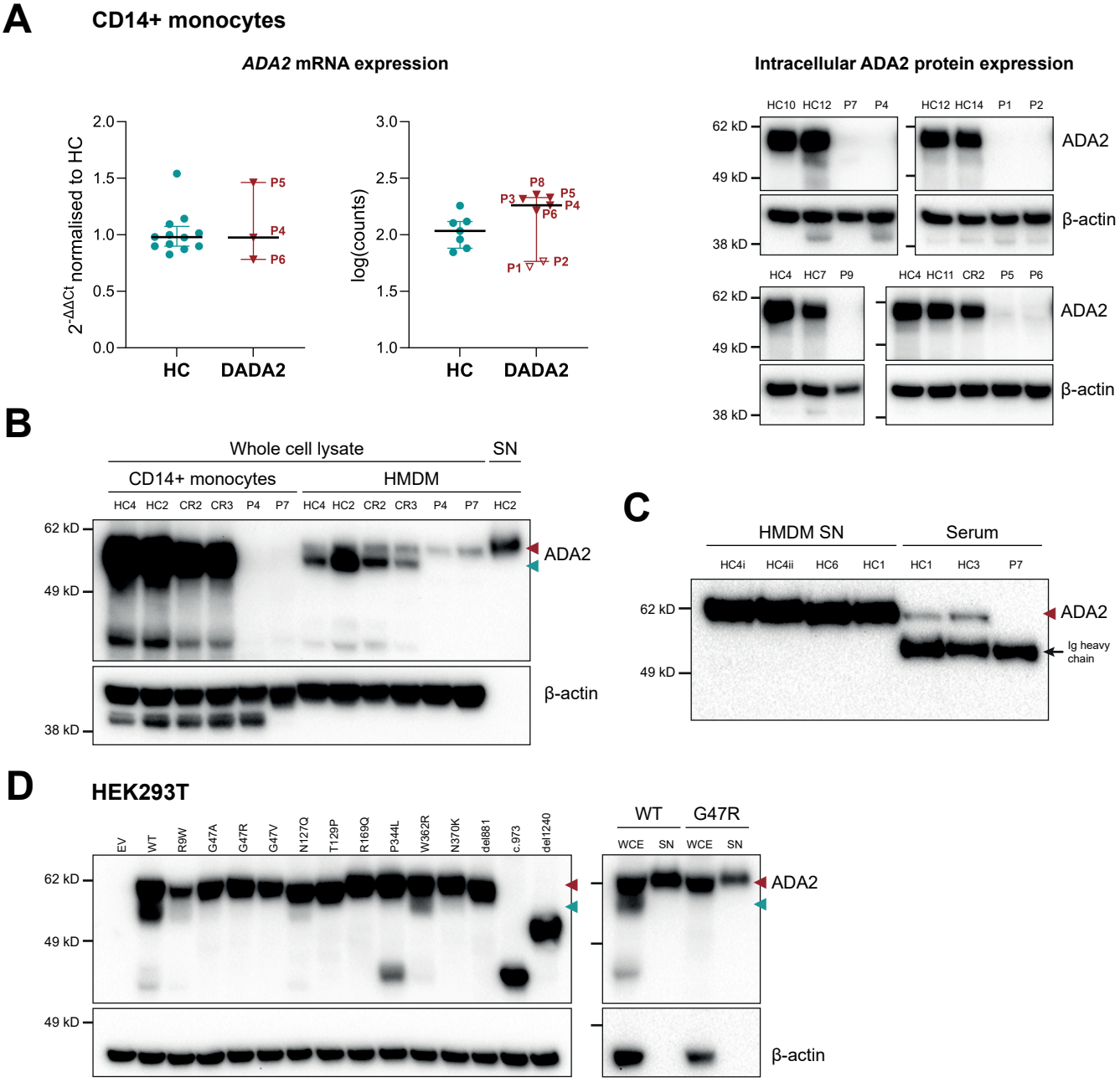

Figure 1: Expression pattern of pathogenic ADA2 variants. (A) mRNA and intracellular protein expression of ADA2 in CD14+ monocytes from healthy controls (HC) and DADA2 patients (P) by qPCR or bulk RNA-sequencing and western blot of whole cell lysates, respectively. The left plot shows mRNA expression determined by qPCR. *ADA2* mRNA expression was normalized to *GAPDH* and is depicted relative to the mean of HC samples. The right plot shows counts determined by RNA-sequencing. Dot plots show median and interquartile range. (B) ADA2 protein expression in whole cell lysates and supernatant (SN) of CD14+ monocytes and GM-CSF differentiated human monocyte-derived macrophages (HMDM) from healthy controls (HC), carriers (CR) and DADA2 patients (P) was determined by western blot. (C) ADA2 protein expression in supernatants (SN) from healthy control human monocyte-derived macrophages (HMDM) and serum samples from healthy controls (HC) and DADA2 patients (P) by western blot using VeriBlot for detection. (D) ADA2 protein expression in whole cell lysates of HEK293T cells after transfection with wild-type (WT) ADA2 and different pathogenic variants (left) and comparing whole cell lysates (WCE) and supernatant (SN) by western blot (right). The anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam) was used for detection in all depicted western blots. Triangles indicate HMW-ADA2 (red) and LMW-ADA2 (green). Legend: Ig, immunoglobulin.

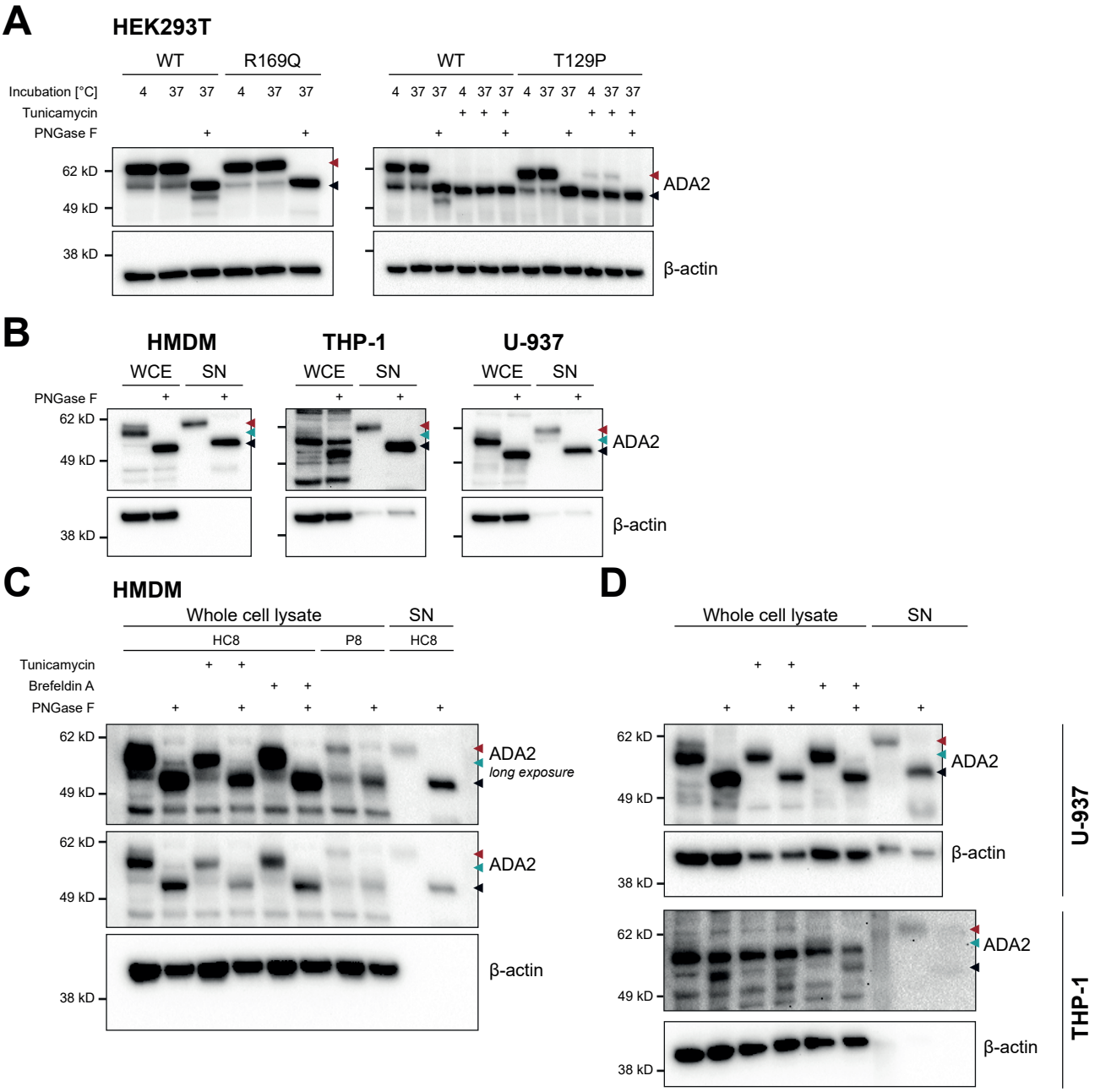

Figure 2: N-glycosylation pattern of ADA2. (A) Western blot of wild-type (WT) ADA2 and the pathogenic variants p.R169Q and p.T129P transfected into HEK293T cells after incubation with or without 2.5 µg/mL tunicamycin (TM) for 24h. Glycan removal was performed by incubation with PNGase F for 1 hour at 37°C under denaturing conditions. (B) ADA2 protein expression by western blot in whole cell lysates (WCE) and supernatants (SN) of human monocyte-derived macrophages (HMDM), U-937 cells and THP-1 cells. Glycan removal was performed by incubation with PNGase F for 1 hour at 37°C under denaturing conditions. (C) ADA2 protein expression in whole cell lysates and supernatants (SN) from GM-CSF-differentiated HMDM of healthy control HC8 and DADA2 patient P8 left untreated or treated with 5 µg/mL tunicamycin or 1 µg/mL brefeldin A for 24h. Glycan removal was performed by incubation with PNGase F for 1 hour at 37°C under denaturing conditions. (D) ADA2 protein expression in whole cell lysates and supernatants (SN) from U-937 and THP-1 cells left untreated or treated with 2.5 µg/mL tunicamycin or 1 µg/mL brefeldin A for 24h. Glycan removal was performed by incubation with PNGase F for 1 hour at 37°C under denaturing conditions. ADA2 was detected using anti-ADA2 (#HPA007888, Sigma-Aldrich) in all shown western blots. Triangles indicate HMW-ADA2 (red), LMW-ADA2 (green) and glycan-free ADA2 (dark blue).

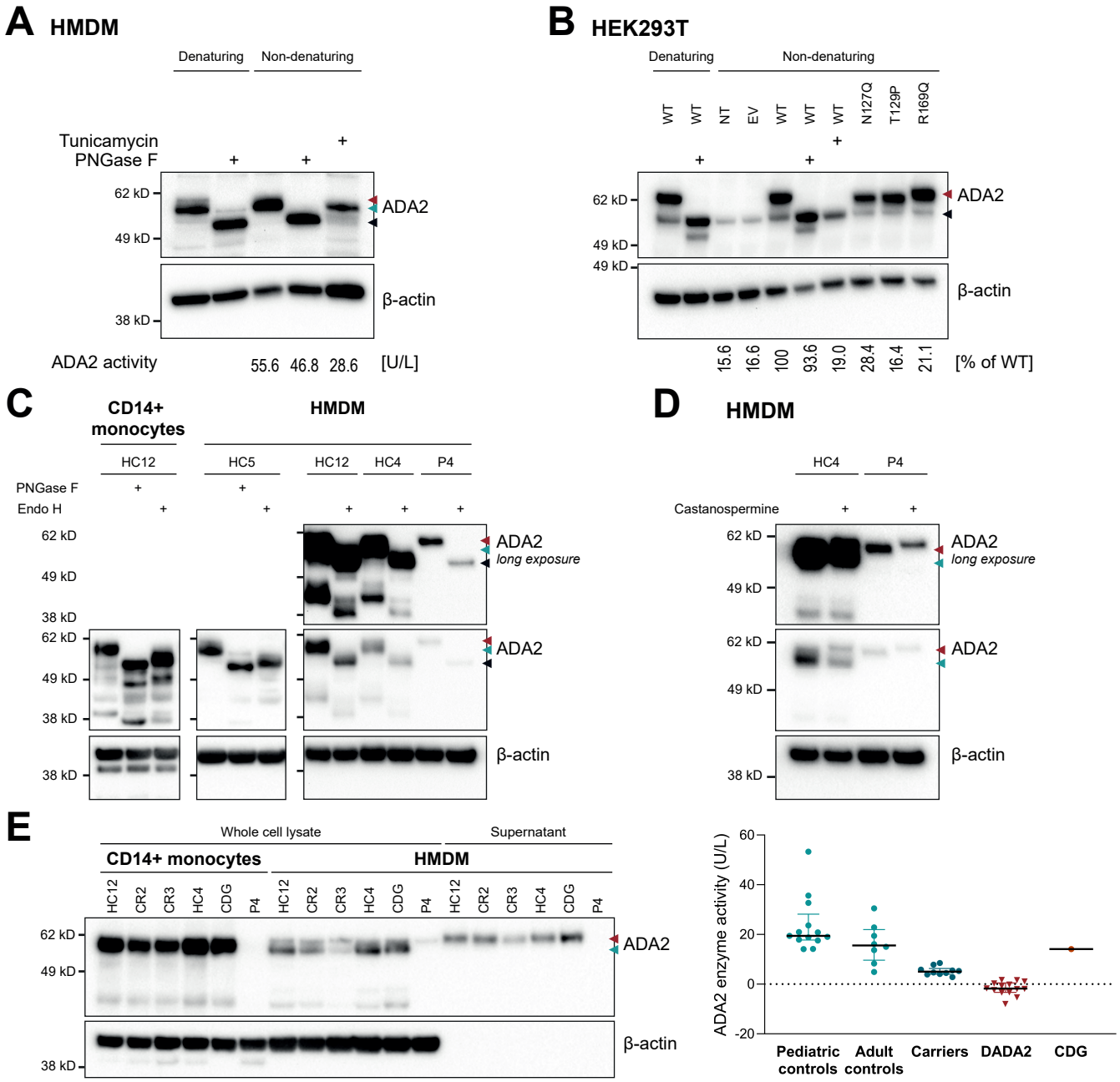

Figure 3: ADA2 expression and activity upon modification of N-glycosylation. (A) GM-CSF-differentiated healthy control HMDM were treated with or without 5 µg/mL tunicamycin for 24h. Glycan removal was performed by incubation with PNGase F at 37°C under denaturing (1h) and non-denaturing (4h) conditions. ADA2 enzyme activity was determined in whole cell lysates handled under non-denaturing conditions. (B) HEK293T cells transfected with wild-type (WT) ADA2 or pathogenic ADA2 variants were treated with or without 2.5 µg/mL tunicamycin for 24h. Glycan removal was performed by incubation with PNGase F at 37°C under denaturing (1h) and non-denaturing (4h) conditions. ADA2 enzyme activity was determined in whole cell lysates handled under non-denaturing conditions. (C) ADA2 protein expression by western blot after glycan removal in CD14+ monocytes or GM-CSF-differentiated HMDM from healthy controls (HC) and DADA2 patient P4 by incubation with PNGase F or Endo H for 1 hour at 37°C under denaturing conditions. (D) ADA2 protein expression by western blot in GM-CSF-differentiated HMDM from healthy control HC12 and DADA2 patient P4 after incubation with 100 µg/mL castanospermine for 24h. (E) *Left:* ADA2 protein expression by western blot in monocyte and HMDM whole cell lysates as well as HMDM supernatants from healthy controls (HC), DADA2 heterozygous carriers (CR), DADA2 patient P4 and a patient with *PMM2*-congenital disorder of glycosylation (CDG). *Right:* ADA2 enzyme activity was

determined in the serum of pediatric and adult healthy controls, DADA2 heterozygous carriers, DADA2 patients and the *PMM2*-CDG patient. Dot plots show median and interquartile range. In figures A+B and C (left) ADA2 was detected using anti-ADA2 (#HPA007888, Sigma-Aldrich; in figures C (right) and D+E anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam) was used. Triangles indicate HMW-ADA2 (red), LMW-ADA2 (green) and glycan-free ADA2 (dark blue). Legend: CDG, congenital disorder of glycosylation; EV, empty vector; NT, non-transfected.

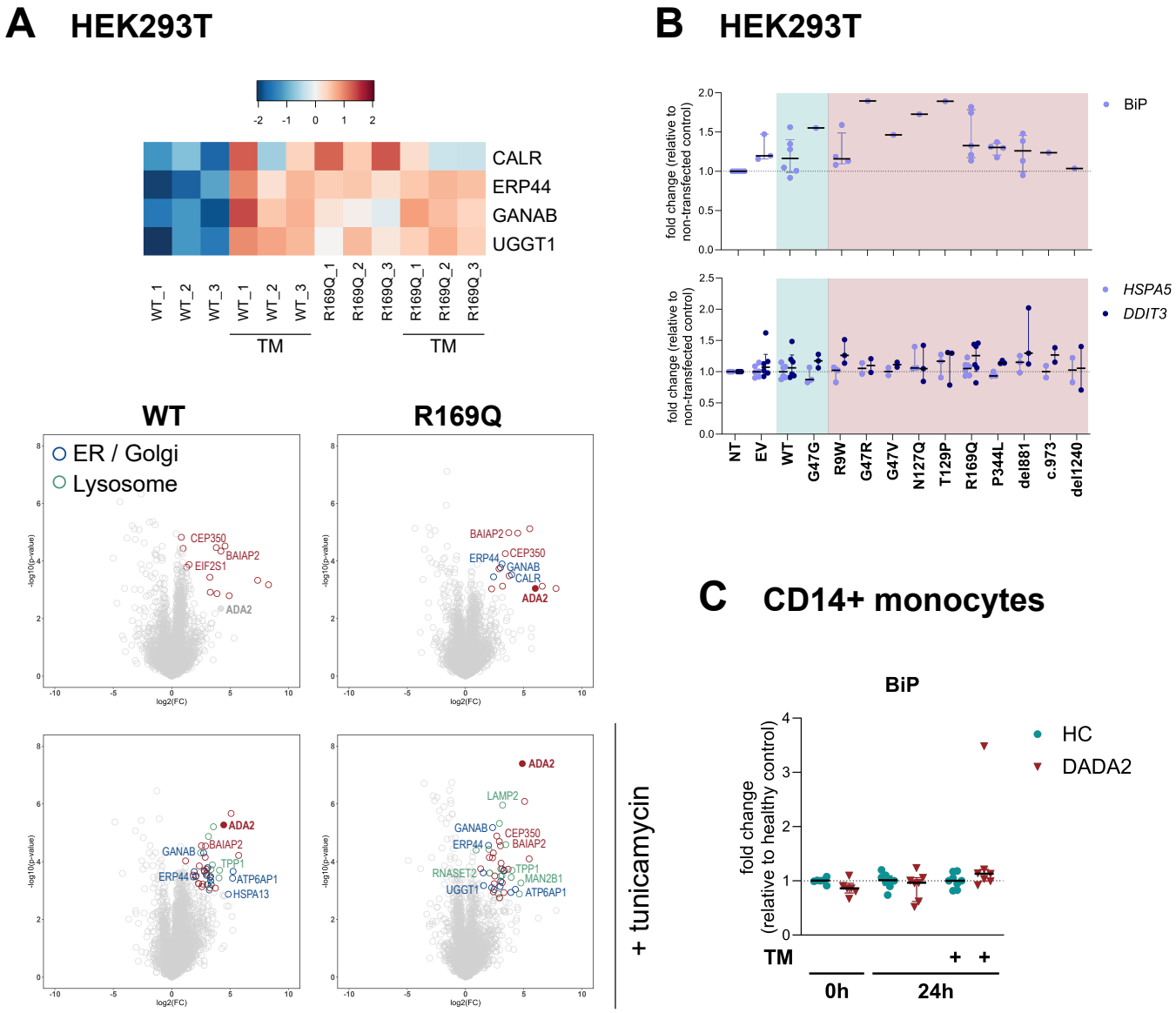

Figure 4: ER stress in DADA2. (A) Interactome analysis of wild-type (WT) ADA2 and the pathogenic variant p.R169Q overexpressed in HEK293T cells treated with or without 2.5 µg/mL tunicamycin (TM) for 24h was performed by immunoprecipitation followed by mass spectrometry. Heatmap shows z-score of protein abundance. Binders significantly pulled down compared to isotype control with a false discovery rate of 5% are highlighted by colored circles. Localization of the interacting proteins to the ER/Golgi (blue) or lysosome (green) is indicated by color. (B) ER stress response in HEK293T cells transfected with wild-type (WT) ADA2 and different pathogenic variants. Protein expression of BiP (*top*) and mRNA expression of *HSPA5* and *DDIT3* (*bottom*) were determined 48h after transfection. A representative western blot is shown in **Supplemental Figure S5C**. mRNA expression was normalized to *GAPDH* and is depicted as $2^{-\Delta\Delta Ct}$ relative to non-transfected control samples. Results from 6 independent experiments are shown. Dot plots show median and interquartile range. (C) ER stress response was evaluated by protein expression of BiP in whole cell lysates of CD14+ monocytes at baseline or after 24h incubation with or without 1 µg/mL tunicamycin (TM). BiP expression was normalized to β-actin. Dot plots show median and interquartile range. The corresponding western blots are shown in **Supplemental Figure S6**. Legend: EV, empty vector; FC, fold change; NT, non-transfected.

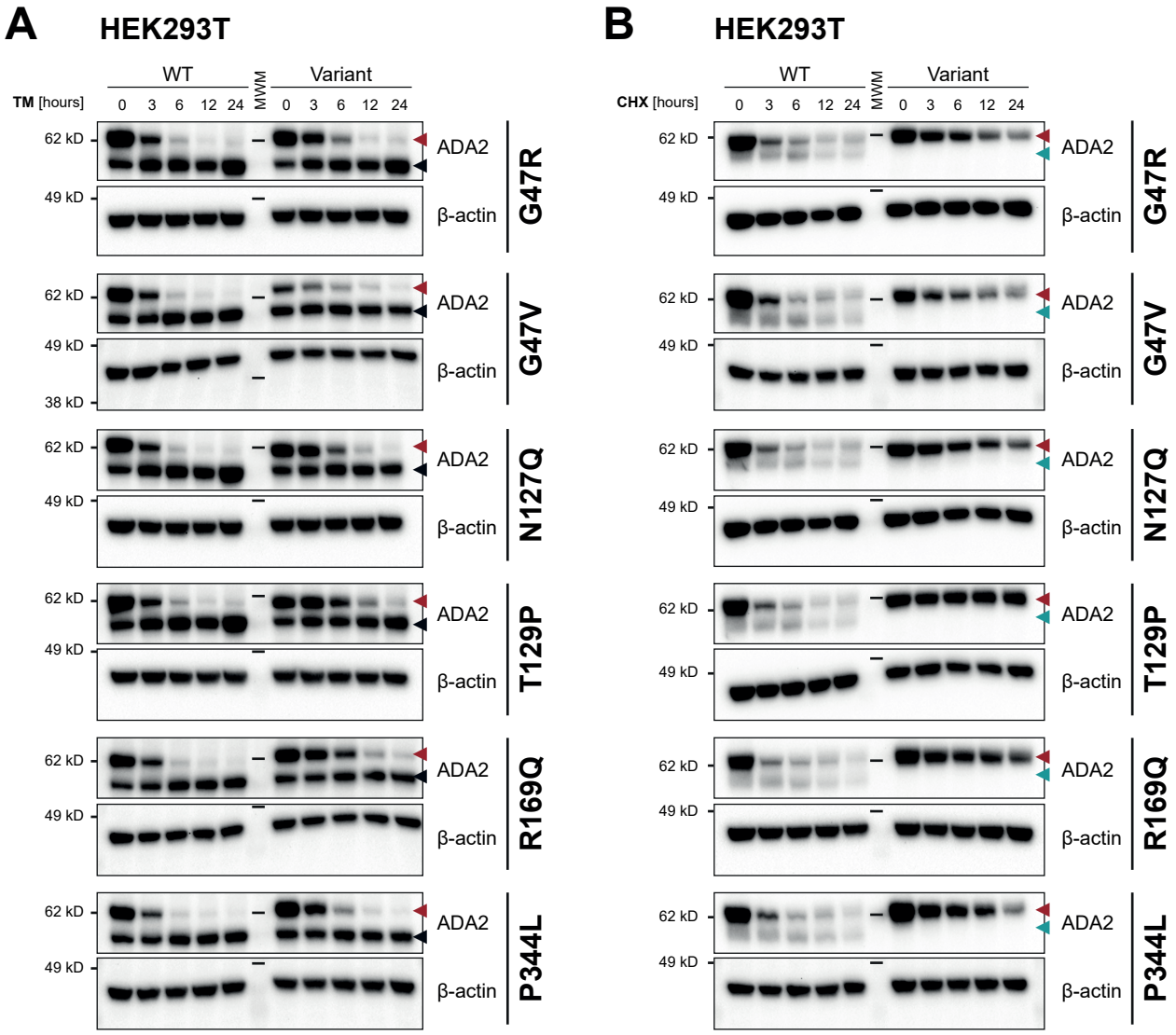

Figure 5: Glycosylation and stability of ADA2. (A) HEK293T cells were transfected with wild-type (WT) ADA2 or different pathogenic variants and treated with 2.5 µg/mL tunicamycin (TM) over 24h. Whole cell lysates were generated at the indicated time points and immunoblotted for ADA2 expression. (B) HEK293T cells were transfected with wild-type (WT) ADA2 or different pathogenic variants and treated with 500 µg/mL cycloheximide (CHX) over 24h. Whole cell lysates were generated at the indicated time points and immunoblotted for ADA2 expression. Triangles indicate HMW-ADA2 (red), LMW-ADA2 (green) and glycan-free ADA2 (dark blue). In figure A, ADA2 was detected using anti-ADA2 (#HPA007888, Sigma-Aldrich); in figure B anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam) was used. Legend: MWM, molecular weight marker.

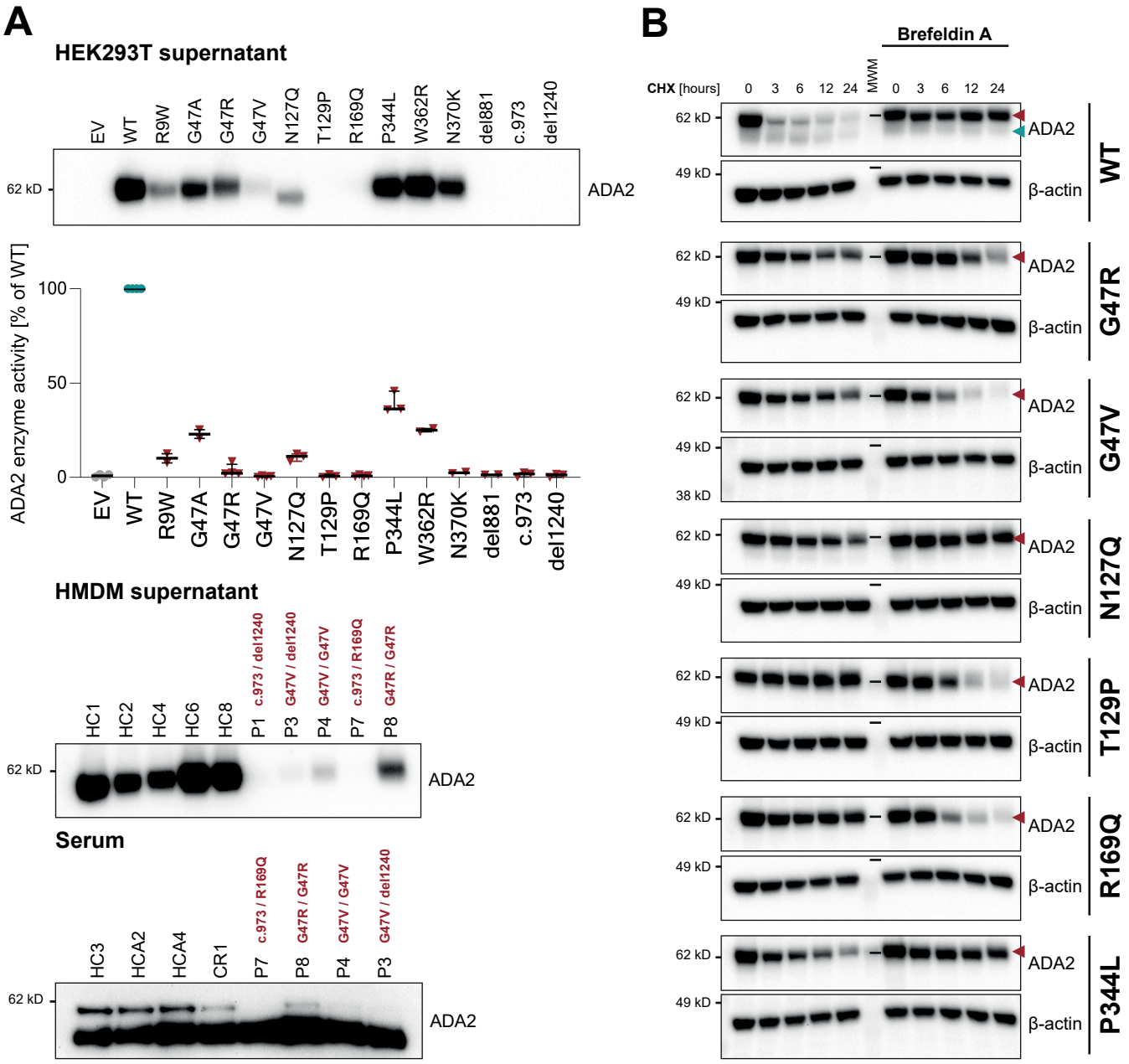

Figure 6: Trafficking of ADA2. (A) ADA2 protein levels were determined in supernatants of M-CSF-differentiated human monocyte-derived macrophages (HMDM) from healthy controls (HC) or DADA2 patients (P) or HEK293T cells transfected with wild-type (WT) ADA2 or different pathogenic ADA2 variants. The patients' genotypes are indicated above. ADA2 enzyme activity was determined in the supernatant. Data are shown from n=2-4 independent experiments. Dot plots show median and interquartile range. Western blot of serum samples from healthy controls (HC), heterozygous DADA2 carriers (CR) and DADA2 patients (P) detected by anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam) and VeriBlot. (B) HEK293T cells were transfected with wild-type (WT) ADA2 or different pathogenic variants and treated with 500 µg/mL cycloheximide (CHX) ± 1 µg/mL brefeldin A (BFA) over 24h. Whole cell lysates were generated at the indicated time points and immunoblotted for ADA2 expression. Triangles indicate HMW-ADA2 (red), LMW-ADA2 (green) and glycan-free ADA2 (dark blue). ADA2 was detected using anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam). Legend: EV, empty vector; MWM, molecular weight marker.

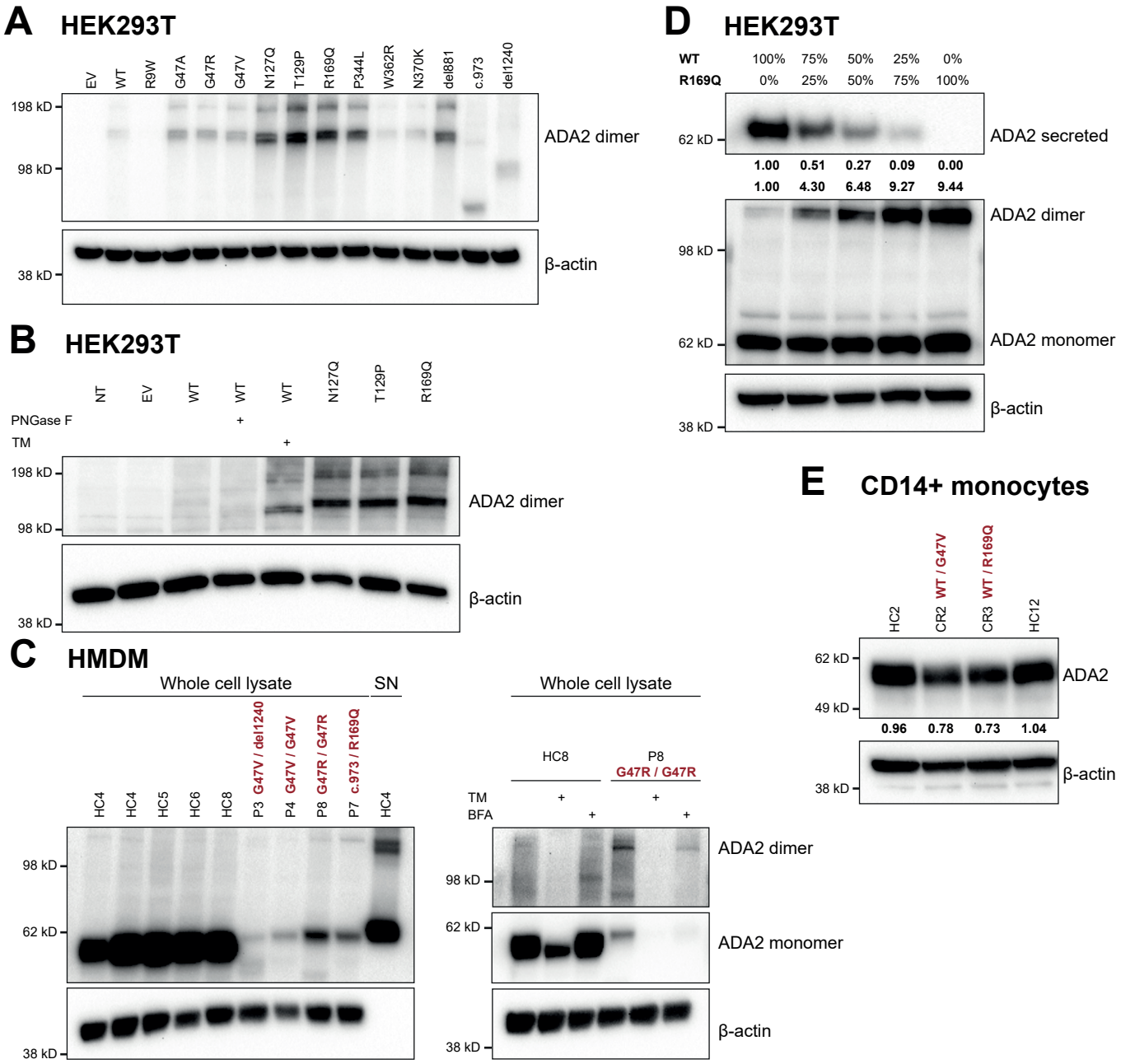

Figure 7: Intracellular dimer formation and secretion of mutant ADA2. (A) Formation of intracellular ADA2 dimers in HEK293T cells transfected with wild-type (WT) ADA2 and different pathogenic ADA2 variants. The blot is equivalent to the one shown in **Figure 1D**. (B) Formation of intracellular ADA2 dimers in HEK293T cells transfected with wild-type (WT) ADA2 and different pathogenic ADA2 variants with or without treatment with 2.5 µg/mL tunicamycin (TM). ADA2 was detected using anti-ADA2 (#HPA007888, Sigma-Aldrich. (C) ADA2 protein expression and intracellular dimer formation in GM-CSF (*left*) or M-CSF (*right*) differentiated human monocyte-derived macrophages from healthy controls (HC) and DADA2 patients (P) with or without treatment with 5 µg/mL tunicamycin (TM) or 1 µg/mL brefeldin A (BFA) for 24h. (D) HEK293T cells were transfected with a total of 100 ng plasmid DNA of WT ADA2 or the pathogenic variant p.R169Q at the indicated ratios. ADA2 protein levels were determined in supernatant (*upper blot*) or whole cell lysate (*middle blot*) using anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam) and anti-DDK (FLAG) clone OTI4C5 (#TA50011, OriGene Technologies), respectively. (E) ADA2 protein expression in whole cell lysates of CD14+ monocytes from healthy control (HC) and DADA2 heterozygous carriers (CR) by western blot, normalized to β-actin. In figures A, C and E, ADA2 was detected using anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam). Legend: NT, non-transfected; EV, empty vector.

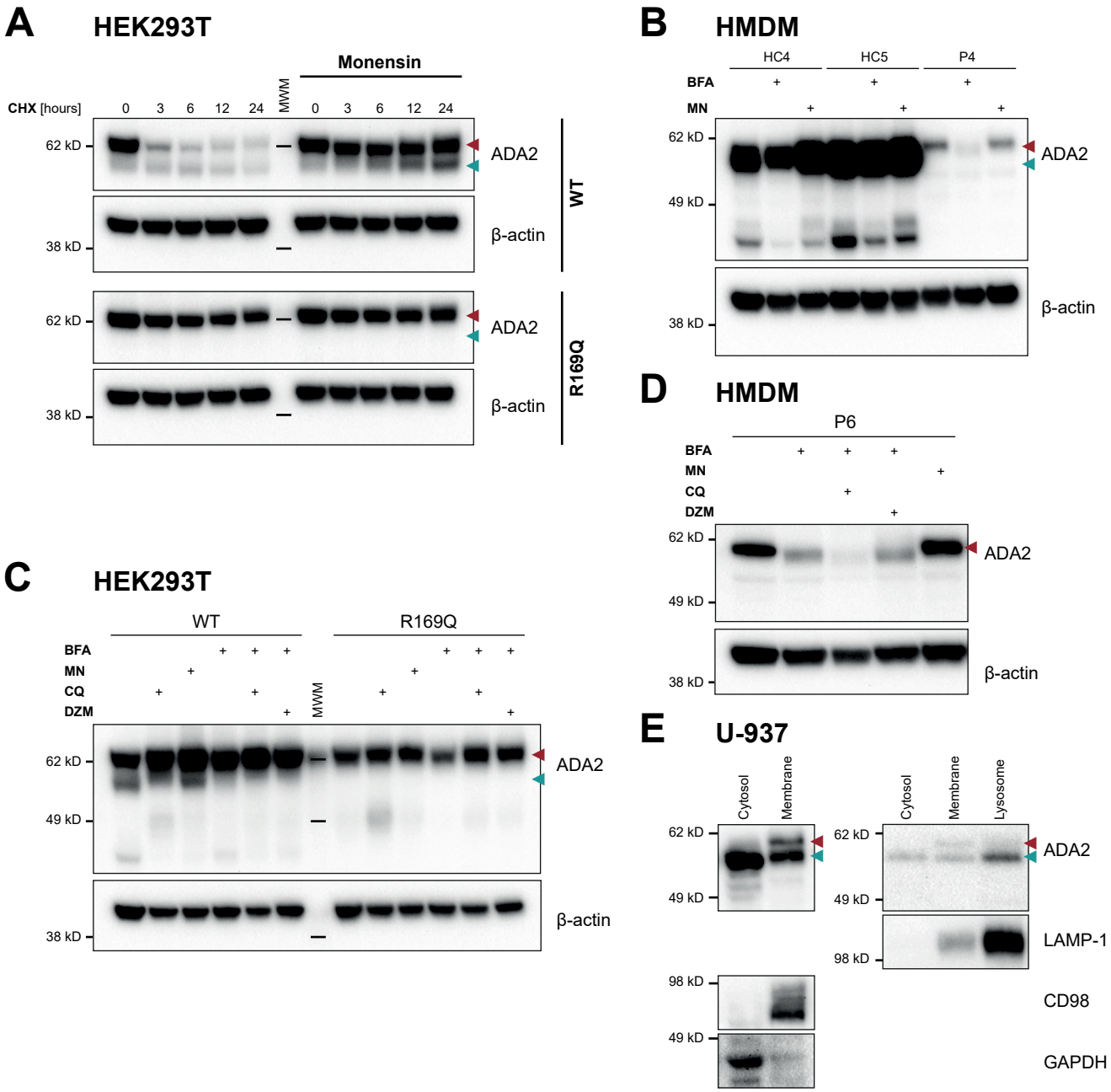

Figure 8: Trafficking and localization of HMW- and LMW-ADA2. (A) HEK293T cells were transfected with wild-type (WT) ADA2 or the pathogenic variant p.R169Q and treated with 500 µg/mL cycloheximide (CHX) ± 2 µg/mL monensin for 24h. Whole cell lysates were generated at the indicated time points and immunoblotted for ADA2 expression. (B) ADA2 protein expression in GM-CSF-differentiated human monocyte-derived macrophages from healthy controls (HC) or DADA2 patient P4 left untreated or treated with 1 µg/mL brefeldin A (BFA) or 2 µg/mL monensin (MN). (C) HEK293T cells were transfected with wild-type (WT) ADA2 or the pathogenic variant p.R169Q and treated with the following reagents for 24h as indicated: 50 µM chloroquine (CQ), 2 µg/mL monensin (MN), 1 µg/mL brefeldin A (BFA), 200 nM delanzomib (DZM). Whole cell lysates were immunoblotted for ADA2 expression. (D) HMDM from DADA2 patient P6 were generated by differentiation with 20 ng/mL GM-CSF for 10 days followed by 24-hour incubation with the following reagents: 1 µg/mL brefeldin A (BFA), 50 µM chloroquine (CQ), 200 nM delanzomib (DZM), 2 µg/mL monensin (MN). Whole cell lysates were immunoblotted for ADA2 expression. The western blot is representative of n=2 independent experiments. (E) ADA2 protein expression in cytosolic, membranous and lysosomal fractions of U-937 cells. Triangles indicate HMW-ADA2 (red) and LMW-ADA2 (green). In figure A-D, ADA2 was detected using anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam); in figure E anti-ADA2 (#HPA007888, Sigma-Aldrich) was used. Legend: MWM, molecular weight marker.

Tables

Table 1. Cohort characteristics

ID	Age [years]	Sex	Genotype	Predominant phenotype
P1	12	M	c.973-2A>G/c.del1240-1442; splice site (intron 6)/del exon 9	Vasculitis, hepatosplenomegaly, hypogammaglobulinemia
P2	14	F	c.973-2A>G/c.del1240-1442; splice site (intron 6)/del exon 9	Vasculitis, warts, hypogammaglobulinemia, arthralgia
P3	26	M	c.140G>T/c.del1240-1442; p.G47V/del exon 9	Bone marrow failure, hypogamma-globulinemia, hepatosplenomegaly
P4	26	M	c.140G>T/c.140G>T; p.G47V/p.G47V	Hypogammaglobulinemia, hepatosplenomegaly
P5	6	F	c.140G>T/c.506G>A; p.G47V/p.R169Q	Hypogammaglobulinemia, hepatosplenomegaly, stroke
P6	9	F	c.140G>T/c.506G>A; p.G47V/p.R169Q	Bone marrow failure, hypogamma-globulinemia, hepatosplenomegaly
P7	32	F	c.973-2A>G/c.506G>A; splice site (intron 6)/p.R169Q	Stroke, hypertension
P8	16	M	c.139G>A/c.139G>A; p.G47R/p.G47R	Stroke, vasculitis
P9	10	F	c.973-2A>G/c.973-2A>G; splice site (intron 6)/splice site (intron 6)	Vasculitis, hypogammaglobulinemia
P10	50	F	c.973-2A>G/c.506G>A; splice site (intron 6)/p.R169Q	Vasculitis, leukopenia
CR1	39	F	WT/c. del1240-1442; WT/del exon 9	
CR2	38	F	WT/c.140G>T; WT/p.G47V	
CR3	42	M	WT/c.506G>A; WT/p.R169Q	