Lentiviral correction of enzymatic activity restrains macrophage inflammation in adenosine deaminase 2 deficiency

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Running title: Lentiviral correction of DADA2

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Keywords: adenosine deaminase 2, gene therapy, lentiviral vectors, enzymatic activity, macrophages, inflammation

Text word count: 3948

Abstract word count: 198

Figures number: 7

Tables number: 1

References number: 38

Scientific category: Gene therapy

ABSTRACT

Adenosine deaminase 2 deficiency (DADA2) is a rare inherited disorder caused by autosomal recessive mutations in the ADA2 gene. Clinical manifestations include early-onset lacunar strokes, vasculitis/vasculopathy, systemic inflammation, immunodeficiency, and hematologic defects. Anti-TNF therapy reduces strokes and systemic inflammation. Allogeneic hematopoietic stem-progenitor cell (HSPC) transplantation can ameliorate most disease manifestations, but patients are at risk for complications. Autologous HSPC gene therapy may be an alternative curative option for patients with DADA2. We designed a lentiviral vector (LV) encoding ADA2 to genetically correct HSPCs. Lentiviral transduction allowed efficient delivery of the functional ADA2 enzyme into HSPCs of healthy donors. Supranormal ADA2 expression in human and mouse HSPCs did not affect their multipotency and engraftment potential *in vivo*. The LV-ADA2 induced stable ADA2 expression and corrected the enzymatic defect in HSPCs derived from DADA2 patients. Patients' HSPCs re-expressing ADA2 retained their potential to differentiate into erythroid and myeloid cells. Delivery of ADA2 enzymatic activity in patients' macrophages led to a complete rescue of the exaggerated inflammatory cytokine production. Our data indicate that HSPCs ectopically expressing ADA2 retain their multipotent differentiation ability, leading to functional correction of macrophage defects. Altogether, these findings support the implementation of HSPC gene therapy for DADA2.

INTRODUCTION

Adenosine deaminase 2 deficiency (DADA2) is a rare inherited disorder caused by autosomal recessive mutations in the *ADA2* gene.[1,2] Disease onset is usually in childhood, and a significant proportion of patients die early in life.[1-4] DADA2 patients present with a variable clinical phenotype, including cutaneous and cerebral vasculopathy, ranging from livedo reticularis and polyarteritis nodosa to life-threatening intracranial hemorrhages and lacunar strokes. Systemic inflammation similarly affects the kidney, liver, and gastrointestinal tract. The clinical spectrum also includes hematological and immunological manifestations, such as cytopenias (neutropenia, autoimmune hemolytic anemia, thrombocytopenia, severe pure red cell aplasia)[1,3,5], mild immunodeficiency, hypogammaglobulinemia, low switched memory B cells and low IgM serum levels.[6,7] Features that mimic autoimmune lymphoproliferative syndrome can also occur in DADA2.[8-10]

ADA2 has a partial structural homology of ADA1, a well-known enzyme catalyzing adenosine and deoxyadenosine deamination into inosine and deoxyinosine, respectively. Although ADA1 and ADA2 share adenosine deaminase activity, they differ substantially. ADA1 is a monomeric intracellular protein expressed in most cell types, but significant amounts can also be found in plasma. ADA2 is a secreted homodimeric protein produced by myeloid cells (monocytes, macrophages).[11] ADA2 affinity for adenosine substrate is much weaker than ADA1.[12] Based on these observations, ADA1 should represent the primary regulator of extracellular adenosine levels, while ADA2 should act locally at the site of inflammation, where adenosine levels are much higher. The most striking evidence that the two ADA isoforms have non-redundant biological functions derives from the

observation that ADA1 and ADA2 deficiencies manifest with distinct pathological features. Patients with ADA1 deficiency exhibit a severe combined immunodeficiency with no evidence of vasculopathy, systemic inflammation, and hematological defects typical of DADA2.

How ADA2 regulates the immune system is still enigmatic. Increased ADA2 levels were found in fluids of patients with infections (*M. tuberculosis*[13] and HIV-1[14]) and chronic inflammatory conditions (systemic lupus erythematosus[15], rheumatoid arthritis[16], macrophage activation syndrome[17], Crohn's disease[18], chronic active hepatitis[19]). Defective B cell and T follicular helper cell responses have been reported.[20] Pro-inflammatory cytokines (IL-1β, TNF) were found elevated in the skin and brain biopsies of patients.[1] Loss of ADA2 in patients is associated with increased production of pro-inflammatory cytokines from M1 macrophages and poor differentiation of M2 macrophages.[1] DADA2 is associated with enhanced neutrophil extracellular trap formation.[21] Marked upregulation of neutrophil-expressed genes and an interferon signature in patients' blood leukocytes was also reported.[22,23] All these observations suggest that DADA2 pathophysiology may derive from a dysregulated activation of the myeloid cell compartment, causing endothelial cell activation and vascular disease.

Since inflammation is one of the overarching features of DADA2, the current medical management is based on standard immunosuppression. Anti-TNF therapy reduced vasculitis and prevented strokes.[24,25] Allogeneic hematopoietic stem cell transplantation (HSCT) has shown promise in small cohorts of DADA2 patients with the hematological phenotype.[8,26,27] However, HLA-matched donors are not always available, and the morbidity risk of the HSCT procedure should not be underestimated in DADA2 patients with vasculitis, systemic inflammation, and

immunodeficiency.[27] Therefore, the development of alternative therapeutic approaches for DADA2 is urgently needed. It is reasonable to assume that strategies based on genetic correction and engraftment of autologous hematopoietic stem/progenitor cells (HSPCs) can provide a definitive therapeutic option for DADA2. This study evaluated whether a LV encoding ADA2 can restore enzymatic activity in patients' HSPCs and correct macrophage inflammatory activation.

METHODS

Patients

Patient 1 and 2 (twins) carry heterozygous substitution at c.563T>C (p.Leu188Pro) and exon 7 deletion (IVS6_IVS7del), patient 3 carries a homozygous missense mutation c.1367A>G (p.Tyr456Cys), patient 4 a homozygous substitution c. 139G>A (p.Gly47Arg), patient 5 a deletion in exon 2, c.144del (p.Arg49Glyfs) and the nucleotide substitution c.1085G>A (p.Trp362Ter), patient 6 and 7 (siblings) the missense mutations c.140G>T (p.Gly47Val) in exon 2 and c.1435T>C (p.Ser479Pro) in exon 9. Table I describes patient characteristics.

Study approval

All clinical investigation has been conducted according to Declaration of Helsinki principles. Patients, parents, and healthy controls gave informed consent following a standard ethical procedure (Clinical protocol number Tiget 06) approved by the Ethical Committees of IRCCS Ospedale San Raffaele (Milan, Italy) and IRCCS Ospedale Giannina Gaslini (Genoa, Italy). Bone marrow and peripheral blood were collected from DADA2 patients on the occasion of diagnostic or therapeutic procedures. The relevant institutional review boards approved the research.

Mouse studies were conducted according to protocols approved by the IRCCS San Raffaele Scientific Institute and Institutional Animal Care and Use Committee (IACUC #997), adhering to the Italian Ministry of Health guidelines for the use and care of experimental animals. All efforts were made to minimize the number, pain, and distress of mice during and after experimental procedures.

Mice

NOD.Cg-Kit^{W-41J}Prkdc^{scid}Il2rgtm1Wjl/^{WaskJ} (NSGW41, stock #026497) mice were purchased from the Jackson Laboratory. C57BL/6-Ly5.1 and C57BL/6N mice were purchased from Charles River (Calco, Italy). Mice were maintained in specific pathogen-free conditions at the IRCCS San Raffaele Scientific Institute SPF Animal Facility.

Lentiviral transduction of human CD34$^+$ cells

Bone marrow CD34$^+$ cells isolated from healthy donors (HDs) were purchased from Lonza. Patients' CD34$^+$ cells were purified from BM aspirate using the CD34 MicroBead Kit (Miltenyi Biotech). Cell purity was >97%. Cells (1x10^6 cells/ml, 0.4-1.1x10^5 cells/well) resuspended in serum-free CellGro medium containing human SCF (300 ng/ml), TPO (100 ng/ml), FLT3 (300 ng/ml), and IL-3 (60 ng/ml; all from Peprotech) were incubated in RetroNectin-precoated wells for 22 hours. 16,16-dimethyl-Prostaglandin E_2 (10 μM) was added 2 hours before LV transduction at the indicated multiplicity of infections (MOI). After 16 h transduction, cells were collected, washed, and cultured in IMDM medium supplemented with fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, SCF (100 ng/ml), TPO (100 ng/ml), Flt3L (100 ng/ml), and IL-3 (20 ng/ml) for 14 days.

Colony-forming assay

Purified CD34$^+$ cells were resuspended in complete human Methocult medium and plated at 1,000 cells per plate in 35-mm plates in duplicate per condition. Fifteen days later, erythroid burst-forming units, granulocyte-macrophage colony-forming

units, and granulocyte, erythroid, macrophage, megakaryocyte colony-forming units were scored for number and morphology by light microscopy.

Macrophage transduction and polarization

$CD14^+$ monocytes were purified from peripheral blood mononuclear cells using the CD14 MicroBeads (Miltenyi Biotec), according to the manufacturer's instructions. Monocytes were plated in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-Glutamine in the presence of human recombinant M-CSF (10 ng/ml). For transduction, monocytes were incubated for 2 hours with the accessory viral protein vpl-VPX, followed by overnight transduction at the MOI of 10. Viral-containing supernatant was removed, and cells were incubated with M-CSF (10 ng/ml) in a complete medium for four days. For M1 polarization, macrophages were treated with *E. coli* LPS (1 µg/ml) and human recombinant IFNγ (20 ng/ml) for 48 hours.

U937 cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-Glutamine and differentiated into macrophages by incubation with 12-O-Tetradecanoylphorbol-13-acetate (TPA, 20 ng/ml) for 48 hours. U937 macrophages were cultured in a complete medium devoid of TPA for one day and then polarized into M1-like macrophages with LPS (50 ng/ml) and IFNγ (10 ng/ml) for 48 hours.

Statistical methods

GraphPad Prism version 9 (GraphPad Software) was used to prepare graphics and perform statistical analyses. Mann-Whitney U test was used to compare the distribution of a numerical variable among two independent groups. The rank-based

non-parametric Kruskal-Wallis test determined statistically significant differences between two or more groups and the non-parametric Wilcoxon signed-rank test to compare two matched samples. P-values of ≤0.05 were considered statistically significant.

RESULTS

LV-mediated transduction directed the efficient delivery of ADA2 expression and enzymatic activity in human CD34$^+$ cells without evidence of toxicity

We explored the ADA2 expression profile in various subsets of peripheral blood mononuclear cells and bone marrow (BM) progenitors. ADA2 mRNA and protein were expressed more strongly in CD14$^+$ monocytes and, at a lower level, also in T (CD4$^+$ and CD8$^+$), B (CD19$^+$), NK (CD56$^+$) cells, and BM CD34$^+$ HSPCs (**Figure S1**).

Based on the broad expression profile in immune cells, we generated a LV in which the expression of human ADA2 was driven by the ubiquitous human phosphoglycerate kinase promoter (PGK; LV-ADA2) (**Figure 1A**). The PGK promoter allows stable expression of therapeutic proteins in myeloid cells[28] and has been used at SR-TIGET to transduce CD34$^+$ cells in >40 patients affected by metabolic disorders with a favorable risk-benefit profile for up to 8 years post-gene therapy.[29] A PGK.GFP LV was used as a negative control vector (LV-GFP). Human CD34$^+$ HSPCs isolated from the BM of three independent, healthy donors (HDs) were transduced with the LV-ADA2 or LV-GFP at a multiplicity of infection (MOI) of 10, 50, and 100, in the presence of prostaglandin E_2 as a transduction enhancer.[30,31] Transduction efficiency evaluated on individual vector-positive granulocyte-macrophage colony-forming units (CFU-GM) and erythroid burst-forming units (BFU-E) was on average ≥75% in all conditions (**Figure 1B**). The median vector copy number (VCN) ranged from 1.82 to 2.69 and 1.58 to 3.70 in the CFU-GM and BFU-E colonies, respectively (**Figure 1C**). The viability of CD34$^+$ cells pre and post LV-ADA2 exposure was comparable to cells cultured in the absence (UT) or presence of the LV-GFP (**Figure 1D**). LV-ADA2 transduction did not affect the clonogenic

potential of $CD34^+$ cells at each vector dose (**Figure 1E**), indicating a lack of toxicity even at a high VCN.

Next, we examined ADA2 expression and activity in HD CD34-derived myeloid cells at 14 days post-transduction. An increase in the protein and transcript ADA2 levels was observed in LV-ADA2 but not LV-GFP transduced cells (**Figure 2A, B**). Vector-derived ADA2 protein exhibiting a proper enzymatic activity was also secreted from ADA2-transduced cells in a dose-dependent manner (**Figure 2C, D**). Moreover, pro-inflammatory (M1-like) macrophages differentiated from LV-ADA2 transduced HD $CD34^+$ cells released TNF and IL-6 at similar levels of LV-GFP transduced and untransduced macrophages (**Figure S2**).

These results indicate that the transduction procedure and LV-derived ADA2 expression are well-tolerated and do not influence the capacity of HSPCs to differentiate into myeloid and erythroid cells and that HSPC-derived myeloid cells can mount a proper inflammatory response *in vitro*.

ADA2 transduction did not impact the multilineage engraftment potential of BM $CD34^+$ cells *in vivo*

We examined the engrafting and differentiation potential of ADA2-transduced $CD34^+$ cells *in vivo*. HD $CD34^+$ cells transduced with LV-ADA2 or LV-GFP were transplanted into NSGW41 mice lacking T, B, and NK-cells in the absence of pre-conditioning.[32] Vector-positive leukocytes in the peripheral blood were comparable between ADA2 and GFP mice (**Figure 3A**). The percentage and the absolute number of human $CD45^+$ cells increased over-time in both groups, reaching 0.5-1.0% at 20 weeks post-transplant (**Figure 3B**). The absolute counts and the kinetic of the reconstitution of all peripheral blood leukocyte subsets (B, T, myeloid, and NK cells) were similar in the

ADA2- and GFP-groups (**Figure 3C**). Moreover, uncommitted hematopoietic stem cells (HSC), myeloid, lymphoid, and erythroid progenitors and differentiated cells evaluated in the BM at 20 weeks did not differ among mice receiving ADA2-and GFP-transduced $CD34^+$ cells (**Figure 3D**), indicating that LV-derived ADA2 expression does not impact $CD34^+$ cell ability to engraft, self-renew, and give rise to a multilineage repertoire.

To investigate whether ADA2 over-expression can affect HSPC engraftment and differentiation in a complete null ADA2 background, we generated mouse chimeras, as mice lack an ADA2 ortholog. Lineage-negative cells isolated from the BM of CD45.1 C57BL/6 mice were transduced with LV-ADA2 or LV-GFP and adoptively transferred into lethally irradiated CD45.2 C57BL/6 mice. The median VCN in blood leukocytes was similar between ADA2 and GFP mice and remained stable for up to 19 weeks post-transplant (**Figure S3A**). Plasma enzymatic activity in ADA2 chimeras was within the range of human adult HDs' plasma (7.0-25.2 U/L) (**Figure S3B**). ADA2 chimeras gained normal weight and did not show any sign of distress (**Figure S3C**). Normal tissue architecture and cellularity were observed in the spleen and BM of ADA2 and GFP chimeras (**Figure S3D**). Donor chimerism, myeloid and lymphoid cell counts in peripheral blood were comparable between ADA2 and GFP chimeras (**Figure S4**). We evaluated whether ADA2 overproduction might influence the inflammatory response *in vivo*. Upon LPS treatment, ADA2 and GFP chimeras produced a similar plasma cytokine profile (**Figure S5**).

Altogether, these results indicate that ADA2-expressing human and mouse HSPCs maintain long-term repopulation and multilineage differentiation potential and that ectopic ADA2 overexpression does not exacerbate the inflammatory cytokine response *in vivo*.

The BM of patients with the hematological phenotype of DADA2 exhibits a substantial reduction of HSC and progenitor pools

Despite evidence of hematological alterations, there are currently no reports describing the phenotypic characteristics of the BM cells of patients with DADA2. Thus, we studied in-depth the BM cell composition of adult patients, two presenting with severe pancytopenia and BM aplasia requiring allogeneic HSCT (PT1 and PT3) and one (PT2, twin of PT1) with autoinflammatory manifestations and neutropenia by multiparametric flow cytometry. A cohort of adult HDs was used as a reference dataset for comparative analysis with patients' BM samples. Compared to HDs, patients' BM exhibited a reduced number of mature and immature populations belonging to different hematopoietic lineages. The amount of myeloid, B, and erythroid cells was low in all ADA2-deficient patients (**Figure 4A-C**). Defects in the general hematopoietic output can be attributed to a diminished HSPC number. Indeed, the number of HSCs and committed progenitors of multiple lineages (lymphoid, myeloid, erythroid, and megakaryocytes' progenitors) were significantly reduced in patients' BM compared to HD controls (**Figure 4D, E**). These immunophenotyping data suggest that DADA2, through cell-intrinsic regulation or extrinsic factors (*i.e.,* inflammation, autoreactive antibodies), affect the maintenance of HSCs, giving rise in the long-term to single- or multilineage-cytopenia typical of patients with hematological and immunological manifestations.

LV-mediated ADA2 expression in patients CD34$^+$ cells restores enzymatic activity

We investigated the ability of the LV-ADA2 to restore ADA2 expression and enzymatic activity in patients' CD34$^+$ cells. Purified BM CD34$^+$ cells from PT2 and

PT3 were cultured in the presence or absence of LV-ADA2 and LV-GFP at a MOI of 10 and 50. PT2 and PT3 $CD34^+$ cells exhibited a normal capacity to produce CFU-GM, CFU-GEMM, and BFU-E colonies (**Figure 5A**), suggesting no disruption in their clonogenic capacity following genetic manipulation, despite the overall reduced content. The CFU assay showed a dose-effect relationship between vector dose and transduction efficiency, which reached >75% at the MOI of 50 for both vectors, and the median VCN per genome was ~2.3 at MOI 50 (**Figure 5B, C**).

We next measured ADA2 protein expression in untransduced and transduced patients' $CD34^+$ cells maintained *in vitro* with cytokine support for 14 days. ADA2 was expressed in untransduced HDs' cells but absent in patients' cells (**Figure 6A**), indicating that mutations are associated with a complete loss of ADA2 in HSPCs. $CD34^+$ cell transduction with the LV-ADA2 re-established intracellular ADA2 expression in both patients in a dose-dependent manner (**Figure 6A**). LV-derived ADA2 released from ADA2-transduced PT3's cells exhibited a dose-dependent enzymatic activity by increasing vector dose (**Figure 6B, C**). These findings indicate that LV-ADA2 is an efficient tool to stably deliver ADA2 expression and re-establish enzymatic activity in patients' $CD34^+$ cells.

ADA2 gene transfer corrects the pro-inflammatory profile of patients' macrophages

DADA2 is associated with an increased pro-inflammatory macrophage profile.[1] To study whether LV-derived ADA2 corrects the hyperinflammatory macrophage phenotype, monocyte-derived macrophages from six patients were transduced with the LV-ADA2 and differentiated into M1-like macrophages. ADA2 protein was absent in untreated macrophages from all patients (**Figure 7A**). Macrophage transduction

with the LV-ADA2 re-established extracellular ADA2 enzymatic activity (**Figure 7B**). While uncorrected patients' M1-macrophages showed increased IL-6 and TNF expression despite anti-TNF treatment, LV-mediated ADA2 reconstitution led to a significant reduction of IL-6 expression in all patients, while TNF expression decreased in 3/5 patients (**Figure 7C**). IL-6 and TNF release was significantly reduced in patients' M1 macrophages after ADA2 reconstitution (**Figure 7D**). Similar results were obtained using M1 macrophages differentiated from ADA2-transduced $CD34^+$ cells of PT1 (**Figure S6**). These data provide the first evidence that LV-mediated restoration of ADA2 corrects the inflammatory macrophage defect in DADA2.

Next, we assessed whether macrophage-mediated inflammation is a functional defect caused by loss of ADA2 and whether enzymatic activity acts as an effective brake of macrophage activation, minimizing the risk of unwanted inflammation. To this aim, we used a U937 macrophage cell line deficient for ADA2 transduced with a LV encoding either wild type ADA2 ($ADA2^{WT}$ LV; VCN=2.10±0.16) or L188P mutant ADA2 lacking enzymatic activity ($ADA2^{L188P}$ LV; VCN=2.18±0.03) (**Figure 7E, F**). Upon LPS/IFNγ exposure, untransduced $ADA2^{-/-}$ U937 macrophages expressed and secreted TNF and IL-6 at higher levels than $ADA2^{+/+}$ macrophages, similarly to patients' macrophages (**Figure 7G, H**), confirming that the U937 cell model well-recapitulates a clinically-relevant disease phenotype. The reconstitution of wild type, but not the L188P ADA2 mutant, restored a physiological secretion of TNF and IL-6 (**Figure 7G, H**). These data indicate that the amplified inflammatory response of patients' macrophages is a cell-intrinsic consequence of the loss of ADA2 and that enzymatic activity is essential for the control of macrophage activation.

DISCUSSION

The therapeutic value of allogeneic HSCT for patients with DADA2[27], together with the excellent therapeutic efficacy of gene therapy in patients with ADA-SCID[29], suggests that transplantation of autologous gene-corrected HSPCs may also represent a promising strategy for DADA2. Therefore, we designed a pre-clinical study to evaluate the efficacy of an ADA2-encoding LV in support of its future development for clinical studies. The efficient transduction of myeloid and erythroid progenitors was obtained using the LV-ADA2 and optimized gene transfer conditions. ADA2-transduced HDs' HSPCs expanded as much as HSPCs transduced with the control vector, and the number of CFU-GM and BFU-E colonies derived from ADA2-transduced HSPCs were comparable to the untransduced condition. HSPC transduction with the LV-ADA2 led to a dose-dependent increase of intracellular expression and release of ADA2, which exhibited a proper enzymatic activity. We also employed two mouse models to examine the effect of ADA2 (over)-expression *in vivo*. Immunocompromised NSGW41 mice were transplanted with ADA2-transduced HD $CD34^+$ cells isolated from HD's BM. LV-ADA2 transduced lineage-negative cells of CD45.1 C57BL/6 mice were also adoptively transferred into congenic C57BL/6 recipients, which are naturally null for ADA2. In both models, engraftment of ADA2-transduced HSPCs was efficiently achieved. Human and mouse HSPCs were able to generate a multilineage immune cell repertoire as much as HSPCs transduced with the GFP control vector. Mice did not show remarks of distress or transduction-related toxicity and showed normal spleen and BM tissue architecture. ADA2 over-expression did not change LPS-elicited systemic cytokine response *in vivo*. These results reveal that ADA2 over-expression is well-tolerated *in vivo* and does not alter

myeloid and erythroid progenitors' proliferation and maturation potential, and suggest that tight regulation of ADA2 expression might not be necessary, as also demonstrated in pre-clinical and clinical studies for ADA-SCID.[33,34]

An in-depth analysis of the BM compartment of three DADA2 patients with hematological manifestations revealed a significant reduction of HSC and progenitors, which resulted in insufficient production or premature depletion of mature cells of the myeloid, lymphoid, and erythroid lineage. It is unclear whether HSC loss is a direct consequence of ADA2 deficiency in these cells or extrinsic factors, like chronic inflammation and autoreactive antibodies, contributing to this phenomenon and eventually causing cytopenia in patients with DADA2. Future studies would be necessary to compare the BM cell composition between children at early disease onset and adults and examine whether anti-TNF therapy prevents or slows down HSC loss. Even though the absolute number of patients' HSPCs was low, cell recovery sufficed to examine the ability of LV-ADA2 to restore ADA2 expression and activity in these cells. Clinically relevant transduction levels (≥75%) and vector copies (1-3 per cell) were achieved after a single transduction with the LV-ADA2 in the HSPCs of two patients. LV-ADA2 reconstituted the intracellular ADA2 expression in CD34-derived myeloid cells at normal and supranormal levels after transduction at MOI 10 and 50, respectively, which was accompanied by ADA2 secretion in cell supernatants. Notably, *ex vivo* transduction and ADA2 overexpression did not alter patients' CD34 cell ability to differentiate in myeloid and erythroid cells. Collectively, these results demonstrate that the LV-ADA2 platform and transduction conditions can efficiently deliver the functionally active ADA2 in patients' HSPCs.

From the perspective of a future clinical application, we examined the ability of our LV-ADA2 to correct the exaggerated release of pro-inflammatory cytokines from patients' macrophages. We confirmed in an Italian cohort of patients with a heterogeneous clinical phenotype that ADA2-deficient macrophages release inflammatory TNF and IL-6 cytokines at high levels.[1] LV-mediated ADA2 reconstitution in these cells restored a physiological secretion of TNF and IL-6. These results were well recapitulated in an ADA2-deficient U937 macrophage cell line, indicating that the excessive macrophage inflammatory response is a direct consequence of ADA2 loss rather than a secondary event caused by excessive inflammation occurring in patients. Notably, we demonstrated that the correction of the macrophage inflammatory profile requires ADA2 enzymatic activity.

Several questions need to be answered, including the engraftment level and the conditioning regimen intensity required to achieve disease control, the therapeutic ADA2 range, and the possible selective advantage of transduced cells. HSCT experience shows that full-donor chimerism successfully restored the hematological, immunological, and vascular phenotype in patients with DADA2.[26,27] The highly diverse phenotypic variability reported in DADA2 not always correlates with the residual levels of ADA2 activity.[35] Suboptimal ADA2 function seems to be associated with vasculitis, while a more extensive loss was observed in patients with hematological disease.[36] However, 50% of HD enzymatic activity may be sufficient to revert the clinical manifestations, considering that parents carrying one mutation are typically unaffected. Since a recent study implicated ADA2 in regulating B-cell proliferation and immunoglobulin secretion[20], ADA2 correction in lymphocytes might provide these cells with a growth advantage in patients undergoing HSPC gene therapy. Therefore, a reduced-intensity conditioning regimen may be an option in

these patients to overcome the risk of infections and toxicity. Supranormal levels of ADA2 enzymatic activity may eventually compensate for incomplete correction. On the other hand, in patients with a predominant myelomonocytic defect and inflammatory phenotype, a fully myeloablative conditioning regimen may be needed to fully deplete the diseased compartment since myeloid cells do not usually exhibit a selective advantage, as demonstrated in other myeloid diseases.[37] Finally, the ability of corrected HSPCs to engraft *in vivo* in an inflamed environment following BM failure should be investigated. There may be a need for a bridge treatment with anti-TNF therapy to favor the engraftment of corrected HSPCs. This last measure could also limit post-transplant vascular complications in patients presenting compromised endothelial integrity caused by DADA2.

Which patients will most likely benefit from an HSPC gene therapy approach for DADA2 once pre-clinical safety and efficacy studies would be completed? Gene therapy may be a promising treatment option for young patients with immunodeficiency and inflammation-associated manifestations or at an initial stage of BM failure syndrome. Indeed, we demonstrated efficient transduction and preserved regenerative potential of HSPCs in three adult patients with similar disease characteristics and undergoing anti-TNF therapy at the time of BM harvest. Allogeneic HSCT would remain the most effective treatment for patients, especially young adults and adults, with cytopenias involving single or multiple lineages due to progressive BM failure or reduced regenerative potential of HSPCs. Indeed, these patients may be unable to donate a sufficient amount of autologous HSPCs for gene therapy. However, the use of mobilizing agents, such as G-CSF alone or in combination with Plerixafor, patient's treatment with anti-inflammatory drugs (*i.e.*, anti-TNF agents) before HSPC harvest, and improved *ex vivo* manipulation

combining HSPC transduction and expansion may allow achieving clinically relevant doses for HSPC gene therapy also for these patients.[30,38]

In conclusion, we showed the therapeutic value of the LV-ADA2 platform by demonstrating efficient reconstitution of ADA2 expression and activity in patients' HSPCs and correction of the excessive inflammatory response of patients' macrophages, which is at the basis of some of the disease manifestations. LV-mediated HSPC gene therapy may represent an effective treatment for patients with DADA2, and further studies are warranted in support of its clinical development.

AUTHOR CONTRIBUTIONS

Study concept, design and supervision – A.M., A.A., M.P.C., L.N. Acquisition, analysis and interpretation of data – M.Z., I.B., F.B., S.S., R.J.H., L.B.-R., M.C., E.P. Generation and provision of study material: L.S.S., G.M., P.C., A.L., S.G. Pathology evaluation: F.S. Provision of patients' material: F.B., S.C., F.C., A.P., M.G., M.P.C. Provision of enzyme activity data: P.Y.L. Writing of the manuscript – A.M. Critical revision of the manuscript – M.Z., I.B., F.B., S.S., L.N., M.P.C., A.A.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGEMENTS

This work was funded by grants from the European Commission (Marie Skłodowska-Curie Actions, Individual Fellowship to AM and E-rare EUROCID project to AA), Fondazione Telethon (SR-Tiget Core Grant, Project A5, to AM), Jeffrey Modell Foundation to AA, and partly by the Else Kröner Fresenius Prize for Medical Research 2020 to AA. We thank La Spes organization for supporting fellowships to MZ and IB. The presented work is in partial fulfillment of MZ's Ph.D. thesis. IRCCS Istituto Giannina Gaslini and IRCCS San Raffaele Hospital are part of the European Reference Network (ERN) for Rare Immunodeficiency, Autoinflammatory and Autoimmune Diseases (Project ID No 739543). Selected centers are part of the Italian Onco-Hematology Association (AIEOP) and Inborn Error Working Party of EBMT. We thank all patients, family members, anda staff from all the clinical units that participated in the study. We thank Claudia Sartirana for assistance in patients' sample processing, Anna Kajaste-Rudnitski for obtaining the vpl-VPX plasmid, Luca

Cesana for preparing the vpl-VPX particles, Rossana Norata for preparing the mouse tissue specimens, Paola Rancoita for some statistical analysis, the Flow cytometry Resource, Advanced Cytometry Technical Applications Laboratory (FRACTAL) for assistance with flow cytometry.

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FIGURE LEGENDS

Figure 1. Efficient LV-mediated ADA2 transfer in HDs' HSPCs. (**A**) Schematic representation of the LV-ADA2 and LV-GFP used in the study. (**B, C**) Percentage of transduction and vector copy number (VCN) per genome were evaluated in colonies derived from $CD34^+$ HSPCs of three independent, healthy donors (HDs) transduced with the LV-ADA2 at increasing vector concentrations (MOI=10, 50, 100). Numbers indicate the median VCN for each condition. (**D**) The viability of BM HDs' $CD34^+$ HSPCs was examined before pre-stimulation (Day 0), post overnight transduction (Post-TR) with the LV-ADA2 and LV-GFP, and after *in vitro* expansion for 14 days (Post-LC) in the presence of support cytokines. Numbers indicate the expansion rate between in vitro expanded cells (Post-LC) and cells recovered after transduction (Post-TR). (**E**) Total numbers and percentages of single colonies derived from myeloid (CFU-GM) and erythroid (BFU-E) progenitors in untransduced (UT), ADA2-, and GFP-transduced conditions. **, $p<0.01$; ***, $p<0.001$; Mann-Whitney U test. Data in B, D, and E represent the means ± standard deviation, and data in C as box-and-whisker plots.

Figure 2. Robust ADA2 expression, secretion, and enzymatic activity in CD34-derived cells after LV-ADA2 transduction. (**A, B**) ADA2 expression was measured at transcript (**A**) and protein (**B**) levels in expanded CD34-derived cells from HDs left untreated (UT) or transduced with the LV-ADA2 and LV-GFP at MOI 10, 50, and 100. GAPDH was included as a loading control. Levels of secreted ADA2 were also shown. ADA2 protein (**C**) and enzymatic activity (**D**) were measured in cell-free

supernatants collected from cells transduced with the LV-ADA2 and LV-GFP at MOI 10, 50, and 100. *, $p<0.05$. Data represent the means ± standard deviation.

Figure 3. Normal engraftment and multilineage differentiation of ADA2-transduced HD's CD34$^+$ cells in NSGW41 mice. (**A**) The average of the vector copy number (VCN) per genome was evaluated in ADA2- and GFP-transduced HSPCs expanded in vitro (LC), in total peripheral blood leukocytes at 7, 14, and 20 weeks post-transplant, and at 20 weeks in the BM and spleen of NSGW41 mice receiving LV-ADA2 or LV-GFP transduced lineage-negative cells (n=6 mice per group). (**B, C**) Longitudinal analysis of human cell chimerism (CD45$^+$ cells), B, T, myeloid, and NK cells in peripheral blood at 7, 14, and 20 post-transplantation. (**D**) Flow cytometry analysis showing the total number of HSCs and various progenitor populations in the BM of NSGW41 mice. **, $p<0.01$; ***, $p<0.001$. Data represent the means ± standard error.

Figure 4. Patients' BM contains a reduced number of HSCs and progenitors. (**A-E**) The absolute number of various cell lineages and progenitors was estimated in the BM of three patients with DADA2 by multidimensional flow cytometry and compared to a cohort of 12 adult HDs. iPMN, immature polymorphonucleated cells; PMN, mature polymorphonucleated cells; DC, dendritic cells; HSPC, hematopoietic stem and progenitor cells; HSC, hematopoietic stem cells; MPP, multipotent progenitors; MLP, multilymphoid progenitors; ETP, early T-cell progenitors; Pre-BNK, pre-B and NK progenitors; CMP, common myeloid progenitors; GMP, granulocyte–monocyte progenitors; MEP, megakaryocyte–erythroid progenitors; MKp,

megakaryocyte progenitors; EP, erythroid progenitors. *, p<0.05; **, p<0.01. Data distribution are represented as violin plots.

Figure 5. Efficient transduction of patients' CD34$^+$ cells using the ADA2-LV. (**A**) The clonogenic potential of CD34$^+$ cells of two patients and two HDs left untransduced (UT) or transduced with LV-ADA2 and LV-GFP at the MOI of 10 and 50 was evaluated as number and percentage of myeloid (CFU-GM), erythroid (BFU-E), and mixed (CFU-GEMM) colony-forming units. (**B, C**) Transduction efficiency was calculated as the proportion of vector-positive colonies (**B**) and average vector copy number (VCN) per genome evaluated on positive colonies (**C**) by droplet digital PCR. *, p<0.05; **, p<0.01: ***, p<0.001.

Figure 6. LV-ADA2 restores ADA2 expression and enzymatic activity in patients' CD34-derived cells. (**A, B**) ADA2 expression was evaluated in CD34-derived cells (**A**) and supernatants (**B**) of two HDs and two PTs transduced with LV-ADA2 or LV-GFP at the MOI of 10 and 50. β-actin expression was included as a loading control. (**C**) ADA2 enzymatic activity was quantified in cell-free supernatants from ADA2- and GFP-transduced CD34-derived cells from one HD and one patient.

Figure 7. **LV-mediated ADA2 correction in patients' macrophages restored ADA2 enzymatic activity and suppressed cytokine hyperproduction.** (**A**) ADA2 protein expression was assessed in monocyte-derived macrophages from six patients with DADA2 and HDs by immunoblot analysis. β-actin expression was included as a loading control. (**B**) ADA2 enzymatic activity was measured in cell-free supernatants from patients' (n=5) and HDs' (n=7) macrophages transduced or not

with the ADA2-LV. (**C, D**) IL-6 and TNF expression and release were assessed in untransduced and ADA2-transduced M1-macrophages of patients and HDs by quantitative RT-PCR (**C**) and ELISA (**D**), respectively. HDs, n=5; patients, n=6 except for RT-PCR for TNF expression n=5. (**E**) Immunoblot analysis of ADA2 expression and secretion of $ADA2^{+/+}$ and $ADA2^{-/-}$ U937 macrophages transduced with LV encoding wild-type ADA2 ($ADA2^{WT}$) or the ADA2 mutant L188P lacking enzymatic activity ($ADA2^{L188P}$). (**F**) ADA2 enzymatic activity was assessed in cell-free supernatants of untransduced $ADA2^{+/+}$ and $ADA2^{-/-}$ U937 macrophages transduced with $ADA2^{WT}$ and $ADA2^{L188P}$ (**G, H**) Expression and secretion of TNF and IL-6 were measured in $ADA2^{-/-}$ U937 macrophages expressing ADA2 wild-type or L188P mutant by quantitative RT-PCR (**G**) and ELISA (**H**). ns, not significant, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Data in F, G, and H represent the means ± standard deviation. (UT, untransduced)

Table I. Characteristics of the patients included in the study

ID	Gender	Age (y)	Mutations	Phenotype	IS Therapy*	G-CSF
1	F	23	p.Leu188Pro / IVS6_IVS7del*	2 strokes (2-3y), hypogammaglobulinemia (3y), recurrent infections (since 13y), livedo reticularis (13y), neutropenia (14y), TLGL (21y)	Etanercept, PD	yes
2	F	23	p.Leu188Pro / IVS6_IVS7del*	2 strokes (8-14y), neutropenia (8y), hypogammaglobulinemia (8y), livedo reticularis (10y), hypothyroidism (22y)	Etanercept	yes
3	F	31	p.Tyr456Cys / p.Tyr456Cys	Hodgkin Lymphoma (28y), severe prolonged neutropenia after chemotherapy (30y)	MPD	no
4	F	18	p.Gly47Arg / p.Gly47Arg	Livedo reticularis (early infancy), recurrent giardiasis (3-4y), PAN (5y), hemorrhagic stroke (10y), intracranial aneurysm rupture (17y), hypertension, systemic inflammation	Etanercept	no
5	F	63	p.Arg49Glyfs* / p.Trp362Ter	Oral candidiasis (1m), RRI (infancy), hypogammaglobulinemia (14y), neutropenia (20y), recurrent pneumonias (45-50y), TLGL (61y)	Etanercept, Rituximab	yes
6	F	24	p.Gly47Val / p.Ser479Pro	Livedo (7y), neurological symptoms (8y), PAN and hypertension (9y), pericarditis (10y), peripheral neuropathy (12y), hypo IgM (24y), systemic inflammation	Etanercept	no
7	M	25	p.Gly47Val / p.Ser479Pro	Neurological symptoms (2y), hypertension (10y), PAN (11y), stroke (11y), hypogammaglobulinemia (25y), systemic inflammation	Etanercept	no

G-CSF, granulocyte colony stimulating factor; IS, immunosuppressive; MPD, methylprednisolone; PAN, panarteritis nodosa; PD, prednisone; RRI, recurrent respiratory infections; SAH, subarachnoid hemorrhage; TLGL, T large granular lymphocytes. *, ongoing treatment at the time of blood collection.

Figure 1

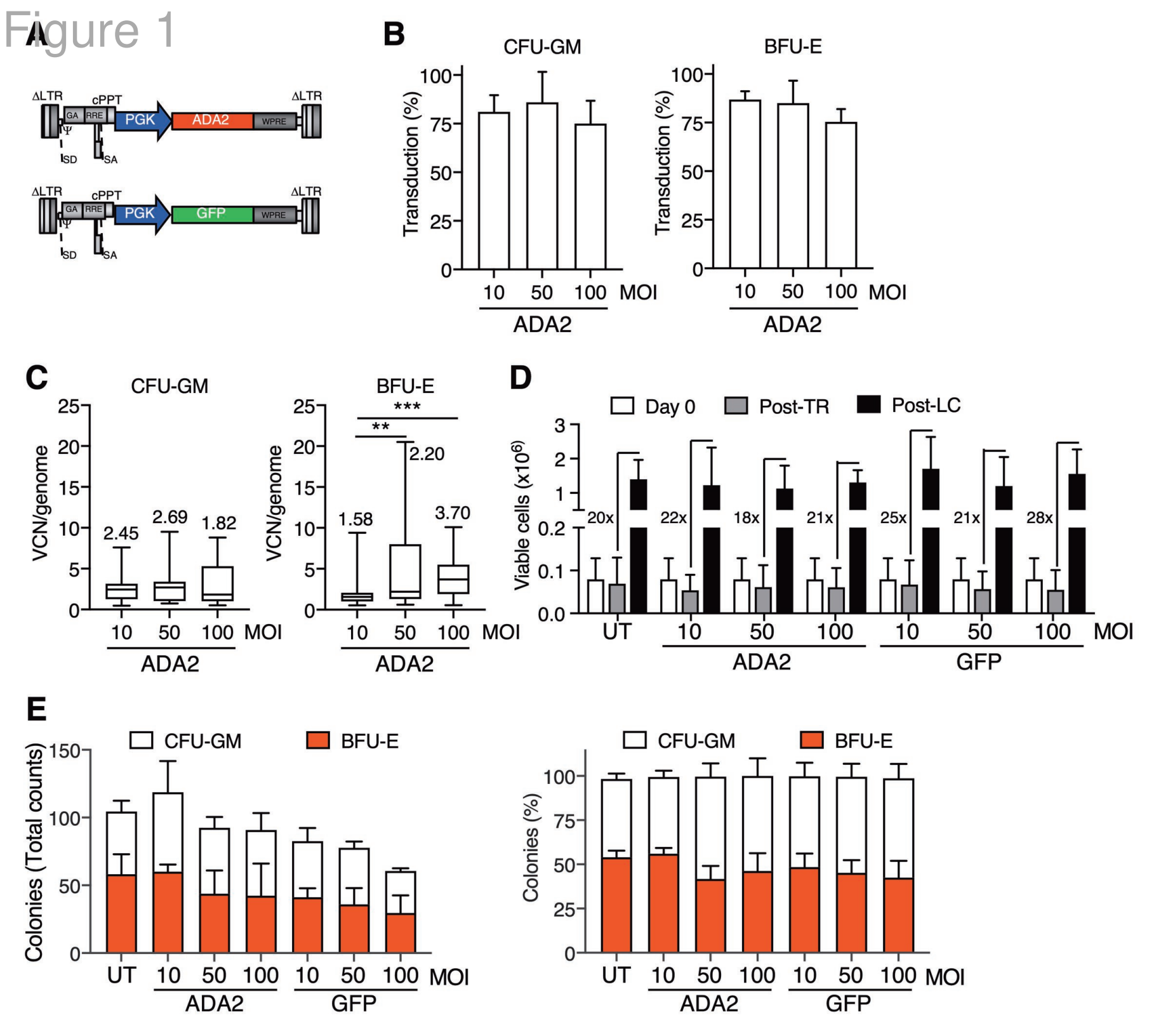
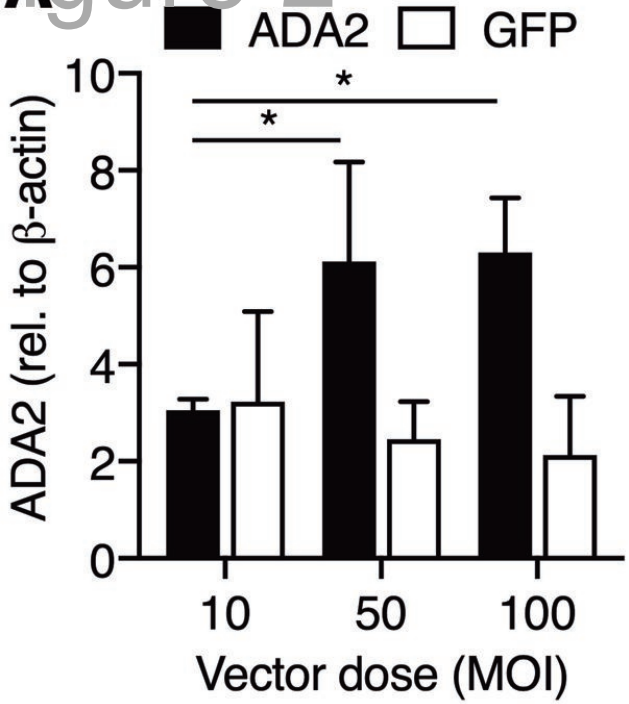
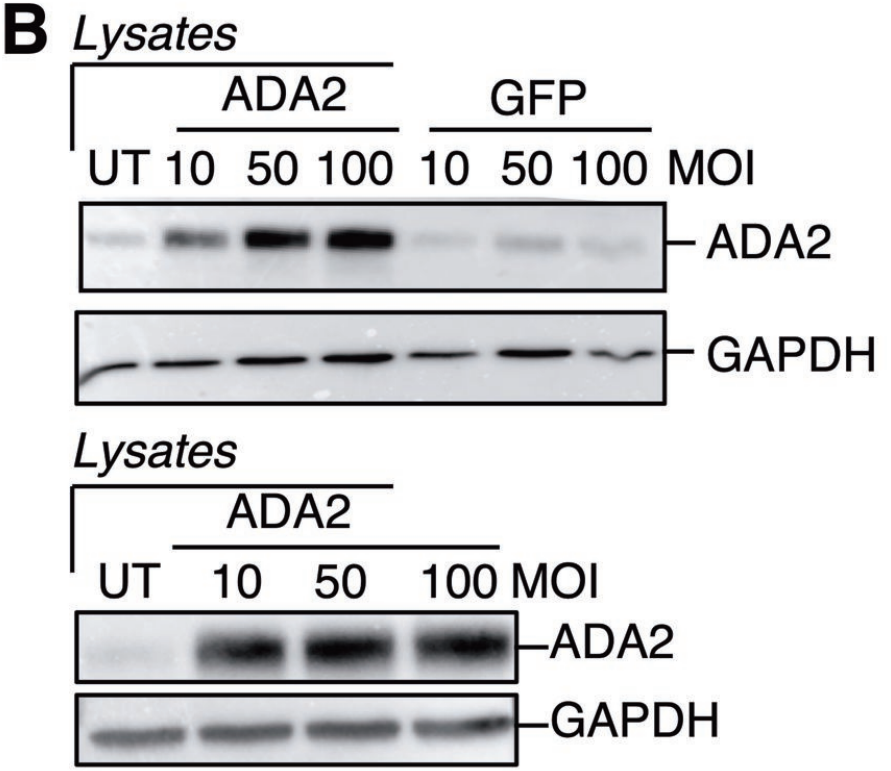
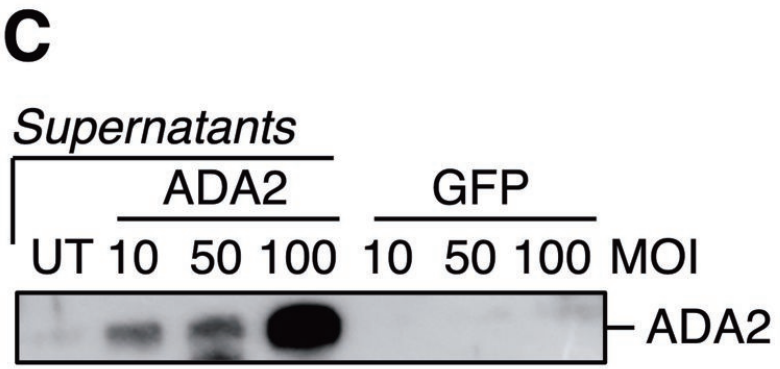

A
ΔLTR
cPPT
GA
RRE
Ψ
SD
SA
PGK
ADA2
WPRE
GFP
B
CFU-GM
BFU-E
Transduction (%)
MOI
C
VCN/genome
2.45
2.69
1.82
1.58
2.20
3.70
***
**
D
Day 0
Post-TR
Post-LC
Viable cells (x10^6)
20x
22x
18x
21x
25x
21x
28x
UT
GFP
E
CFU-GM
BFU-E
Colonies (Total counts)
Colonies (%)

Figure 2


A
ADA2
GFP
ADA2 (rel. to β-actin)
10
8
6
4
2
0
*
*
10
50
100
Vector dose (MOI)


B
Lysates
ADA2
GFP
UT 10 50 100 10 50 100 MOI
ADA2
GAPDH
Lysates
ADA2
UT 10 50 100 MOI
ADA2
GAPDH


C
Supernatants
ADA2
GFP
UT 10 50 100 10 50 100 MOI
ADA2

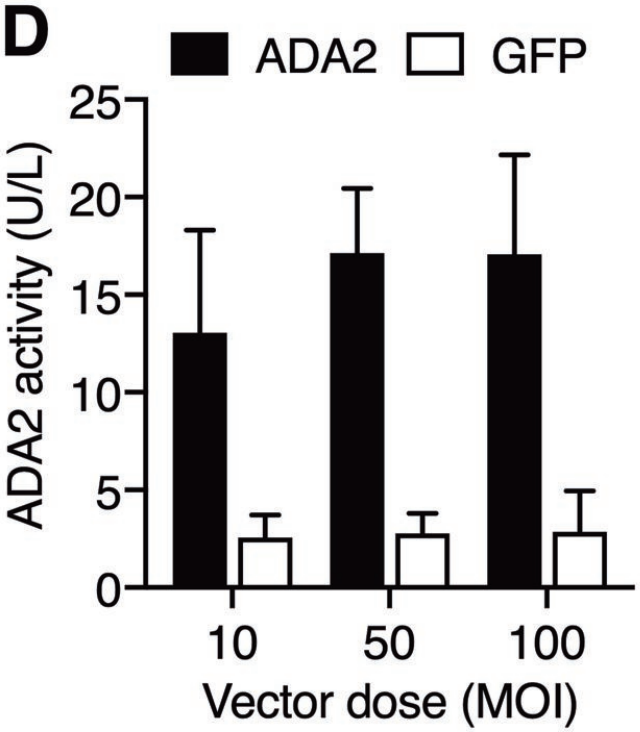

D
ADA2
GFP
ADA2 activity (U/L)
25
20
15
10
5
0
10
50
100
Vector dose (MOI)

Figure 3

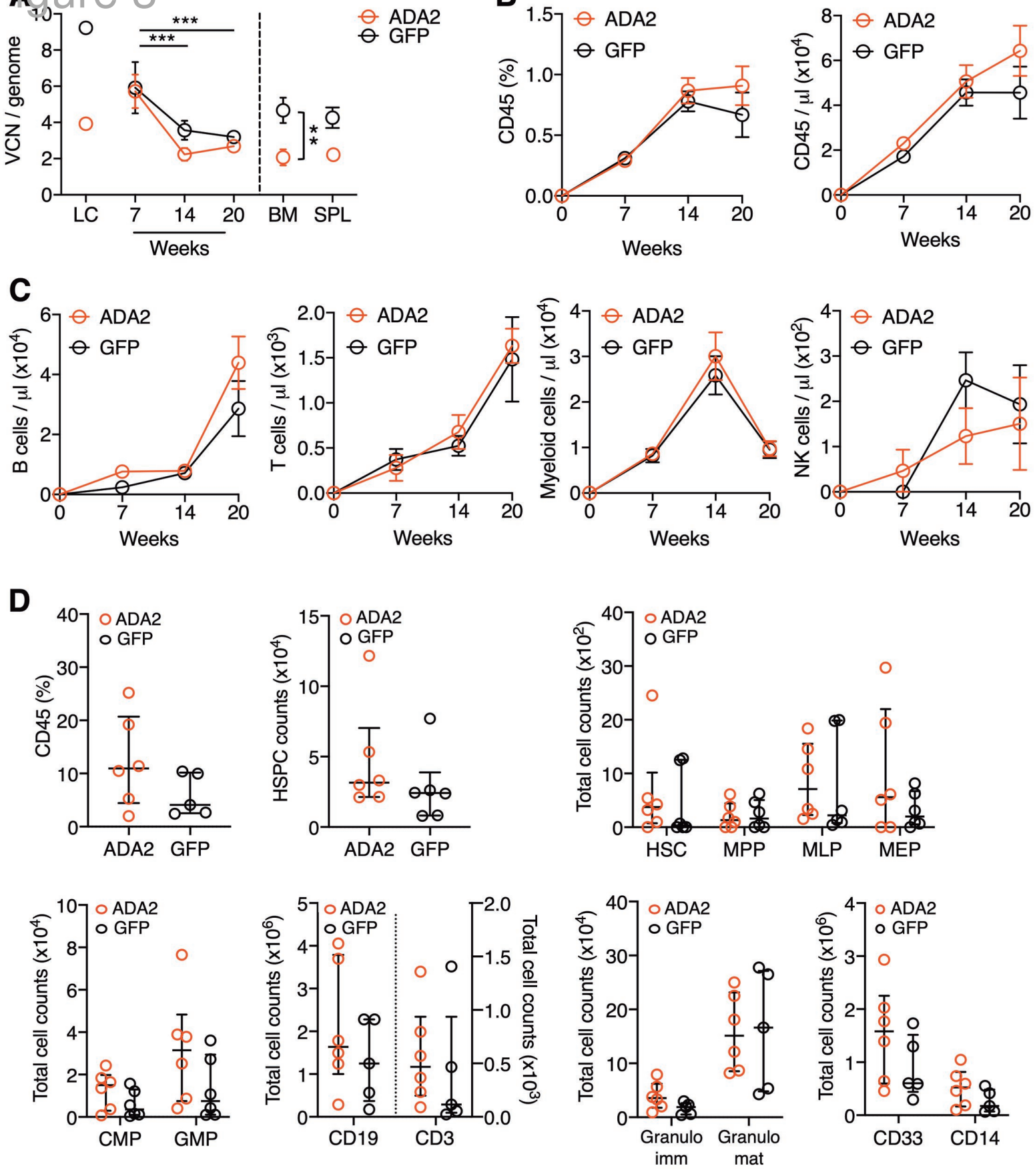

A
VCN / genome
ADA2
GFP
***
***
**
LC
7
14
20
BM
SPL
Weeks
B
CD45 (%)
CD45 / µl (x10⁴)
Weeks
C
B cells / µl (x10⁴)
T cells / µl (x10³)
Myeloid cells / µl (x10⁴)
NK cells / µl (x10²)
D
CD45 (%)
HSPC counts (x10⁴)
Total cell counts (x10²)
HSC
MPP
MLP
MEP
Total cell counts (x10⁴)
CMP
GMP
Total cell counts (x10⁶)
CD19
CD3
Total cell counts (x10³)
Granulo imm
Granulo mat
CD33
CD14

Figure 4

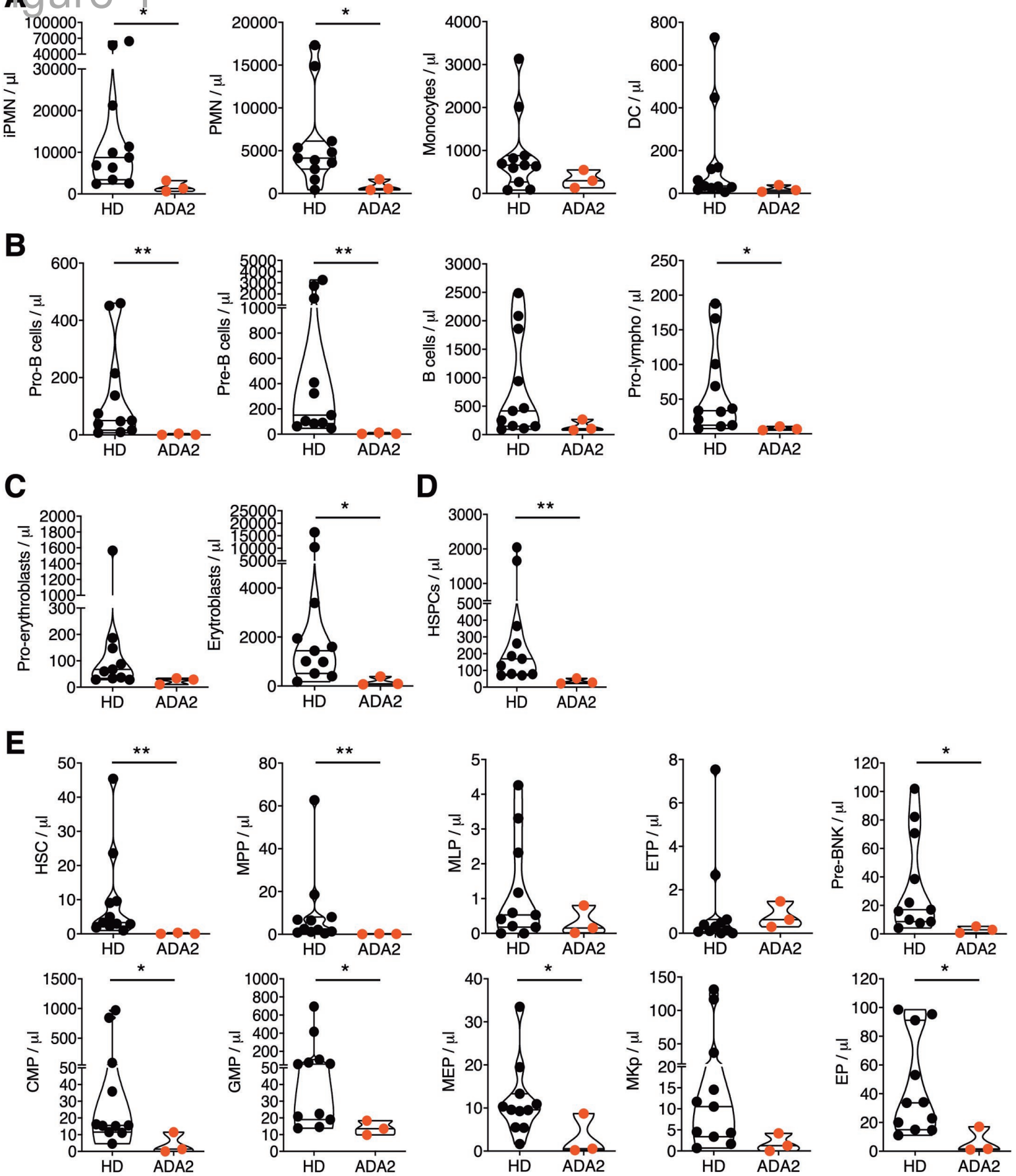

A
iPMN / µl
PMN / µl
Monocytes / µl
DC / µl
B
Pro-B cells / µl
Pre-B cells / µl
B cells / µl
Pro-lympho / µl
C
Pro-erythroblasts / µl
Erytroblasts / µl
D
HSPCs / µl
E
HSC / µl
MPP / µl
MLP / µl
ETP / µl
Pre-BNK / µl
CMP / µl
GMP / µl
MEP / µl
MKp / µl
EP / µl
HD
ADA2

Figure 5

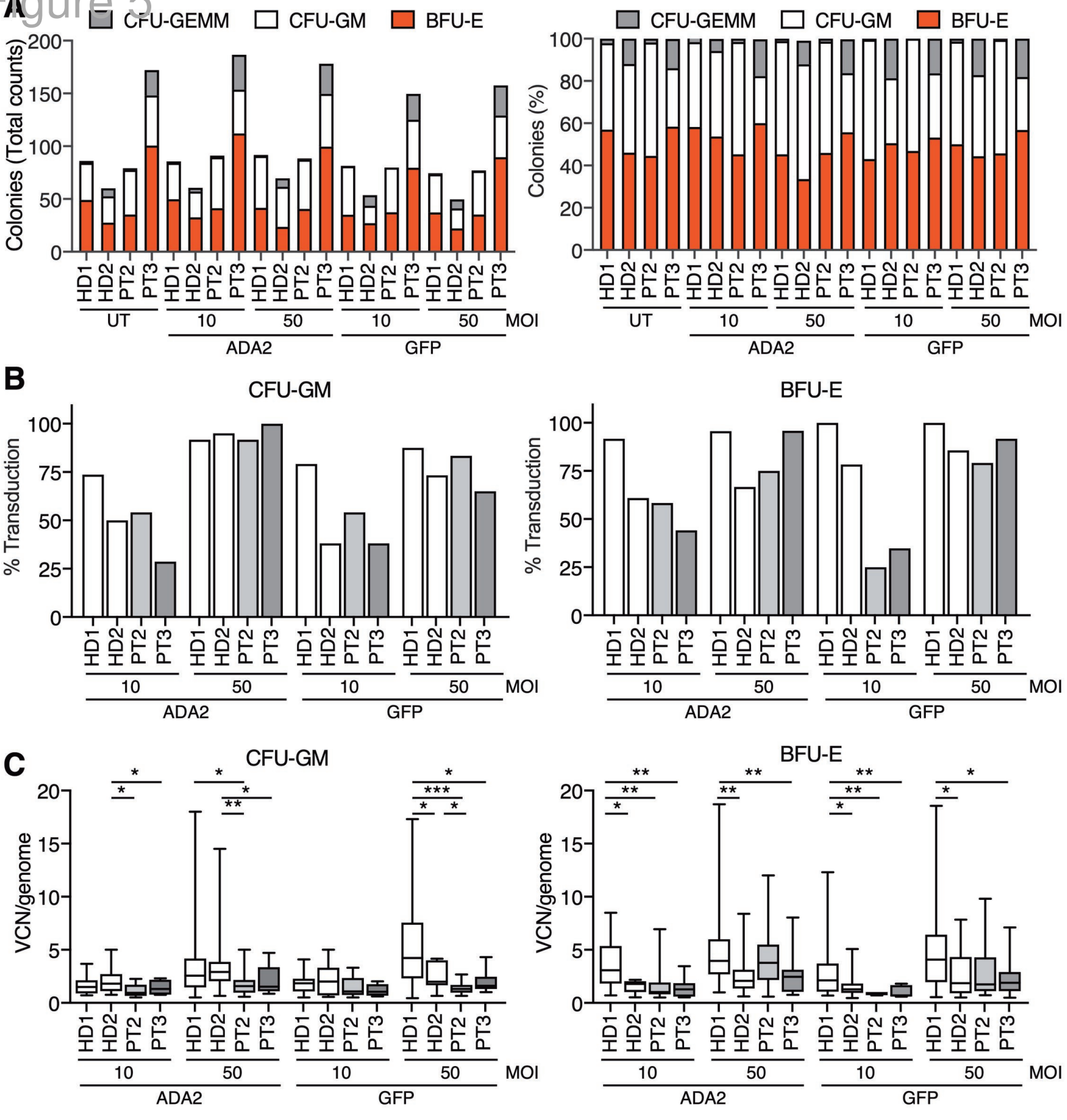

A
CFU-GEMM
CFU-GM
BFU-E
Colonies (Total counts)
Colonies (%)
HD1
HD2
PT2
PT3
UT
10
50
MOI
ADA2
GFP
B
CFU-GM
BFU-E
% Transduction
C
VCN/genome

Figure 6

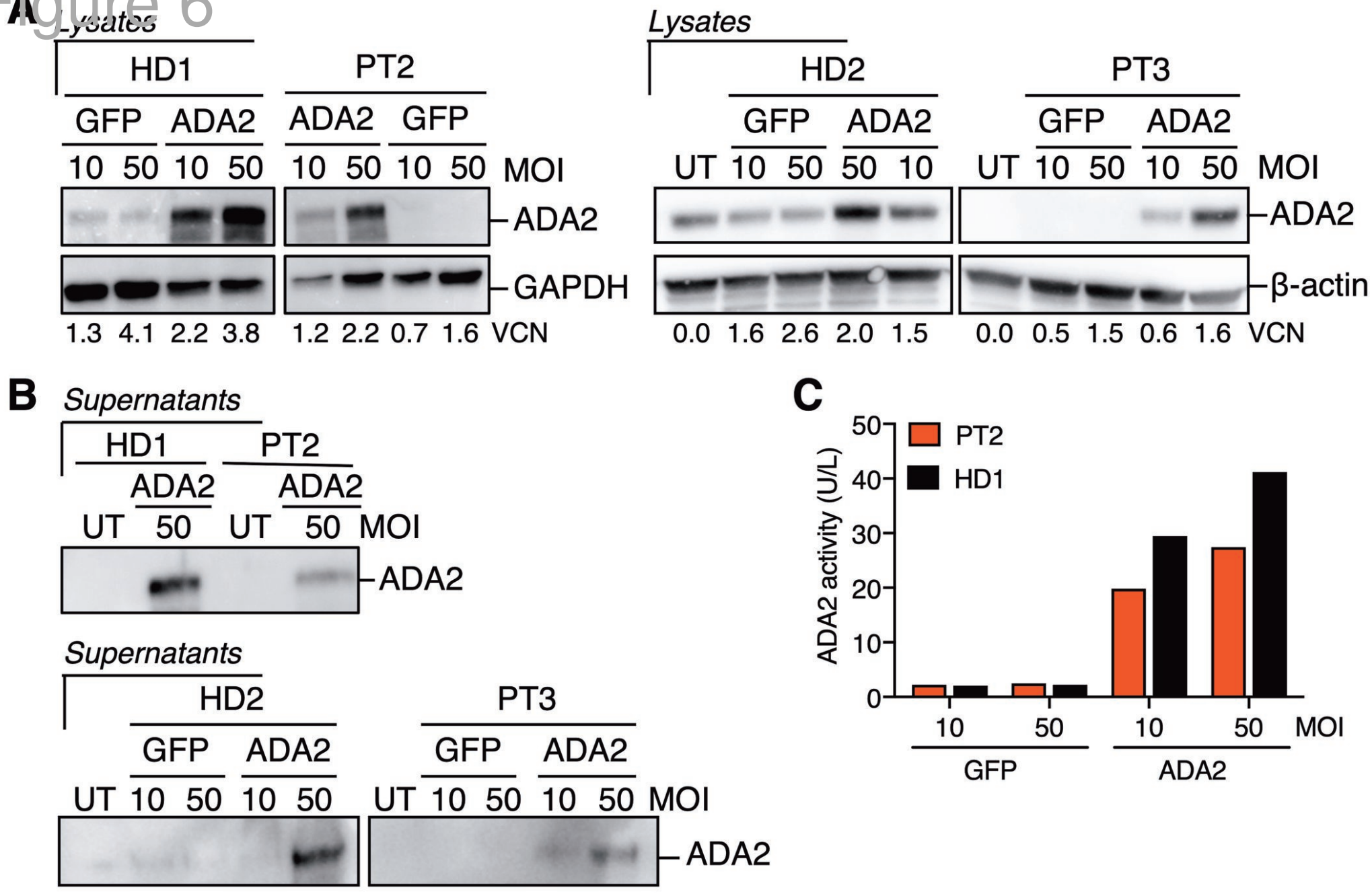
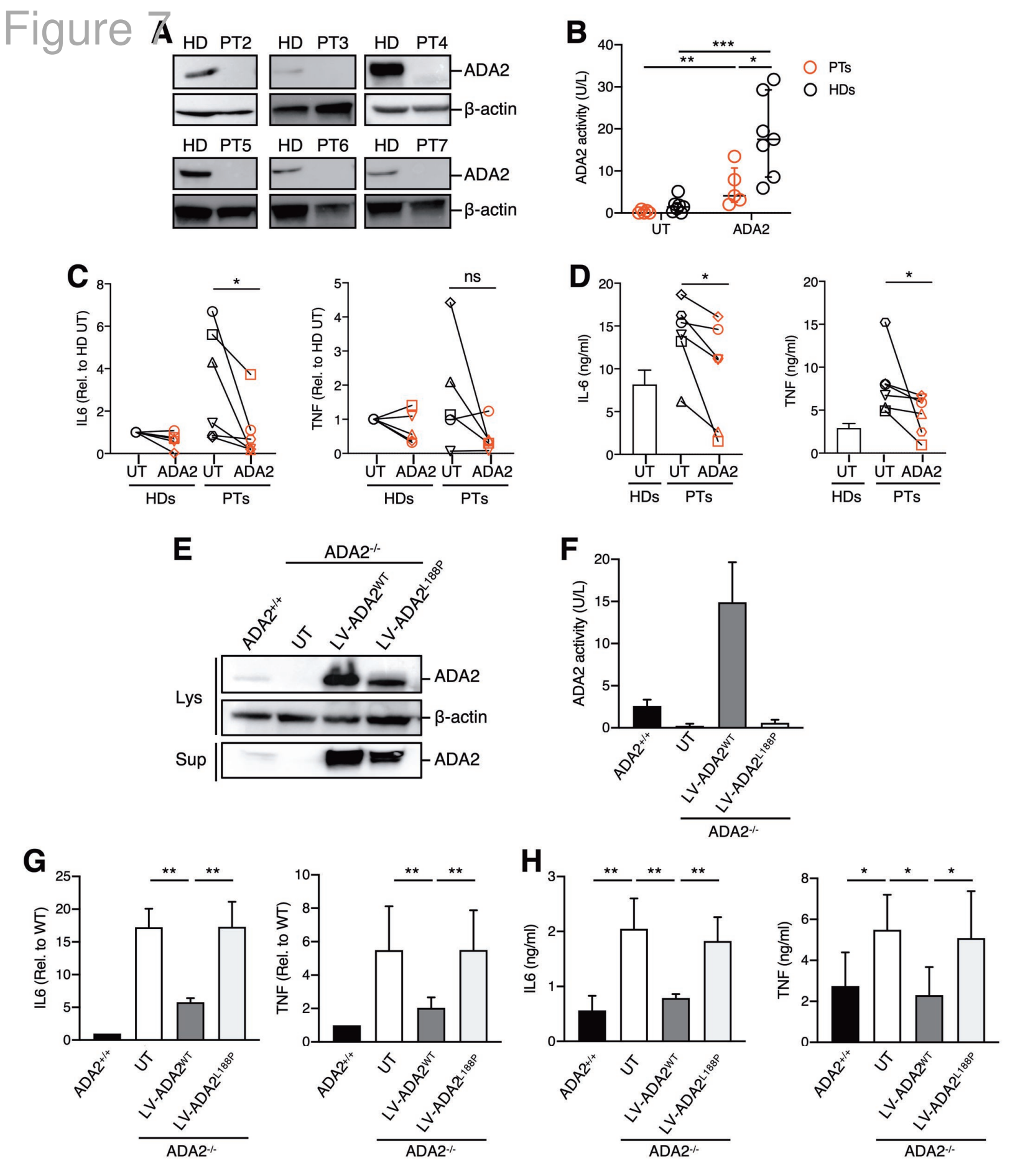

A
Lysates
HD1
PT2
GFP
ADA2
ADA2
GFP
10 50 10 50 10 50 10 50 MOI
ADA2
GAPDH
1.3 4.1 2.2 3.8 1.2 2.2 0.7 1.6 VCN
Lysates
HD2
PT3
GFP
ADA2
GFP
ADA2
UT 10 50 50 10 UT 10 50 10 50 MOI
ADA2
β-actin
0.0 1.6 2.6 2.0 1.5 0.0 0.5 1.5 0.6 1.6 VCN
B
Supernatants
HD1
PT2
ADA2
ADA2
UT 50 UT 50 MOI
ADA2
Supernatants
HD2
PT3
GFP
ADA2
GFP
ADA2
UT 10 50 10 50 UT 10 50 10 50 MOI
ADA2
C
ADA2 activity (U/L)
50
40
30
20
10
0
PT2
HD1
10 50 10 50 MOI
GFP
ADA2

Figure 7


A
HD PT2 HD PT3 HD PT4
ADA2
β-actin
HD PT5 HD PT6 HD PT7
ADA2
β-actin
B
ADA2 activity (U/L)
PTs
HDs
UT
ADA2
C
IL6 (Rel. to HD UT)
TNF (Rel. to HD UT)
UT ADA2 UT ADA2
HDs PTs
ns
D
IL-6 (ng/ml)
TNF (ng/ml)
E
ADA2-/-
ADA2+/+ UT LV-ADA2WT LV-ADA2L188P
Lys
Sup
ADA2
β-actin
F
ADA2 activity (U/L)
G
IL6 (Rel. to WT)
TNF (Rel. to WT)
H
IL6 (ng/ml)
TNF (ng/ml)