ADA$^{-/-}$ mice

ADA deficient mice have been described previously.[17] All animals were bred and maintained in a specific pathogen-free animal facility. Procedures were performed according to protocols approved by the Committee for Animal Care and Use of San Raffaele Scientific Institute (IACUC 406). PEG-ADA: ADA$^{-/-}$ mice were injected intraperitoneally with PEG-ADA (Adagen, Enzon Inc., Piscataway, USA) at 1000U (1 Unit is defined as the amount of enzyme necessary to convert 1μM of adenosine to inosine per min at 25°C) per kg body weight. Injections were started on postnatal day 10 and were given once a week. BMT: Following 3Gy (split dose) total body irradiation, neonatal ADA$^{-/-}$ mice were injected into the temporal vein with 5×10^6 total BM cells freshly harvested from tibiae and femuri of congenic ADA$^{+/+}$ mice as described.[18] GT: Gene therapy treatment of neonatal ADA$^{-/-}$ mice was performed using total BM cells harvested from congenic 2-week-old ADA$^{-/-}$ mice. After RBC lysis, cells were cultured (1×10^6/mL) for 24 hours in StemSpan serum-free expansion medium (StemCell Technologies, Vancouver, BC, Canada) in the presence of 50ng/mL murine stem cell factor (SCF), 10ng/mL human Flt3 ligand (Flt3-L), 20ng/mL human interleukin-6 (IL-6), 10ng/mL murine IL-3 (Peprotech, Rocky Hill, USA), 20ng/mL murine thrombopoietin (Tpo) (R&D Systems, Minneapolis, MN), 4μg/mL polybrene, and PGK-ADA (0.4μg of viral p24 per 10^6 cells, corresponding to a multiplicity of infection [MOI] of 4–12). 5×10^6 transduced BM cells were infused by injection through the temporal vein of irradiated (3Gy split dose) neonatal ADA$^{-/-}$ mice.

Immunohistochemical studies

Analyses were performed on thymi of 3 weeks old ADA$^{+/+}$ and ADA$^{-/-}$ mice. 4μm thick sections from formalin-fixed, paraffin-embedded tissues were taken on a microtome and subjected to routine hematoxylin and eosin staining. Development of Tregs was studied by staining sections with monoclonal rat anti-human Foxp3 antibody (eBioscience, San Diego, CA, USA). T cells were stained with rat anti-CD3ε (Valter Occhiena). Signal was revealed after incubation with a HRP-conjugated secondary antibody (ChemMATE Envision Rabbit/Mouse, DAKO Cytomation, Glostrup Denmark) by diaminobenzydine, and slides were counterstained with hematoxylin. Images were acquired with an Olympus DP70 digital camera mounted on an Olympus Bx60 microscope, using CellF imaging software (Soft Imaging System).

Thyroids from 18 weeks old treated ADA$^{-/-}$ and control mice were collected upon sacrifice and stained with cleaved caspase-3 (AbD Serotec) antibody. The slides were developed using a HRP-conjugated secondary antibody and were counterstained with hematoxylin. Positive nuclei were scored by means of Aperio image Analysis Nuclear algorithm (Aperio Technologies, Vista USA).

FACS analyses

FACS stainings on murine lymphocytes were performed using the following antibodies: FITC-conjugated anti-CD3, FITC-conjugated anti-CD8, FITC-conjugated anti-CD11b, FITC-conjugated anti-IgM, PE-conjugated anti-CD4, PE-conjugated anti-Gr1, PE-conjugated anti-NK, PE-conjugated anti-CD25, PE-conjugated anti-CD39, PE-Cy5 conjugated anti-CD8, PE-Cy5 conjugated anti-CD45, PE-Cy5 conjugated anti-CD25,

PE-Cy5 conjugated anti-B220, PE-Cy7 conjugated anti-CD73, APC-conjugated anti-CD4, PerCP-Biot conjugated anti-CD45RB (BD Pharmingen), APC-Cy7 conjugated anti-CD3 (BD Pharmingen), Pacific Blue-conjugated anti-CD4 (all BD Pharmingen). Intracellular staining was performed using PE-conjugated anti-CTLA4 (BD Pharmingen) and FITC or APC-conjugated anti-FoxP3 (Biolegend) antibodies.

Human peripheral blood mononuclear cells were purified by standard density gradient technique and stained with PerCP- or PE-Cy7–conjugated anti-CD4 and APC-conjugated anti-CD25 antibodies in combination with PE-conjugated anti-CD127, PE-conjugated anti-CD39 or PE-conjugated anti-CD73 antibodies (all BD Pharmingen). Intracellular detection of human FOXP3 was performed after incubation for 30min at RT, using a Alexa 488-conjugated (Biolegend) and Alexa488-conjugated IgG1 isotype control (Biolegend), according to manufacturer's protocol. The PE-conjugated anti-CD73 antibody was again added to the intracellular staining to detect both extracellular and intracellular protein. All samples were acquired using a BD FACS Canto and analyzed using FlowJo software (TreeStar Inc).

ATP hydrolysis assay

Immunomagnetic bead (Miltenyi)-sorted $CD4^+CD25^+$ cells (70.000/well) were incubated in flat-bottom 96-well plates for 40 min with various concentrations of exogenous ATP (50μM or 100μM) (Sigma Aldrich). As control some wells were pre-incubated with ARL67156 (Sigma Aldrich), an ectonucleotidase inhibitor, at the final concentration of 200μM for 30 min prior to the addition of exogenous ATP. The concentration of consumed ATP, produced AMP and adenosine was determined by HPCE[21] and expressed as difference between timepoints 0 and 40min.

***In vitro* suppression assays**

For suppression assays using murine cells, $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ responder cells were isolated from $ADA^{+/+}$ and $ADA^{-/-}$ spleen by immunomagnetic beads (Miltenyi); Purity was ≥85%. 50,000 $CD4^+CD25^-$ $ADA^{+/+}$ responder T cells, stimulated with plate-bound anti-CD3 (0.2μg/ml) and soluble anti-CD28 (0.2μg/ml) were cocultured with 50,000 $ADA^{+/+}$ or $ADA^{-/-}$ $CD4^+CD25^+$ Treg cells (1:1 ratio).

After enrichment of $CD4^+$ cells (Miltenyi) $CD4^+CD25^{high}CD127^-$ human Treg cells and $CD4^+CD25^-$ responder T cells from patients and healthy controls were isolated by FACS sorting. Purity was ≥90%. Autologous and allogeneic suppression was performed plating 10,000 responder cells and 10,000 Tregs (1:1 ratio). Cells were co-stimulated with an equal number of allogeneic accessory cells (APCs), irradiated at 30Gy and 1μg/mL of soluble anti-CD3 (Janssen-Cilag, Milan, Italy).

To confirm their anergic state, isolated Tregs were cultured alone or with stimuli. In order to reverse Treg anergy, 100U of IL-2 was added. Only suppression assays showing anergy of the isolated Tregs and its reversion in the presence of IL-2 were considered. After 96 hours of coculture, murine and human suppression assays were pulsed for 16 hours with 1 μCi ^{3}Hthymidine/ well (Amersham Biosciences). Cells were

harvested and counted in a scintillation counter. Percentage of suppression was calculated in comparison to effector T cells' proliferation.

Anti-ADA and anti-platelet ELISA

Mouse sera were analyzed to evaluate the presence of immunoglobulin specific for ADA or platelets. 96-well polystyrene plates were coated over night at 4°C with 0.1µg/ml of bovine ADA (Calbiochem, San Diego, USA). Alternatively, proteic extract from platelets was used as coated antigen. The platelets were isolated from blood collected with EDTA and diluted with a same volume of 1× tyrode buffer (5mM HEPES, 137mM NaCl, 2.7mM KCl, 0.4mM NaH2PO4, 2.8mM Dextrose, pH7.4). The blood was centrifuged at 800rpm for 7min, the platelet-rich plasma was transferred to a second tube and centrifuged at 5700rpm for 7min. The supernatant was eliminated and total protein was extracted from the platelet pellet. 10µg/mL of proteic extract was coated on a 96-well plate and incubated overnight at 4°C. Diluted serum (1:100 and 1:1000 in blocking buffer) was added and incubated for 2 hours. Positivity was revealed by staining with secondary goat anti-mouse IgG-AP antibody (1:2000 in blocking buffer) followed by pNPP conversion by alkalinephosphatase (Sigma-Aldrich, St Louis, USA). The assay was read at 405nm on a 680microplate reader (BioRad, Richmond, USA).

Anti-dsDNA ELISA

For anti-dsDNA ELISAs, polystyrene plates were coated with poly–L-lysine (Sigma) and DNA from calf thymus (Sigma). After post-coating with 50µg/mL of polyglutamic acid for 45min and blocking with PBS 3%BSA, serial dilutions of serum from 1:100 to 1:3200 were incubated overnight. Specific antibodies were detected with alkaline phosphatase-conjugated goat anti- mouse IgG (Southern Biotech).

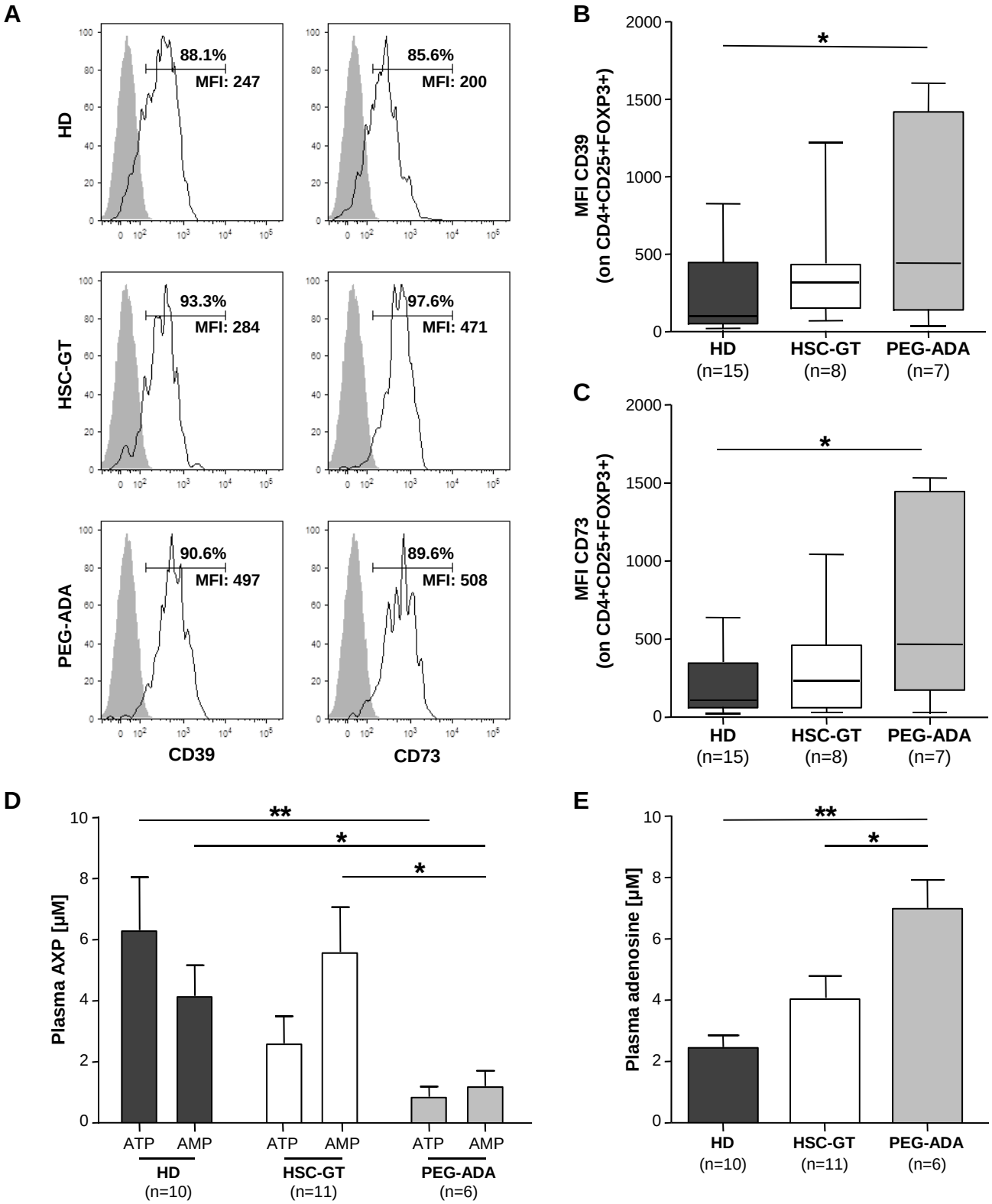

Figure S1. Expression and function of CD39 and CD73 in ADA-SCID patients following PEG-ADA or HSC-gene therapy

(A) Representative histograms for the expression of CD39 and CD73 in Tregs of HD, HSC-GT and PEG-ADA treated patients. Percentages of expression and MFI are indicated. Grey filled histogram represents the Isotype control. (B) MFI of CD39 and (C) CD73 in 15 HD, 8 HSC-GT and 7 PEG-ADA treated patients. Box and Whiskers graphs representing the minimum and the maximum values; Student's t-Test: **p*<0.05. (D) AXP levels divided into ATP and AMP illustrate ATP conversion in plasma samples of 10 pediatric HD, 11 HSC-GT and 6 PEG-ADA treated patients; mean +SD (E) Adenosine levels in corresponding plasma samples of 10 pediatric HD, 11 HSC-GT and 6 PEG-ADA treated patients; mean +SD.

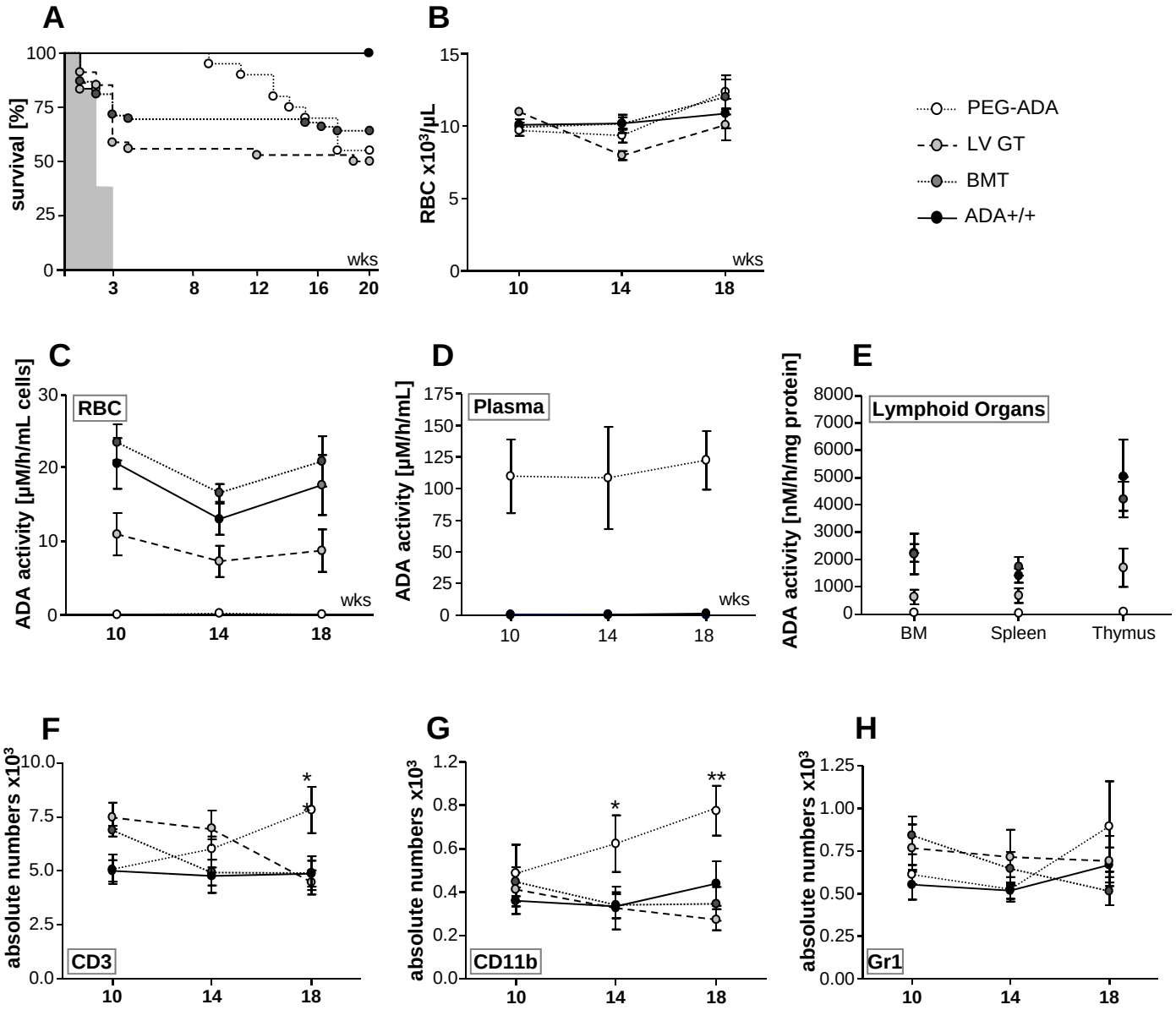

Figure S2. Immunological and metabolic reconstitution in ADA-/- mice treated with PEG-ADA, GT and BMT

(A) Kaplan-Meier survival curves for ADA-/- mice treated with PEG-ADA (n=20), GT (n=33) or BMT (n=52) in comparison to ADA+/+ mice (n=11) and untreated ADA-/- (n=17, highlighted in grey). (B) Red blood cell (RBC) counts in PEG-ADA treated mice (n=12) in comparison with GT (n=8) or BMT (n=15) treated ADA-/- mice and ADA+/+ mice (n=17); mean ±SD. (C) ADA activity in RBC was detected in ADA-/- mice treated with BMT (n=15) or lentiviral GT (n=8) and in ADA+/+ mice (n=11), but not in mice treated with PEG-ADA (n=8). (D) ADA activity in the plasma was exclusively detectable in mice treated with PEG-ADA. (E) ADA activity was measured in total cells from bone marrow (BM), spleen and thymus. No ADA activity was detected in RBC and lymphoid tissues from untreated ADA-/- mice. (F-H) Absolute numbers of peripheral blood lymphocyte populations measured by FACS analyses as percentage of WBC counts, at weeks 10, 14 and 18; mean ±SEM. (F) CD3+ T cells. (G) Monocytes. (H) Granulocytes. *p<0.05; **p<0.005.

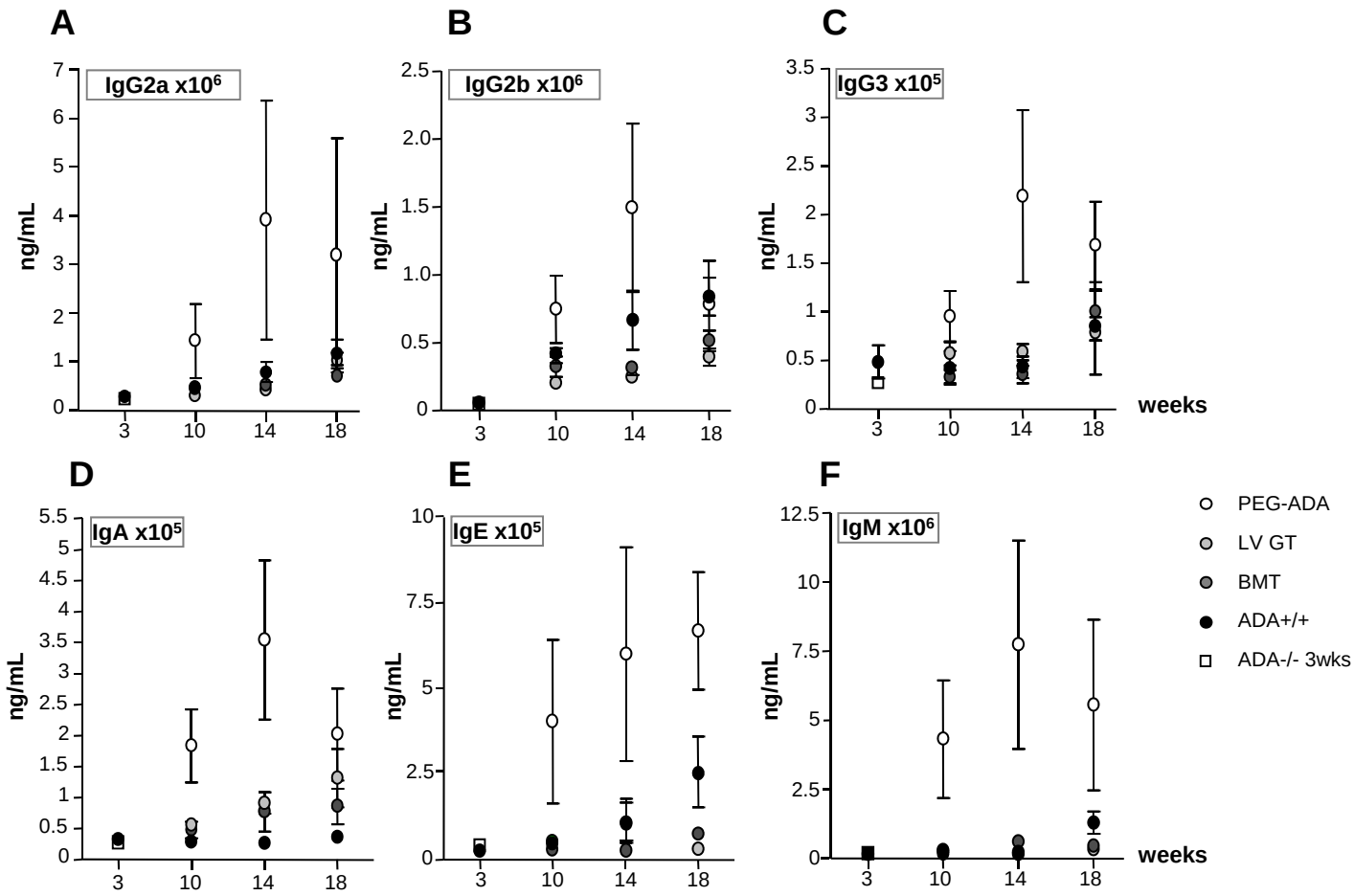

Figure S3. Serum immunoglobulin levels in ADA-/- mice treated with PEG-ADA, GT and BMT.

Multiplex analyses on PEG-ADA (n=7), GT (n=6) or BMT treated ADA-/- mice (n=10) in comparison with ADA+/+ (n=7) and 3 weeks old ADA-/- (n=6) and ADA+/+ (n=6) mice; Mean ±SD. (A) IgG2a [ng/mL x10^6]. (B) IgG2b [ng/mL x10^6]. (C) IgG3 [ng/mL x10^5]. (D) IgA [ng/mL x10^5] (E) IgE [ng/mL x10^5]. (F) IgM [ng/mL x10^6].

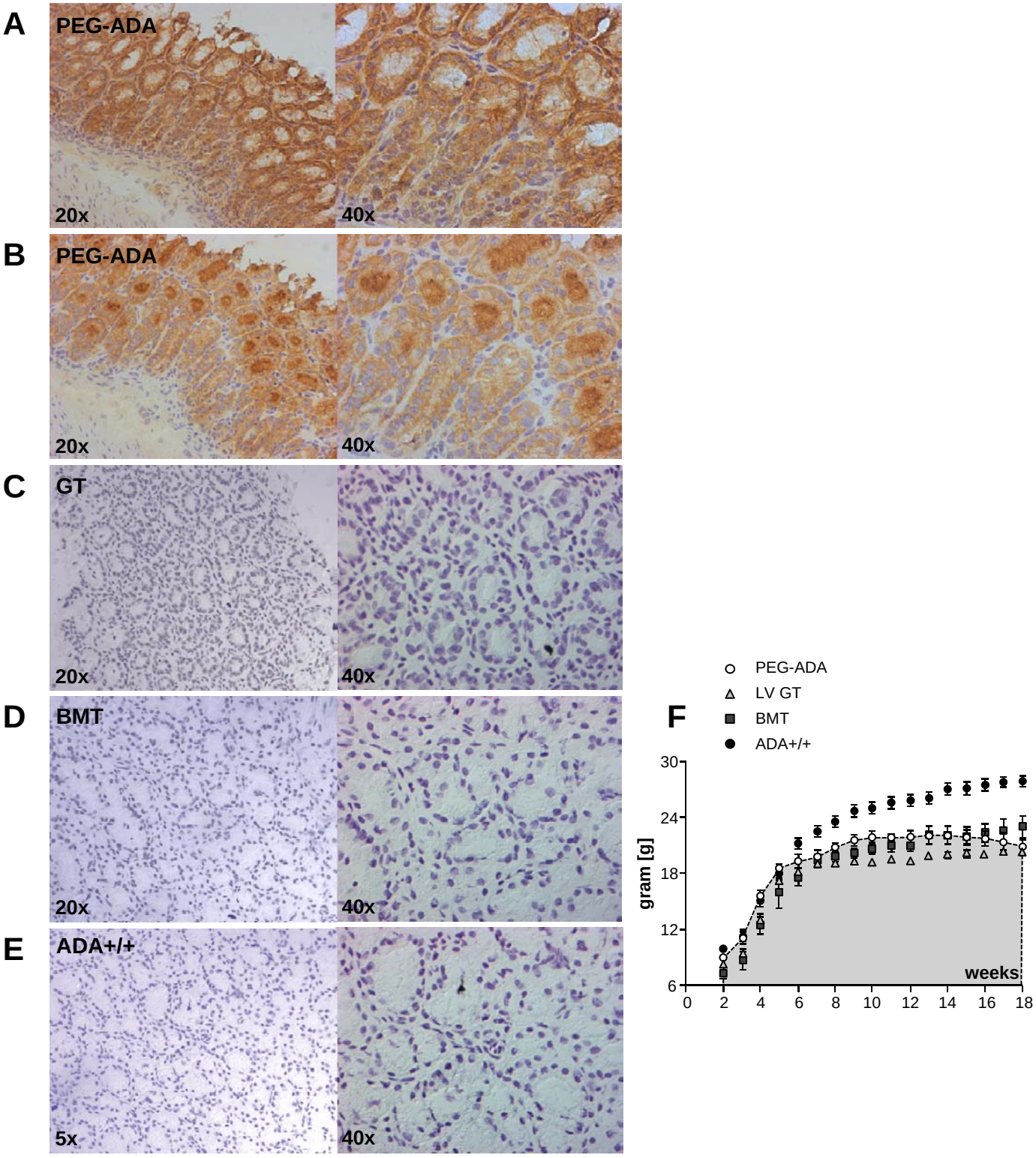

Figure S4. Autoantibodies against the gastrointestinal tract in PEG-ADA treated mice

(A+B) PEG-ADA treated mice. ADA-/- mice treated with (C) GT or (D) BMT. (E) ADA+/+ control. (F) PEG-ADA treated mice gain weight quickly upon initiation of treatment, but after long-term PEG-ADA they more frequently loose weight as compared to BMT or GT treated mice; mean ±SEM.