

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Aiuti A, Cattaneo F, Galimberti S, et al. Gene therapy for immunodeficiency due to adenosine deaminase deficiency. *N Engl J Med* 2009;360:447-58.

Supplementary material

Supplementary Methods

Patients' diagnosis. Diagnosis was performed by biochemical studies carried out at local centers or by Dr. M. Hershfield (Duke University, NC) and confirmed by ADA mutation analysis carried out by Dr R. Hirschhorn (New York University, NY) or Correlagene Diagnostic Inc. (Cambridge, MA).

Patients' treatment. Patients received standard prophylaxis with fluconazole or itraconazole, cotrimoxazole and acyclovir, and i.v. substitution with immunoglobulins. Cotrimoxazole was switched to Pentamidin before bone marrow harvest and maintained until hematological reconstitution.

The area under the serum concentration-time curve (AUC) after the first administration of busulfan was measured in 8 patients by HPLC (Suppl. Table 1). Pt3 and Pt6 displayed an elevated busulfan concentration after the first dose (5110 and 6000 ng/ml*h, respectively), and in Pt6 the total dose was adjusted to 2 mg/Kg.

Clinical protocols. The studies Hadassah and SR-I began in September 2000 and March 1992, respectively. The studies are closed and patients continue the follow up according to national guidelines. Study SR-II began in October 2002 and is expected to be completed by October 2011.

Study design. Dr. Aiuti, Bordignon, Valsecchi and Roncarolo designed the study. Mr. Callegaro gathered the data; Dr. Galimberti, Aiuti and Roncarolo analyzed the data. Dr. Aiuti, Valsecchi and Roncarolo vouch for the completeness of the data and analyses and wrote the manuscript with input from all the coauthors.

Laboratory studies

Frequency of transduced cells. Cell subpopulations were purified using antibody-coated microbeads or FACS sorting.¹ The frequency of transduced cells and vector copy number were determined on genomic DNA by quantitative PCR analysis for NeoR vector sequences, normalized for DNA content.¹

Immunological studies. All immunostaining and flow cytometry were performed as described.² In vitro T-cell responses to mitogens and antigens were measured by thymidine incorporation in proliferative assays.² Specific antibodies were analyzed by ELISA, following patients' immunization with toxoid vaccines, conjugated or bacterial polysaccharide antigens, or measles vaccine.³ Cytotoxic NK activity was measured by a modification of a flow cytometric assay.⁴ TREC were measured in PB T cells by PCR² and the TCR V β repertoire was analysed by FACS analysis using a mix of directly conjugated antibodies corresponding to 24 different TCR V β specificities (IOtest Beta Mark kit; Immunotech, France) and by spectratyping analyses.⁵ Expression of ADA protein was assessed in patients PB mononuclear cells by flow cytometry⁶ using an anti-human ADA monoclonal antibody (kindly provided by Dr. M. Hershfield) revealed by a secondary goat anti-mouse Alexa 488 antibody (Southern Biotechnology, USA). Values for healthy subjects were obtained from laboratory references, published literature^{2,3 7-9}, or healthy control donors.

Biochemical studies. ADA activity in cell lysates was measured by an adenosine to inosine conversion assay, followed by high-performance-capillary-electrophoresis (HPCE).¹⁰ Total adenine ribo-(AXP) and deoxyribonucleotides (dAXP) in erythrocytes were measured by HPCE.¹⁰

Supplementary Results

Six severe infections requiring hospitalization were recorded in five patients. The patients were hospitalized — for 2, 5, 6, 6, and 21 days (total days for two episodes), respectively (see also Table 2, main manuscript).

Two children showed eating disorder before gene therapy. In Patient 3 the disorder progressively improved after scholarization, while in Patient 7 it required implantation of a percutaneous endoscopic gastrostomy and psychological support.

Patient 8, although not evaluated for efficacy, showed an increase in T-cell counts (from 0.01 to 0.15 x10⁹/L) and transduced cell engraftment in all hematopoietic lineages. The patient is currently

on PEG-ADA and suffering from recurrent infections and persistent autoimmunity requiring chronic steroid treatment.

Supplementary Discussion

Busulfan was included in our protocol because of its potent effects on primitive hematopoietic progenitors and the lack of organ toxicity at reduced doses in nonmyeloablative regimens for hematopoietic stem cell (HSC) transplant.^{2, 11} Studies in non human primates indeed showed that low dose busulfan provides a strong competitive advantage for the survival and proliferation of transplanted HSC during suppression of endogenous hematopoiesis.¹²

In addition to the points discussed in the main text, we cannot exclude that the slower kinetic of T-cell reconstitution is due to a lower number of “true” gene corrected repopulating HSC in the infused autologous graft, as compared to unmanipulated HSC from a normal donor in the context of an allogeneic bone marrow transplant.

Several possible explanations may account for the differences between the ADA-SCID and the X-linked SCID gene therapy trials^{13, 14}. First, ADA is a constitutively expressed enzyme of purine metabolism which does not need regulation and favors survival of cells rescued by gene therapy. The IL2RG is a signal transducing receptor chain which is upregulated upon T-cell activation and induces T-cell proliferation when combined with the receptors of one of the six different cytokines (IL2, IL4, IL7, IL9, IL15 and IL21). Although transgenic IL2RG was not overexpressed in mature T cells after gene therapy and STAT5 was not constitutively phosphorylated,^{14, 15} retroviral vector-mediated expression of IL2RG was not regulated in mature T cells of X-linked SCID patients treated with gene therapy. In addition, in transduced thymocytes IL2RG may be expressed at inappropriate levels in different stages of differentiation. It should be noted that the kinetics of T-cell reconstitution was substantially different between the two group of patients, being the one of ADA-SCID more slow and progressive than those observed in the X-linked SCID trials. Furthermore, as shown in the mouse model, the IL2RG-deficient background may favor the

accumulation of mutations in progenitor cells blocked in their development or in gene corrected thymocytes following their rapid expansion after restoration of IL2RG expression¹⁶. Finally, combinatorial mechanisms of leukemogenesis may result from the interaction of IL2RG with cellular proto-oncogenes activated by vector insertions, such as LMO2¹⁷.

The different outcome of the CGD trial compared to our study may be due to the use of a viral promoter highly active in hematopoietic progenitors, the nature of the transgene, and the use of mobilized peripheral blood CD34+ cells. For diseases carrying an intrinsic high risk due to gene product and disease background, self-inactivating vectors with improved safety profile, such as lentiviral vectors,¹⁸ may improve the safety of gene therapy approaches.

Supplementary References

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Supplementary Table 1: Patients' treatment and myelosuppression

Pt	Collected CD34 ⁺ cells [°]	AUC after first dose (ng x h/ml)	Total AUC (ng x h/ml)	Nadir of ANC§	Days of neutropenia#
1	4.1	n.d.	n.d.	0.15	11
2	1.1	n.d.	n.d.	0.40	1
3	3.5	5111	30664	0.05	38
4	4.7	2090	18640	0.71	0
5	7.7	2689	17724	0.23	21
6	10.2	6000	n.a.	0.55	0
7	15.0	1403	11181	0.29	18
8	15.5	3292	23072	0.20	33
9	13.6	1936	16427	0.10	15
10	14.7	2156	19532	0.22	22
Mean (SD)	9.0 (5.5)	3085 (1639)		0.29 (0.21)	15.6 (13.3)

[°](x10⁶/Kg); §(x10⁹/L); absolute neutrophil counts (ANC). #days of ANC <0.5x10⁹/L. For Patient 1 and Patient 2 busulfan plasma levels were not measured (not done: n.d.). N.a.: not applicable; the total area under the curve (AUC) was not calculated because busulfan dosage was reduced to half due to the elevated levels observed after the first dose.

Supplementary Figure Legends

Suppl. Figure 1. Hematopoietic reconstitution following gene therapy and low dose busulfan.

Mean \pm standard deviation (SD) of absolute neutrophil counts (left) and platelet counts (right) in the peripheral blood during the first year of follow up. Six patients received red blood cell (RBC) transfusions after bone marrow harvest. Patient 3 experienced platelet counts $<20 \times 10^9/L$ for 40 days, requiring platelet transfusions. Neutrophil counts: mean values (shaded area) and 5th percentile (dashed line) for healthy controls are shown.¹⁹ Platelets counts: mean values (dotted lines) with 95th and 5th percentile (dashed lines) are shown.¹⁹

Suppl. Figure 2A. Analyses of T-cell receptor V β families by FACS.

The proportion of specific TCR V β families within CD3⁺ T cells (See Supplementary Methods) is reported for each patient at the last follow up, in comparison to a group of healthy controls.

Suppl. Figure 2B. ADA protein expression by FACS analyses.

Representative analyses of ADA expression in Patient 6 (3 years post-gene therapy) and Patient 7 (2 years post-gene therapy) in comparison to a normal donor control (ND). The expression of ADA was analysed by FACS on gated subpopulations of peripheral blood T cells, B cells and monocytes. Shaded area: isotype control; bold line: anti-ADA monoclonal antibody, revealed by anti-mouse ALEXA 488.

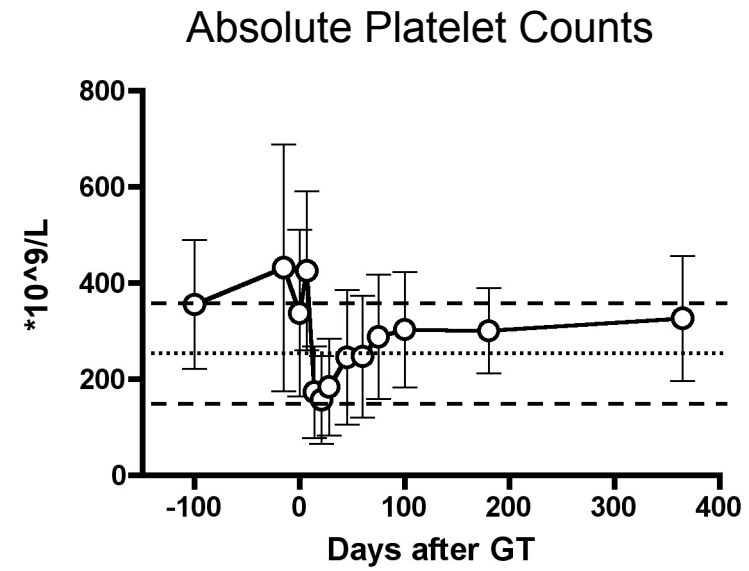
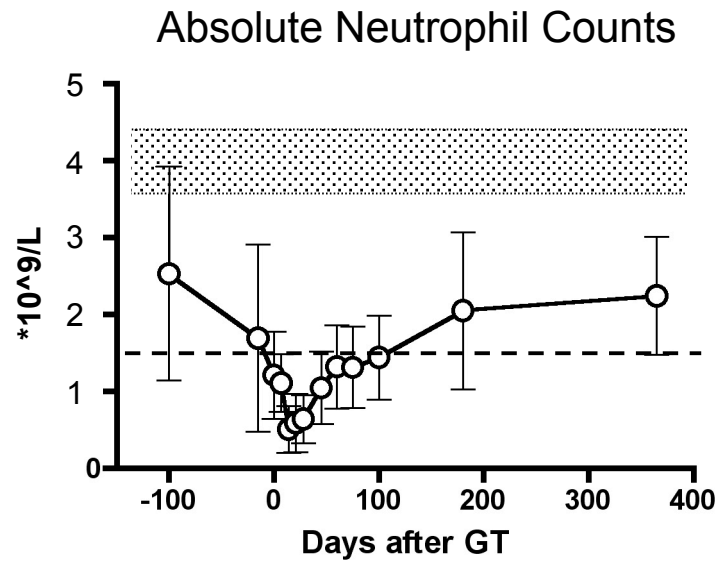
Suppl. Figure 3. Recovery of thymic activity.

A) Median numbers of TREC/100 ng of DNA during follow-up. Box and-Whisker plot for patients receiving bone marrow transplant and healthy controls are shown. B) Median numbers of CD4⁺ CD45RA⁺ naïve T cells. Median and 10th percentile for age matched healthy controls are shown.⁹

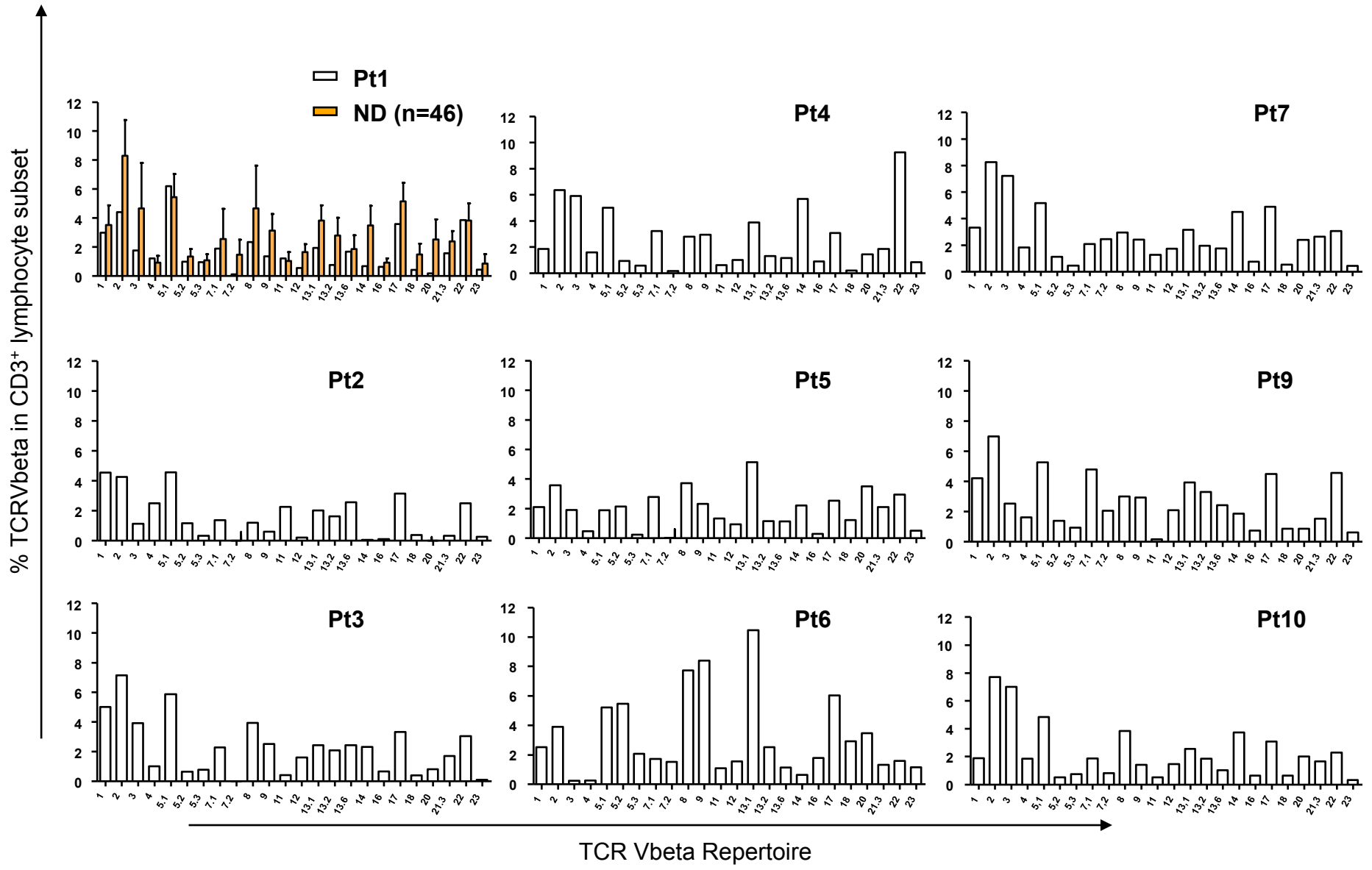
Suppl. Figure 4. Improvement of patient's growth.

Box-and-Whisker plot of patient's weight (left) and height (right) percentiles before and at 1 and 2 years after gene therapy.

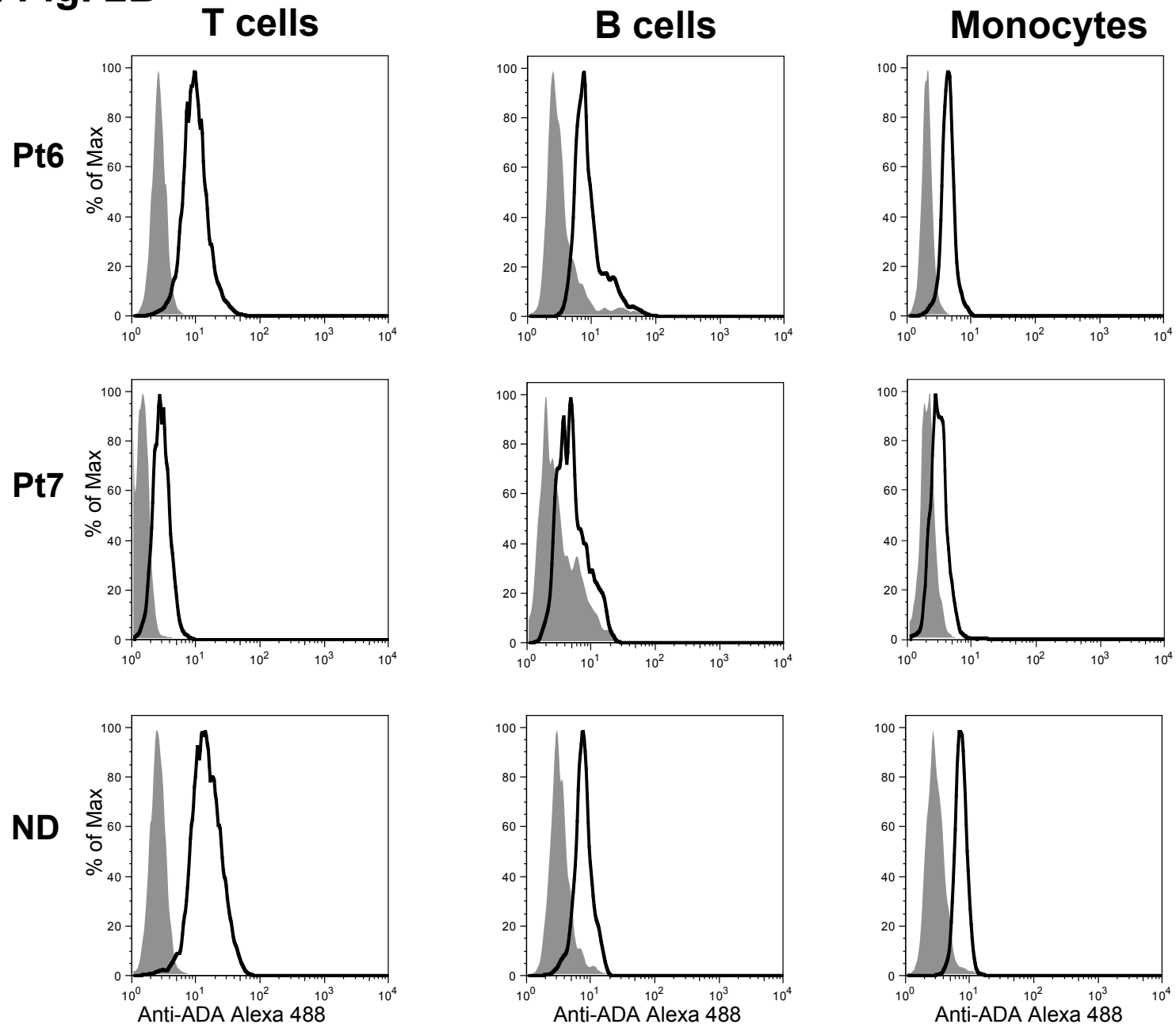
Suppl. Fig.1



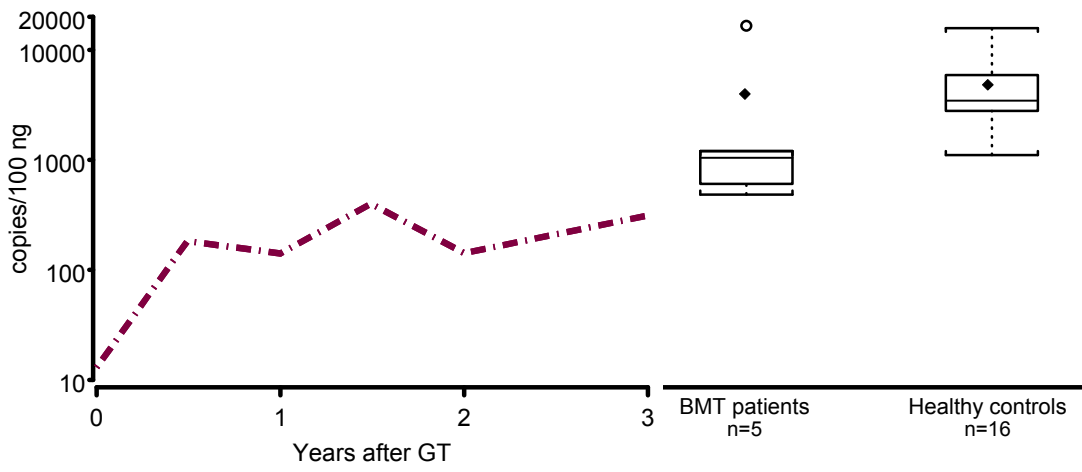
Suppl. Fig.2A



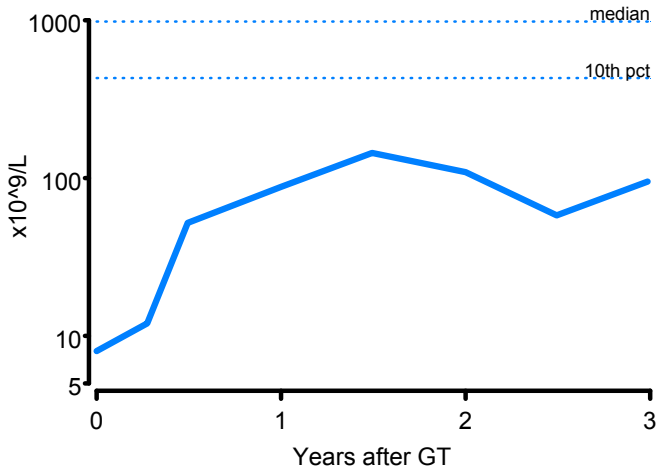
Suppl. Fig. 2B



Suppl Fig. 3A



Suppl Fig. 3B



Suppl. Figure 4

