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Outcomes in Two Japanese Adenosine Deaminase-Deficiency Patients Treated by Stem Cell Gene Therapy with No Cytoreductive Conditioning

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Abstract

Objective

We here describe treatment outcomes in two adenosine deaminase (ADA)-deficiency patients (pt) who received stem cell gene therapy (SCGT) with no cytoreductive conditioning. As this protocol has features distinct from those of other clinical trials, its results provide insights into SCGT for ADA deficiency.

Patients and Methods

Pt 1 was treated at age 4.7 years, whereas pt 2, who had previously received T-cell gene therapy, was treated at age 13 years. Bone marrow $CD34^+$ cells were harvested after enzyme replacement therapy (ERT) was withdrawn; following transduction of *ADA* cDNA by the γ-retroviral vector GCsapM-ADA, they were administered intravenously. No cytoreductive conditioning, at present considered critical for therapeutic benefit, was given before cell infusion. Hematological/immunological reconstitution kinetics, levels of systemic detoxification, gene-marking levels, and proviral insertion sites in hematopoietic cells were assessed.

Results

Treatment was well tolerated, and no serious adverse events were observed. Engraftment of gene-modified repopulating cells was evidenced by the appearance and maintenance of peripheral lymphocytes expressing functional ADA. Systemic detoxification was moderately achieved, allowing temporary discontinuation of ERT for 6 and 10 years in pt 1 and pt 2, respectively. Recovery of immunity remained partial, with lymphocyte counts in patients 1 and 2, peaked at 408/mm^3 and 1248/mm^3, approximately 2 and 5 years after SCGT. Vector integration site analyses confirmed that hematopoiesis was reconstituted with a limited number of clones, some of which were shown to have myelo-lymphoid potential.

Conclusions

Outcomes in SCGT for ADA-SCID are described in the context of a unique protocol, which used neither ERT nor cytoreductive conditioning. Although proven safe, immune reconstitution was partial and temporary. Our results reiterate the importance of cytoreductive conditioning to ensure greater benefits from SCGT.

Key words

Gene therapy, adenosine deaminase (ADA), severe combined immunodeficiency (SCID), primary immunodeficiency (PID), retroviral vector(s), hematopoietic stem cell(s)

Introduction

Adenosine deaminase (ADA) is a ubiquitously expressed enzyme critical in the purine salvage pathway. Genetic loss of this enzyme leads to defects in lymphocytes due to accumulated toxic metabolites, resulting in immunodeficiency [1, 2]. Patients having *ADA* gene mutations compatible with severe combined immunodeficiency (SCID) have substantial risks of early death due to overwhelming infection. ADA deficiency is also considered a metabolic disease, with many tissues other than the hematopoietic system affected by the enzyme defect [3]. The ideal curative treatment should therefore enable not only long-term hematopoietic/immune reconstitution but also life-long systemic detoxification. Hematopoietic cell transplantation (HCT) from an HLA-identical sibling donor may fulfill such requirements, but is not necessarily available to all patients [4, 5]. The risks that still inhere in HCT under other conditions, *e.g.*, from an HLA-matched unrelated donor, often limit its use upon thorough risk-to-benefit estimation [6]. Since the development of enzyme replacement therapy (ERT), many patients have benefited from the systemic detoxification and protective immunity provided by the infusion of polyethylene glycol-conjugated bovine ADA (PEG-ADA) [3]. Despite its great value in sustaining life, ERT often yields only partial immune reconstitution [7], so that development of other curative treatment options is desirable.

Gene therapy has been studied as a possible such option, in which a basic aim is reconstitution of normal hematopoietic/immune systems by infusing autologous hematopoietic stem cells (HSCs) equipped, using viral vectors, with an *ADA* cDNA expression cassette [8, 9]. Although pioneering trials yielded minimal gene-marking in peripheral blood cells with no visible clinical benefits, they did prove the safety of such emerging technology [10-12]. With improvement in several techniques, a new series of SCGT was initiated in the late 1990s, following which Aiuti *et al*. reported marked immune reconstitution in two ADA-SCID patients via SCGT, ascribing good outcome to a protocol modified in two major respects [13]. The first, absence of concomitant use of PEG-ADA is believed to have conferred a selective advantage after SCGT upon gene-corrected cells, allowing them to survive and expand better than uncorrected counterparts [14]. The second, the use of cytoreductive conditioning

with low-dose busulfan, is believed to have increased the space into which gene-modified HSCs can engraft within bone marrow (BM), or, more specifically, the availability of stem-cell niches. Almost all subsequent SCGT protocols for various types of genetic diseases have used some degree of cytoreductive conditioning, with success being reported for increasing numbers of patients [15-24]. ADA-SCID continues to be a leading target for SCGT, which yields notable clinical benefits and is established as safe, with no cases of insertional leukemogenesis reported [8, 9].

In 2003-2004, we conducted in two ADA-SCID patients a clinical trial of SCGT that in some respects can be recognized as distinct. Although nonmyeloablative conditioning is now believed indispensable for the positive long-term outcome in SCGT for ADA-SCID, the rationale had not completely been established. We thus decided not to use cytoreductive treatment before infusing gene-modified BM $CD34^+$ cells, based on individualized risk-benefit assessment. As PEG-ADA was withdrawn approximately 5 weeks before BM harvest, the protocol was unique in combining two conditions, *i.e.*, no conditioning and no ERT. In addition, pt 2 had long-lasting *ADA*-marked T cells at SCGT, cells derived from a previous clinical trial in which peripheral T lymphocytes were the target of gene transfer [25, 26]. In this exceptional instance, T cells and HSCs thus were independently targeted by distinguishable γ-retrovirus vectors. We here describe SCGT outcomes in these two unique patients.

Methods

Patients

Patient characteristics have been described [14, 26], and are summarized in table 1. Both patients had been treated by weekly intramuscular injection of PEG-ADA for the times shown. HLA-matched sibling donors were not available. Of note is that pt 2 had repeatedly received infusions of autologous T cells transduced with the γ-retrovirus vector LASN, harboring *ADA* cDNA, in a clinical trial of gene therapy begun at age 4 years [26]. While on PEG-ADA, pt 2 had maintained ~5-10% gene-marking levels in peripheral T cells, persisting even 10 years later when he underwent SCGT.

Treatment details

After written informed consent was obtained, PEG-ADA replacement was withdrawn ~ 5 weeks before BM harvest. BM $CD34^+$ cells purified using the Isolex 300i cell separation system (Baxter, Deerfield, MA) were pre-stimulated for 2 days in serum-free X-VIVO15^{TM} medium (Lonza, Walkersville, MD) supplemented with 1% human serum albumin and a cocktail of cytokines consisting of 50 ng/ml stem cell factor (SCF), 50 ng/ml thrombopoietin (TPO), 300 ng/ml Flt3-ligand, 100 ng/ml interleukin (IL)-6, and 500 ng/ml soluble IL-6 receptor (sIL-6R; all from R&D Systems, Minneapolis, MN). Pre-stimulated cells on fibronectin fragment CH296 (Takara Bio, Otsu, Japan) were transduced 3 times during the next 3 days with the retroviral vector GCsapM-ADA [27]. On the day after final transduction, cells were harvested, washed, and infused intravenously into patients. Cytoreductive reagents were not used. Cell transduction is summarized in table 2, with detailed methods described in supplemental text. All studies were conducted with ethical and regulatory approval of both institutional committees and governmental authorities.

Measurement of ADA enzyme activity and adenine nucleotide content

Thin-layer chromatography analysis of ADA enzyme activity is described [26]. A small aliquot of transduced BM $CD34^+$ cells was subjected to such analysis to confirm functional reconstitution. After SCGT, mononuclear cells were archived and later used for analysis. The values are represented as units, defined as [one unit = activity estimated to produce one nmol (inosine + hypoxanthine) per min by 10^8 cells (nmol/min/10^8 cells)]. Where indicated, granulocytes were separated and used for measurement. To monitor ADA activity and the level of metabolic detoxification after SCGT, dried blood spots were prepared using a piece of Guthrie filter paper following an established method, by which ADA activity, AXP, and dAXP extracted from erythrocytes were measured at Duke University Medical Center [28].

Assessment of immune functions

T cell proliferative responses were assessed with a standard ^{3}H-thymidine incorporation assay. Phytohemagglutinin and concanavalin-A were used as mitogens. Diversity in T cell receptor recombination was assessed by complementarity-determining region 3 (CDR3) spectratyping analysis as described [29].

Flow-cytometry analysis

Immunophenotyping of cell surface markers in hematopoietic cells was conducted by flow-cytometry analysis in Hokkaido University Hospital. ADA protein expression was assessed by multi-color flow-cytometry analysis as described [30]. Detailed methods are described in supplemental text.

Vector copy number assessment

Vector copy number (VCN) was quantified by quantitative polymerase chain reaction (qPCR). The sequences of primers and probes used are shown in supplemental text. Although we designed two sets of primer / probe pairs for the specific identification of different vectors, *i.e.*, LASN and GCsapM-ADA, we found it difficult to ensure specificity for LASN detection. We thus decided to use another primer / probe pair that could quantify the sum of VCN based on both vector sequences. Estimation of LASN-VCN was then feasible by measuring GCsapM-ADA VCN simultaneously in the same samples. A copy number reference control was obtained from the 293 cell clone, which we established by modifying it so that the GCsapM-ADA proviral sequence was present at 2 copies/cell. Genomic DNA samples were prepared from each cell population indicated, then subjected to qPCR analysis. Values were normalized by referring to the control and were shown as average VCN per cell.

Vector integration site analysis

To determine vector integration sites in samples, linear amplification mediated (LAM)-PCR analysis was conducted following a published protocol [31] with some modifications. Precise integration sites of each vector integrant were determined after

excision of selected bands from agarose gels (Fig. 7), followed by sequencing analysis (supplemental text). To prove that integration sites shared between lymphoid- and myeloid-lineage cells existed, peripheral blood mononuclear cells (PBMNCs) and purified granulocytes were assessed. BM cell samples, available only from pt 1, were used as total cells without further purification.

Results

Treatment summary

After each patient entered hospital, regular administration of PEG-ADA was stopped ~5 weeks before BM harvest. BM aspirates were collected under general anesthesia on day -5. $CD34^+$ cells were purified from these; the purified cells' characteristics are shown in table 2. Following pre-stimulation with a cocktail of cytokines, cells were transduced with the GCsapM-ADA γ-retroviral vector [27] in CH296-precoated culture bags at ~24-hour intervals for the following 3 consecutive days. On day 0, highly viable cells were obtained, and slowly infused into the patient intravenously. No immediate reactions were observed. No cytoreductive treatment, such as administration of busulfan or melphalan, was given to the patients. Pt 1 and pt 2 received the transduced cells at doses of ~1.38×10^6 cells/kg and 0.92×10^6 cells/kg, respectively. Transduction efficiency was estimated to be ~40% (pt 1) and ~50% (pt 2) when assessed in primitive cell colonies derived from the final cell products. Of note is that patient $CD34^+$ cells acquired supra-normal levels of ADA enzyme activity after gene transduction (table 2). Overall, treatment was well-tolerated despite both patients experiencing some gastrointestinal symptoms during the periods when systemic detoxification seemed insufficient. Both patients were discharged from hospital ~6 months after SCGT with no need to resume PEG-ADA administration.

Systemic metabolic detoxification

Following withdrawal of ERT, both patients became anorexic, likely reflecting "ADA-deficient" status. Consistent with this observation, serum activities of liver transaminases (AST and ALT) increases ~ 3 weeks after ERT stopped (Fig. 1a and

supplemental Fig. 1a). More direct evidence of ADA deficiency was demonstrated by an increase in %dAXP values measured in erythrocytes [28], with kinetics like those of AST and ALT (Fig. 1b and supplemental Fig. 1b). These signs of metabolic abnormality, however, improved following SCGT, with liver enzyme values normalized within 6-8 weeks (supplemental Figs. 1a and b). In pt 1, once erythrocyte %dAXP values stabilized at ~ 8 weeks, they maintained that level at ~10% for another year. Interestingly, %dAXP decreased gradually even during the second year, indicating that gene-corrected $CD34^+$ cell-derived hematopoiesis had slowly but steadily contributed to detoxification at least within the hematopoietic system in this ADA-deficient patient (Fig. 1b). Improvement in %dAXP levels exhibited slower kinetics in pt 2, with values having reached ~10% only 3 years after SCGT (Fig. 1b). However, liver enzyme abnormality became evident again for pt 1 beyond 2 years, indicating that systemic detoxification effects did not last long (Fig. 1a). Overall, single infusions of gene-modified $CD34^+$ cells led to sustainment of partial metabolic detoxification, indicating that hematopoietic compartments capable of providing therapeutic levels of ADA activity were established for short-term even though our SCGT protocol included no cytoreductive conditioning.

Immune / hematopoietic reconstitution

Upon withdrawal of ERT, absolute lymphocyte counts (ALCs) dropped quickly, at nadir reaching values of 70/mm^3 for pt 1 and 220/mm^3 for pt 2 (Fig. 2a and supplemental Fig. 2a). After SCGT, ALCs recovered gradually. Pt 1's ALC reached the peak value of 408/mm^3 at 644 days after cell infusion, whereas pt 2's peak ALC (1248/mm^3) occurred later, at 1884 days after SCGT (Fig. 2a). Both patients experienced mild neutropenia following ERT withdrawal. Their absolute neutrophil counts (ANCs) increased gradually after SCGT with kinetics like those for ALC recovery for the first 2-3 years (supplemental Fig. 2b), and were maintained within a normal range thereafter (Fig. 2b). Consistent with short-term, partial systemic detoxification effects, full immune reconstitution was not achieved, as a gradual decrease in ALC was evident for pt 1, whereas pt 2's ALC maintained subnormal levels for up to 10 years after SCGT (Fig. 2a). Accordingly, we continued

intravenous immunoglobulin (IVIg) replacement therapy for both patients throughout the study period.

Characterization of immune-reconstitution kinetics

Patterns of immune reconstitution in our patients were distinct regarding kinetics of lymphocyte-subset development. In pt 1, both $CD4^+$ and $CD8^+$ T cell counts increased with similar kinetics after SCGT whereas an increase in $CD8^+$ T cells predominated in pt 2 (Fig. 3a and supplemental Fig. 3a). This most likely reflected expansion of preexisting $LASN^+$ T cells along with systemic detoxification partially achieved by SCGT. Emergence of $CD20^+$ B cells and $CD16^+$ / $CD56^+$ NK cells was detectable in pt 1, which became evident 10 months, then plateaued beyond 2 years after SCGT (Fig. 3b). In contrast, B cell recovery did not occur in pt 2, whereas NK cells showed some increase (Fig. 3b). B and NK cell counts, however, did not reach normal ranges in either patient.

Detailed analysis of emerging lymphocytes

ADA expression was monitored in patient $CD3^+$ T cells and $CD56^+$ NK cells over time before and after SCGT by flow-cytometry analysis [30]. As shown in Fig. 4, pt 1's lymphocytes lacked ADA expression before SCGT (pre-GT), whereas virtually all populations in healthy control counterparts expressed substantial levels of ADA (Control). Two peaks clearly distinguishable in fluorescence intensity appeared after SCGT in each plot (6 mo, 9 mo, 18 mo, and 27 mo), with the brighter ones likely representing developing lymphocytes that expressed vector-derived ADA. Gradual increases in %ADA-bright cells were evident for both populations, suggesting that gene-corrected T cells, and also NK cells, had preferentially developed *in vivo* owing to a selective advantage over their non-corrected counterparts. When assessed long-term (6.5 years) after SCGT, however, such an advantage seemed blunted for NK cells while it remained still significant for $CD3^+$ T cells as most of them showed ADA expression despite with dull intensity (supplemental Fig. 4). As two peaks did not appear in pt 2's samples, we could not use this assay to estimate %transduction in T cells / NK cells for this patient (supplemental Fig. 5).

Two years after treatment, T cells in both patients showed non-skewed CDR3 size distribution (supplemental Fig. 6), suggesting no ongoing massive monoclonal or oligoclonal T cell expansion. During the observation period, no evidence of naïve T cell development was obtained for either patient, by immunophenotypic assessment of peripheral T cells ($CD4^+$ / $CD45RA^+$) or by a T-cell receptor excision circle assay (data not shown). Despite the low level of T cell reconstitution, the T cells present were viable and functional as evidenced by improvement in ADA activity when assessed ~5 years after SCGT (Fig. 5a, PBMNCs) and by improved proliferative responses to mitogen stimulation, although such responses were found lost for pt 1 at the later time point (Fig. 5b). Only a slight increase in ADA activity was noted in pt 1's granulocytes (Fig. 5a, Granulocytes). Development of a more specific immune response was demonstrated for pt 1, 2 years and 10 months after SCGT when she had chickenpox, which she cleared with no serious complications. Of note was that a defined assay [32] detected apparent proliferative responses specific to varicella zoster virus (VZV) antigen in her T cells one month after infection (supplemental Fig. 7). Pt 2 developed herpes zoster one month after SCGT, but, unlike those of pt 1, his peripheral T cells did not show a VZV-specific response when assessed 2 years after infection (supplemental Fig. 7).

Quantification of transgene-marked cells

Gene marking levels were assessed by quantifying VCN using a qPCR-based method as described in Methods and supplemental materials. A series of analyses for pt 1 demonstrated steady increase in VCN in PBMNCs within 2 years with kinetics similar to those observed for ALC (Fig. 6a left, refer to Fig. 2a). VCN analysis of cell fractions 2 years after SCGT showed the highest gene marking level in $CD3^+$ T cells (1.55 copies/cell), with the second highest value in $CD19^+$ B cells (0.51 copies/cell) (Fig. 6a right). Gene marking was also detectable in PB granulocytes, yet at a low level (0.04 copies/cell), indicating engraftment of progenitor cells capable of producing this short-lived myeloid population. Consistent with this observation, we could detect substantial levels of gene marking in BM $CD34^+$ progenitor cells (0.06 copies/cell). Overall, significantly higher gene marking levels in peripheral lymphocytes than those

in myeloid cells and BM compartments including $CD19^+$ cells (0.08 copies/cell) confirmed the greater influence on SCGT outcomes of selective advantage for gene-corrected cells in lymphoid lineages.

Because pt 2 had pre-existing LASN-marked T cells derived from T cell-gene therapy, VCN was assessed by two different sets of primer / probe pairs: one could specifically detect the SCGT vector (GCsapM-ADA), whereas the other could quantify *ADA* cDNA copies, thereby assessing both vectors' copy numbers (both vectors) (Fig. 6b). Although the frequency of $LASN^+$ T cells after the initial GT trial was ~5-10%, high VCN values (> 1 copy/cell) were continuously detected by the primer / probe for both vectors in PBMNCs after SCGT, indicating virtually ~100% cells having either vector sequence (Fig. 6b). Since this was true at the earliest time point (5 months) when only low levels of gene-marking was noted for GCsapM-ADA (0.005 copies/cell), $LASN^+$ cells most likely constituted a dominant population in T cells for pt 2 for the period studied (Fig. 6b left). Despite the predominance of $LASN^+$ T cells, emergence of T cells marked by SCGT was visible (GCsap-ADA); reconstitution kinetics of these T cells, however, gradually blunted and eventually resulted in a plateau at low levels (< 0.1 copies/cell determined in PBMNCs). The actual marking level in "purified" T cells was higher than this estimate, reaching 0.29 copies/cell for the SCGT vector and 2.4 copies/cell for both vectors (Fig. 6b right, PB). Although T cell reconstitution was limited, engraftment of progenitor cells by SCGT was apparent for this patient, as evidenced by the presence of GCsapM-ADA^+ cells in BM fractions expressing CD34 (0.09 copies/cell) and CD19 (0.03 copies/cell) 2 years after treatment.

Existence of engrafted progenitor cell clones capable of myelo-lymphoid differentiation

Vector integration sites were analyzed. LAM-PCR analysis demonstrated a polyclonal integration pattern in pt 1 PBMNCs at 16 months, which gradually switched to oligoclonal patterns (Fig. 7). Of note is that analysis of sequentially obtained PBMNC samples (32-68 months) commonly identified at least three major bands, likely shared also by granulocytes and BM samples (shown in white arrowheads). For pt 2, polyclonal integration patterns were maintained in PBMNCs throughout the

observation period (Fig. 7) (latest analysis at ~6 years). This, however, should reflect mostly LASN integration sites, because the analytical method used cannot distinguish the two vectors LASN and GCsapM-ADA in amplifying the sequences containing integration sites. In contrast, analysis revealed oligoclonal integrations in granulocytes, which are considered to derive solely from SCGT. We could identify at least two bands of approximately identical size in both PBMNCs and granulocytes of pt 2 (white arrowheads).

To clarify vector integration sites more precisely, PCR amplicons in the selected bands (9 bands in total shown by white arrowheads for pt 1, and 4 bands for pt 2) were retrieved for DNA sequencing. This analysis identified unique integration sites for both patients' samples (supplemental materials). Using this defined measure, we could detect 5 integration sites shared by PBMNCs and granulocytes obtained from pt 1 at ~6 years after SCGT (68 months), among which 4 sites were also found in BM samples (51 months after SCGT). Furthermore, three integration sites out of 4 were also identified in PBMNCs obtained 4 years earlier (21 months). Two integration sites shared by PBMNCs and granulocytes were found in pt 2's samples as well. These results indicate that our SCGT trial achieved engraftment of progenitor clones that were capable of multilineage differentiation, but with clone numbers likely limited due to the absence of cytoreductive conditioning.

Long-term outcomes

Both patients were clinically well, with freedom from severe infections for 3-4 years after SCGT. However, as gradual loss of SCGT-mediated effects was noted, ERT was restarted for pt 1 7 years after SCGT, when she had pneumonia. Her condition improved upon re-initiation of ERT (detailed treatment outcome to be reported elsewhere). Pt 2 remains off PEG-ADA at writing, but he is also expected to need ERT soon. No leukemic transformation has been noted to date in either patient. Overall, these two patients moderately benefited from SCGT, with evidence of engraftment of multipotent progenitor cells expressing ADA, but the effects were transient and limited, with only partial immune reconstitution achieved.

Discussion

SCGT is recognized as one of the most advanced forms of experimental medicine [8, 9]. Among all target diseases, especially primary immunodeficiency disorders (PIDs), ADA-SCID has the longest history of gene therapy trials and has been most successfully managed, with more than 38 patients reportedly treated safely worldwide [19, 20, 33, 34]. Even using γ-retroviral vectors, which are inherently unfavorable owing to leukemogenesis by insertional mutagenesis, as shown in SCGT trials for other PIDs [18, 24, 35], no such events have occurred in similarly treated ADA-SCID patients. In line with these observations, we too so far have not observed any leukemic transformation of long-term progenitor cells that harbor inserted vectors. Although the limited numbers of clones engraftable in our subjects may have lowered risk, our results provide additional support for the idea that ADA-SCID trials are safer than are SCGT trials for other PIDs. Following a trend pushed forward by the promising results in recent SCGT trials [15, 36], safety in SCGT will be further enhanced by the use of vector systems with better safety characteristics, such as self-inactivating lentiviral vectors [37].

Since the epochal SCGT trial reported by Aiuti *et al*. [13], two modifications that they introduced, 1) non-myeloablative conditioning and 2) lack of concomitant ERT, have been considered critical determinants of treatment efficacy. They thus have been incorporated into other ADA-SCID trials [19, 20]. In the 1990s, a series of pioneering SCGT trials were conducted with no cytoreductive conditioning and with continuation of ERT; they were clinically ineffective [38-40]. As a result, two series of SCGT trials differed in two variables, thus impeding clarification of each variable's contribution to treatment efficacy. Carbonaro *et al*. addressed this issue in murine studies to determine the extent to which each protocol variable contributed to therapeutic efficacy and concluded that cytoreduction was important for the engraftment of gene-corrected HSCs, but cessation of ERT might not be necessary to achieve clinical benefits [41]. In addition, the idea has emerged that ERT at some points after SCGT may benefit patient outcomes [19, 34, 41], thus suggesting another combination of two variables for future clinical protocols (*i.e.*, with cytoreductive conditioning, while on PEG-ADA). Although several other variables in the protocol should also be considered, our trials are

unique, with ADA-SCID patients treated after cessation of ERT but with no cytoreductive conditioning. We thus believe that our results provide information valuable for future clinical studies of ADA-SCGT [34].

Although the data are limited to those for relatively short-term, engraftment of multipotent progenitors demonstrated in both patients after SCGT without conditioning supports the idea that to generate an empty niche by cytoreductive treatment may not be absolutely necessary for HSC engraftment in an autologous setting, as indicated in murine experiments [42]. Although the extent and kinetics of hematopoietic reconstitution were far less greater than those in other successful cases, maintenance of low-level %dAXP values in erythrocytes indicated significant ADA activity continuously provided within the mass of hematopoietic cells expressing transferred *ADA*. Moreover, the multilineage potential of the engrafted progenitors was demonstrated in the form of vector integration sites shared between lymphoid- and myeloid-lineage cells. One must nevertheless note that clonal analysis could identify only limited numbers of such clones actively contributing to hematopoietic / immune reconstitution in our patients. Each reconstituted T cell that appeared relatively short-term after SCGT seemed normal in terms of ADA expression and activity and functions such as proliferative responses, but absolute values never normalized. In addition, no evidence of thymic reconstitution was obtained in either patient (data not shown), in contrast to other trials that used low-intensity conditioning [19, 20, 33]. We observed apparent B and NK cell development in pt 1, yet with very low numbers and consequently continued IVIg therapy for both patients. Overall, clinical benefits were moderate as exemplified by partial systemic detoxification, and gradually lessened beyond the third year. We thus conclude that cytoreductive conditioning is critical in enabling gene-modified HSCs to engraft in numbers sufficient to achieve and to maintain immune reconstitution that frees patients from ERT and IVIg replacement long-term.

Our results appear to support the idea that ERT at the time of SCGT may blunt the selective advantage that ADA-expressing lymphocytes have over their defective counterparts. Flow-cytometry analysis demonstrated a gradual increase in ADA-bright cells in both T and NK cell compartments, likely reflecting selective

survival / expansion of cells expressing intracellular ADA during reconstitution in the absence of ERT. Whether withdrawal of ERT enhanced engraftment of long-term progenitors remains uncertain. Relatively high VCN values in BM $CD34^+$ cells (0.06 and 0.09 copies/cell for pts 1 and 2, respectively) 2 years after SCGT may indicate enhancement of HSC engraftment by ERT cessation, although long-term results are necessary before drawing conclusions.

Pt 2 is unique in having received two types of transduced cells in separate trials. This allowed separate tracking of reconstitution kinetics of two cell types utilizing differences between vector constructs, *i.e.*, LASN in T cells and GCsapM-ADA theoretically in all hematopoietic lineages. While on ERT, pt 2 maintained ~5-10% LASN-expressing T cells. Of interest is that virtually ~100% of T cells contained the LASN sequence as early as 5 months after SCGT when only a marginal level of marking by GCsapM-ADA was detectable. This indicates that the initial increase in T cells observed after SCGT in pt 2 is most likely attributable to the expansion of LASN-T cells upon enrichment by cessation of ERT, followed by proliferation in the presence of favorable bystander detoxification effects brought about by SCGT. However, dominance of LASN-positivity in T cells continued for the initial 2 years; perhaps the success of this sub-population impeded expansion of GCsapM-ADA^+ T cells, for which VCN values remained below 0.3 copies/cell when last measured. Competition between similar cells thus must be considered when patients receive gene therapy more than once, an increasingly likely scenario with wider deployment of SCGT.

Finally, some other variables in our protocol should be taken into consideration when comparing outcomes with those in other trials. Among them is patient age. This is considered critical in determining benefits of SCGT in PIDs [43, 44]. It may have affected outcome in our patients adversely, especially due to aged thymic environment. We also must note that doses of infused $CD34^+$ cells (/kg) in our trial were low, lying in a range at which complete immune reconstitution failed in some patients in other successful trials [19, 20, 33]. Patient age and cell dose interplay with one another, of course, and with other variables including culture conditions. We used IL-6 and sIL-6R in addition to the basic cocktail of the three cytokines SCF, TPO,

and Flt3-ligand, anticipating favorable outcomes based on established results [45]. In the absence of control data, however, one can only speculate on how this unique cytokine cocktail contributed to overall outcomes. HSC expansion techniques still remain a hot issue in transplantation medicine [46, 47]. Future SCGT trials may be able formally to compare newly defined culture systems, deploying recent advances in analysis that now enable tracking human HSCs and progeny via comprehensive vector insertion site analysis [15, 36].

Conclusions

This is the first report to describe outcomes of SCGT in ADA-SCID patients conducted after cessation of ERT and with no use of cytoreductive conditioning. Although engraftment of repopulating gene-modified cells was detectable, recovery of patient immunity was partial and transient due to limited HSC engraftment. Our results reinforce the observation that SCGT for ADA-SCID is safe and efficient, but also emphasizes that appropriate cytoreductive conditioning is necessary to maximize clinical benefits.

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Conflict of Interest Disclosures

The authors have no conflict of interest in relation to this article to disclose.

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Figure legends

Fig. 1 *Kinetics of systemic detoxification.* **(a)** Activities of liver enzymes aspartate transaminase (AST) and alanine transaminase (ALT) in patient serum before and after (up to ~6 years for pt 1 and ~10 years for pt 2) stem cell gene therapy (SCGT). At 78 months after SCGT, PEG-ADA treatment was re-initiated for pt 1. **(b)** Patient erythrocyte %dAXP values before and after SCGT. To facilitate comparison, the x-axis scale is the same as that of the x axis in (a).

Fig. 2 *Kinetics of hematopoietic reconstitution.* **(a)** Lymphocyte reconstitution. Absolute lymphocyte counts in patient peripheral blood; the time of stem cell gene therapy (SCGT) is indicated. Shown are the data up to ~6 years after SCGT for pt 1, and ~10 years for pt 2. **(b)** Neutrophil reconstitution. Absolute neutrophil counts in patient peripheral blood; *cf.* (a) for manner of data presentation.

Fig. 3 *Lymphocyte subset reconstitution.* **(a)** Absolute $CD4^+$ and $CD8^+$ T cell counts in peripheral blood before and after stem cell gene therapy (SCGT). **(b)** Absolute $CD20^+$ B cell and $CD16^+/CD56^+$ NK cell counts in peripheral blood; *cf.* (a) for manner of data presentation.

Fig. 4 *Development of ADA-expressing T/NK cells.* Shown are flow-cytometry analysis data on frequencies of ADA-expressing cells among peripheral blood $CD3^+$ T cells and $CD56^+$ NK cells (pt 1 only). Dashed histograms, isotype control; gray histograms, ADA-specific fluorescence. Control: Data obtained from a healthy individual. Before stem cell gene therapy (SCGT): Data obtained 2~5 months before SCGT (still receiving enzyme replacement therapy). The first peak in each ADA histogram after SCGT is assumed to represent ADA-negative populations, not dull-expressers. The frequency (%) of ADA-bright cells is calculated based on the separation between two populations.

Fig. 5 *ADA activity and mitogen-mediated proliferative responses in patient cells.* **(a)** ADA activity (U: nmol/min/10^8 cells, see *Methods*) in peripheral-blood mononuclear cells (PBMNCs) and granulocytes is shown. The normal range of values in healthy volunteer samples is shown as a box-whiskers plot (n = 10 and 5 for PBMNCs and granulocytes, respectively). Before (Pre): Values measured while the subjects were still receiving enzyme replacement therapy. After (Post): Values obtained ~5 years after stem cell gene therapy. **(b)** Proliferative responses of patient PBMNCs to phytohemagglutinin and concanavalin A. Values are represented as stimulation index at each time point. Range of "normal control" stimulation index (mean ± 2SD) is as follows: 132.6 ± 62.3 for PHA; 147.6 ± 77.8 for ConA.

Fig. 6 *Vector copy number assessment in hematopoietic cells.* **(a)** Shown are average vector copy numbers (VCN) determined in pt 1 hematopoietic cells by quantitative PCR analysis. Left: Values in peripheral-blood mononuclear cells (PBMNCs) over time. Right: Values in each sorted hematopoietic cell population ~2 years after stem cell gene therapy (SCGT). BM: bone marrow, PB: peripheral blood. **(b)** Average VCN values in pt 2 samples. Note the presence of two different vector sequences in this subject, one derived from T cell-gene therapy (LASN) and the other from SCGT (GCsapM-ADA). Sequence-specific VCN quantification was applicable to the latter (shown as GCsap-ADA), whereas LASN copies were assessed by using a primer / probe combination that detected both vector sequences (both vectors). Left: VCNs determined in PBMNCs over time. Right: Values measured in each sorted population. The VCN was assessed specifically for the GCsap-ADA sequence in BM samples and PB $CD3^+$ T cells (open circles) and also quantified for both vectors in PB $CD3^+$ T cells (closed circles).

Fig. 7 *Vector integration analysis.* Shown are gel images generated in linear amplification - mediated PCR analysis. Each sample was processed in two independent reactions and the final product was run in duplicate to ensure reliability of the assay. M, size marker. Top: Site-specific integrations are represented as amplicons with distinct sizes in pt 1 samples obtained at indicated times. Granulo,

peripheral blood granulocytes; BM, whole bone marrow cells. As the bands shown by white arrowheads were likely shared by three different cell types, they were subjected to sequence analysis. The band shown by a white arrow was retrieved for independent sequence analysis. Bottom: Vector integration site analysis in pt 2 samples over time. Bone marrow samples were not available due to poor cell yields upon aspiration. Because our LAM-PCR system commonly amplified fragments containing either the LASN- or the GCsapM-ADA-integrant, the amplicons visible in peripheral-blood mononuclear cell lanes are derived from both vectors. In contrast, the bands seen in Granulo lanes should represent GCsapM-ADA integrations as the vector was introduced into $CD34^{+}$ progenitor cells. Candidate bands indicated by white arrowheads were considered as likely shared by lymphoid and myeloid lineages and were subjected to sequence analysis.

Table 1. Patient characteristics

	Age at clinical onset	Sex	Mutations	Age at SCGT	PEG-ADA before SCGT	Previous treatment	Immune status before SCGT
Pt 1	15 d	F	Q119X R235Q	4.7 y	4.5 years	Prophylactic only, including IVIg	Dependent on PEG-ADA; T cell counts occasionally < 100/mm^3
Pt 2	8 mo	M	R211H IVS2+1G>A	13.0 y	11.5 years	LASN-transduced T cells given 11 times (age 4.5-6 y)	Dependent on PEG-ADA; T cell counts ~300-500/ mm^3 with ~5-10% marked by LASN

SCGT, stem cell gene therapy; PEG-ADA, polyethylene glycol-adenosine deaminase; IVIg, intravenous replacement therapy of immunoglobulin.

Table 2. Characteristics of patient BM $CD34^+$ cells

	$CD34^+$ cells (x 10^6/kg)		$\%CD34^+$ at infusion	%Transduction Efficiency (CFC)	ADA enzyme activity (U)	
	Harvested	Infused			Mock vector	GCsapM-ADA
Pt 1	1.17	1.38	70.7%	39.5%	1.9	318.2
Pt 2	0.57	0.92	66.6%	50.0%	1.4	299.4

$\%CD34^+$, percentage of cells expressing CD34 cell surface antigen; CFC, colony-forming cells; Mock vector, GALV-pseudotyped GCsap-EGFP vector used as non-therapeutic transduction control.

Figure 1

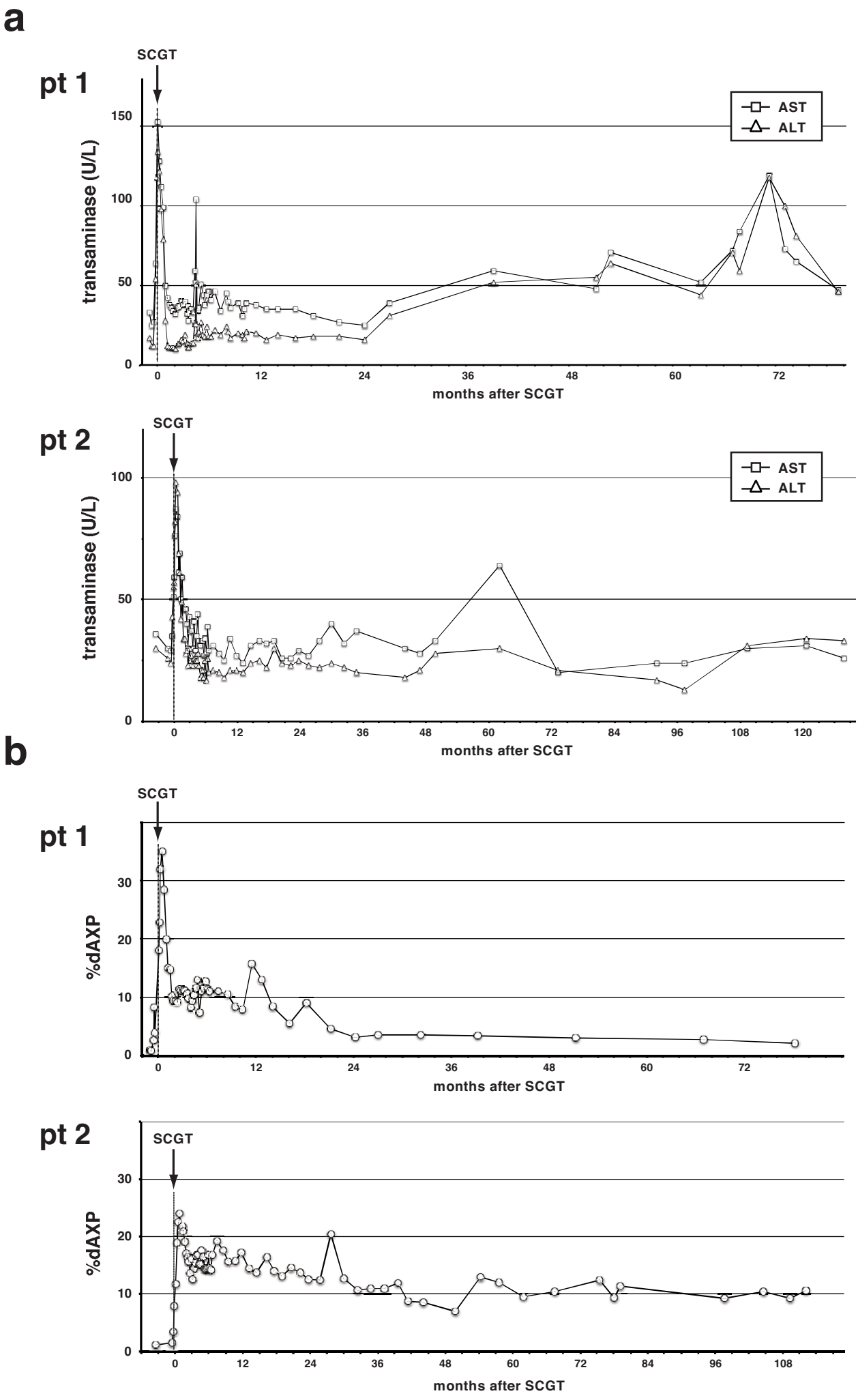
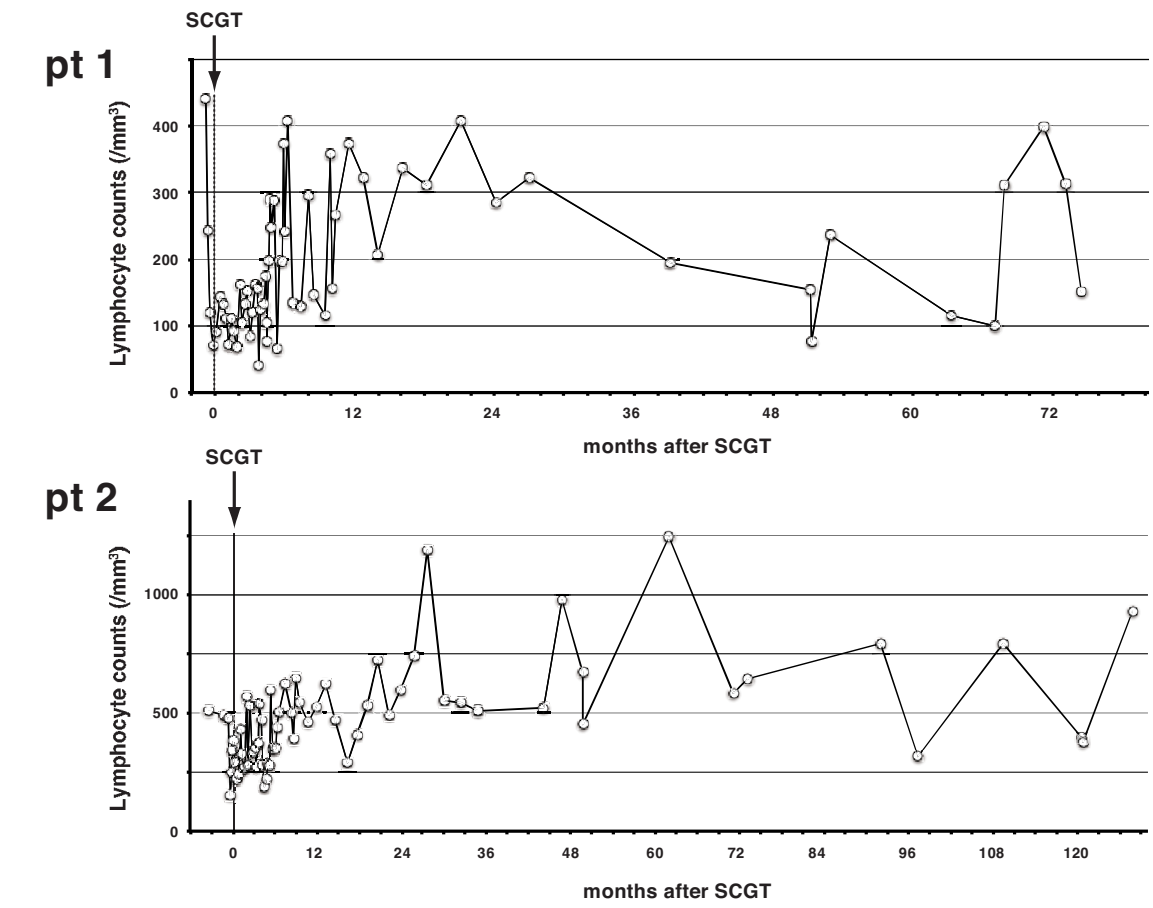

a
pt 1
SCGT
AST
ALT
transaminase (U/L)
150
100
50
0
0
12
24
36
48
60
72
months after SCGT
pt 2
SCGT
AST
ALT
transaminase (U/L)
100
50
0
0
12
24
36
48
60
72
84
96
108
120
months after SCGT
b
pt 1
SCGT
%dAXP
30
20
10
0
0
12
24
36
48
60
72
months after SCGT
pt 2
SCGT
%dAXP
30
20
10
0
0
12
24
36
48
60
72
84
96
108
months after SCGT

Figure 2


a
pt 1
SCGT
Lymphocyte counts (/mm³)
0
100
200
300
400
0
12
24
36
48
60
72
months after SCGT
pt 2
SCGT
Lymphocyte counts (/mm³)
0
500
1000
0
12
24
36
48
60
72
84
96
108
120
months after SCGT

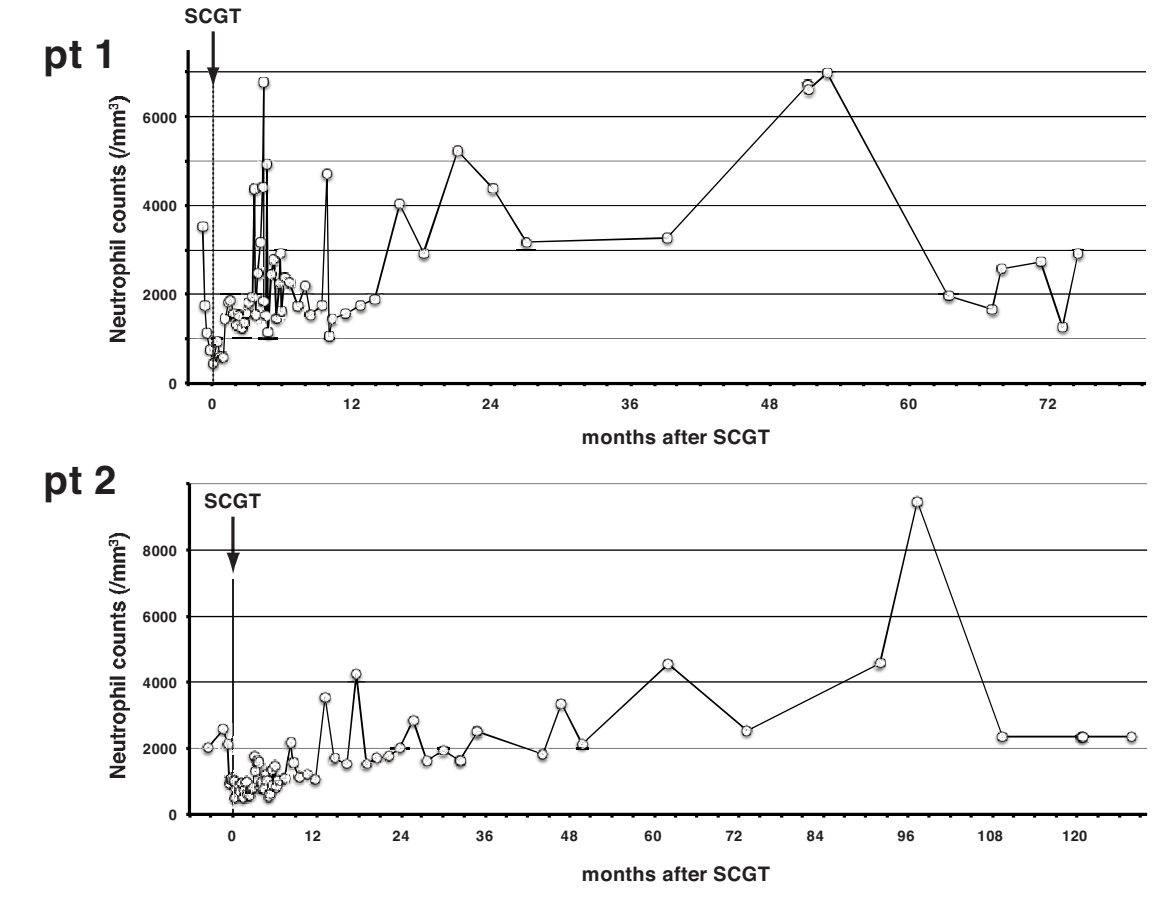
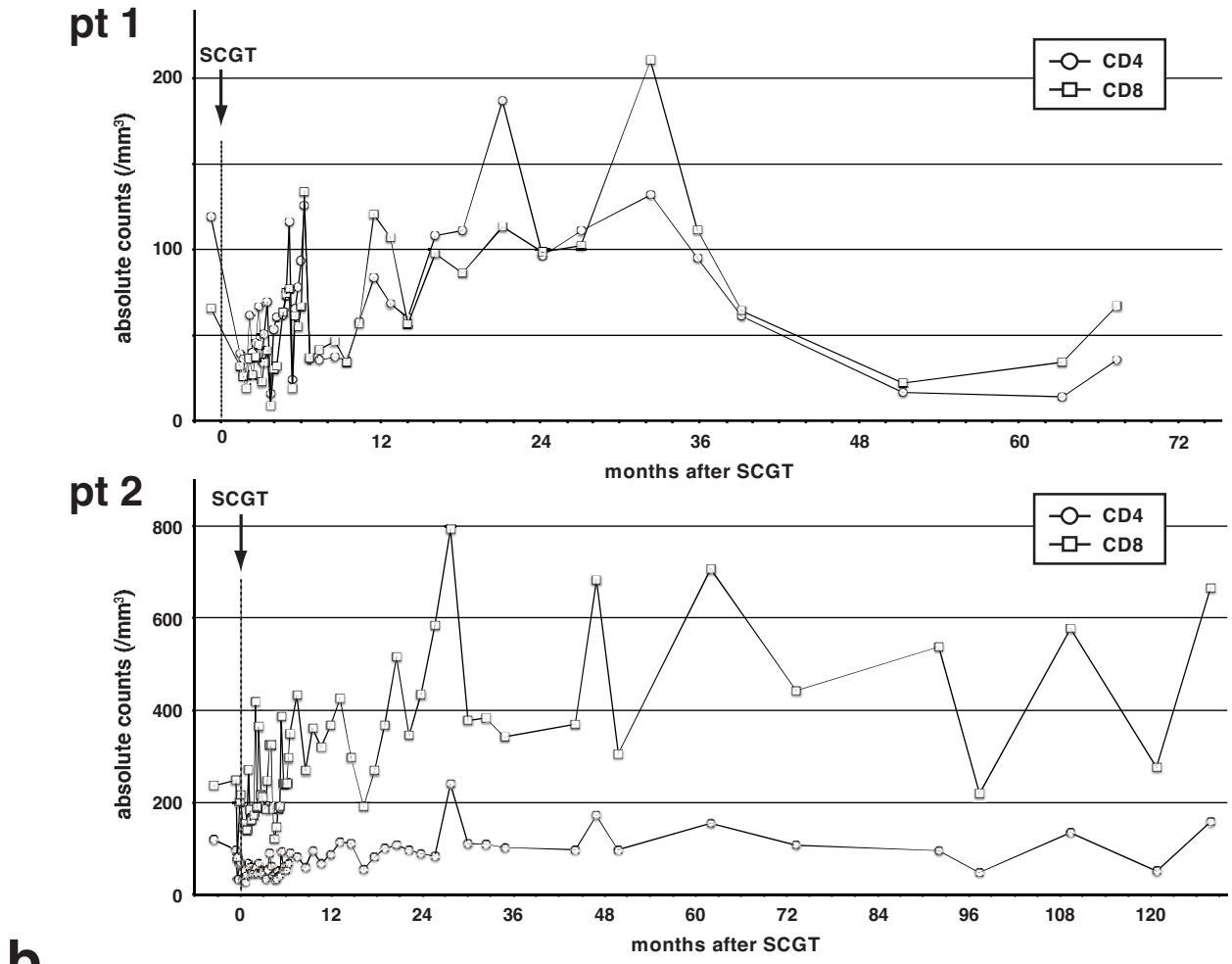

b
pt 1
SCGT
Neutrophil counts (/mm³)
0
2000
4000
6000
0
12
24
36
48
60
72
months after SCGT
pt 2
SCGT
Neutrophil counts (/mm³)
0
2000
4000
6000
8000
0
12
24
36
48
60
72
84
96
108
120
months after SCGT

Figure 3


a
pt 1
SCGT
CD4
CD8
absolute counts (/mm³)
200
100
0
0
12
24
36
48
60
72
months after SCGT
pt 2
SCGT
CD4
CD8
absolute counts (/mm³)
800
600
400
200
0
0
12
24
36
48
60
72
84
96
108
120
months after SCGT

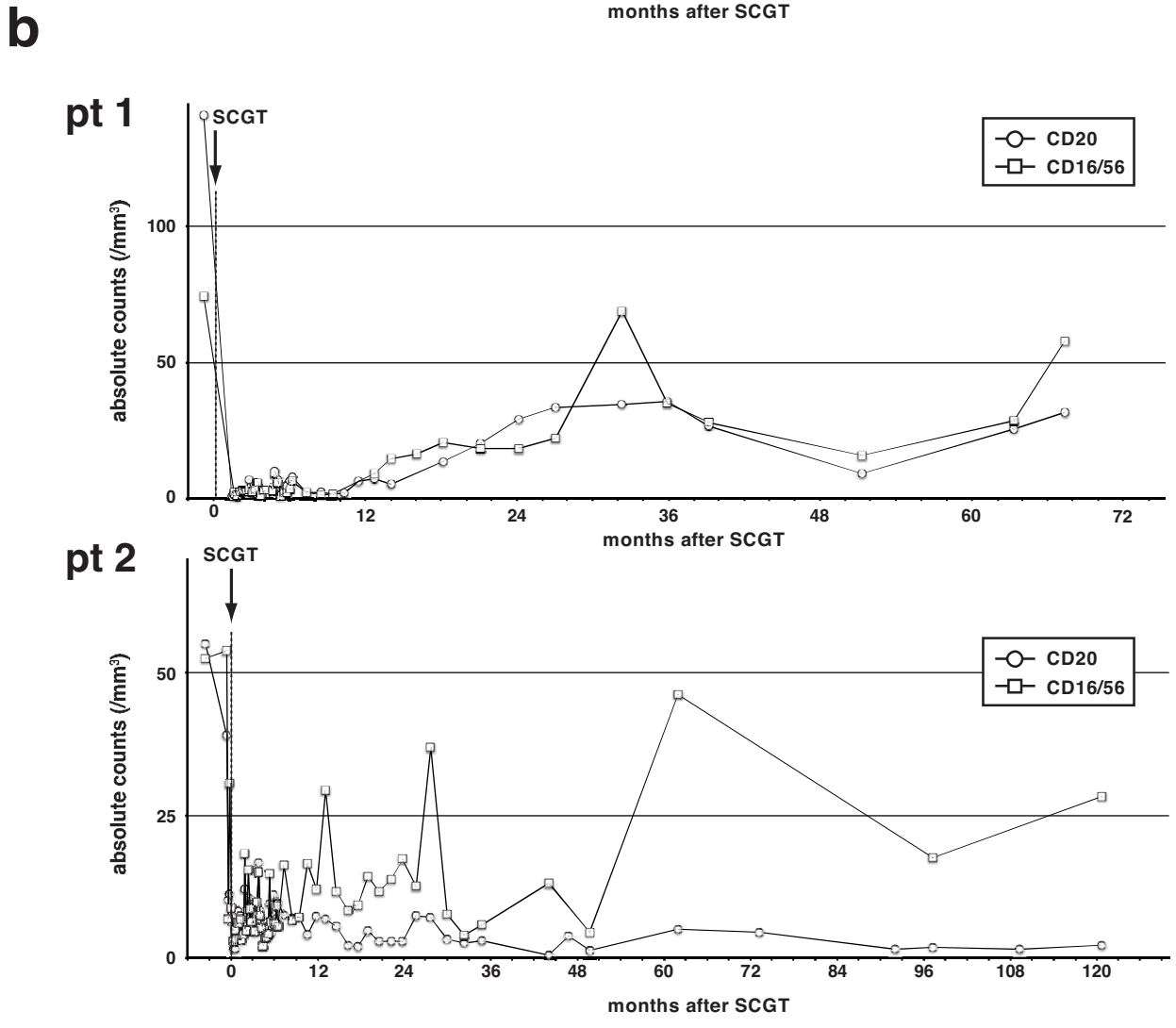

b
pt 1
SCGT
CD20
CD16/56
absolute counts (/mm³)
100
50
0
0
12
24
36
48
60
72
months after SCGT
pt 2
SCGT
CD20
CD16/56
absolute counts (/mm³)
50
25
0
0
12
24
36
48
60
72
84
96
108
120
months after SCGT

Figure 4

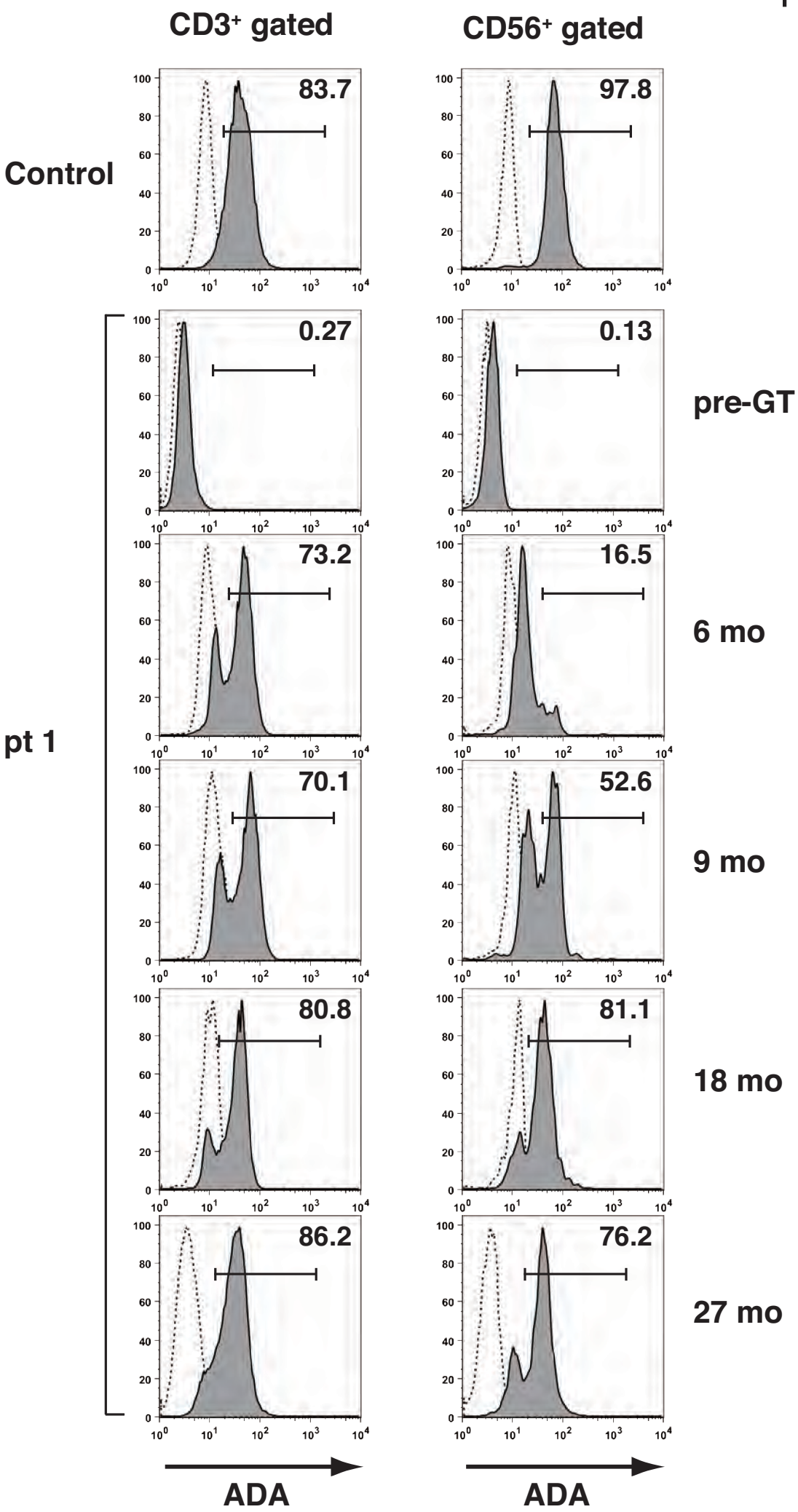

CD3+ gated
CD56+ gated
Control
83.7
97.8
pt 1
0.27
0.13
pre-GT
73.2
16.5
6 mo
70.1
52.6
9 mo
80.8
81.1
18 mo
86.2
76.2
27 mo
ADA
ADA

Figure 5

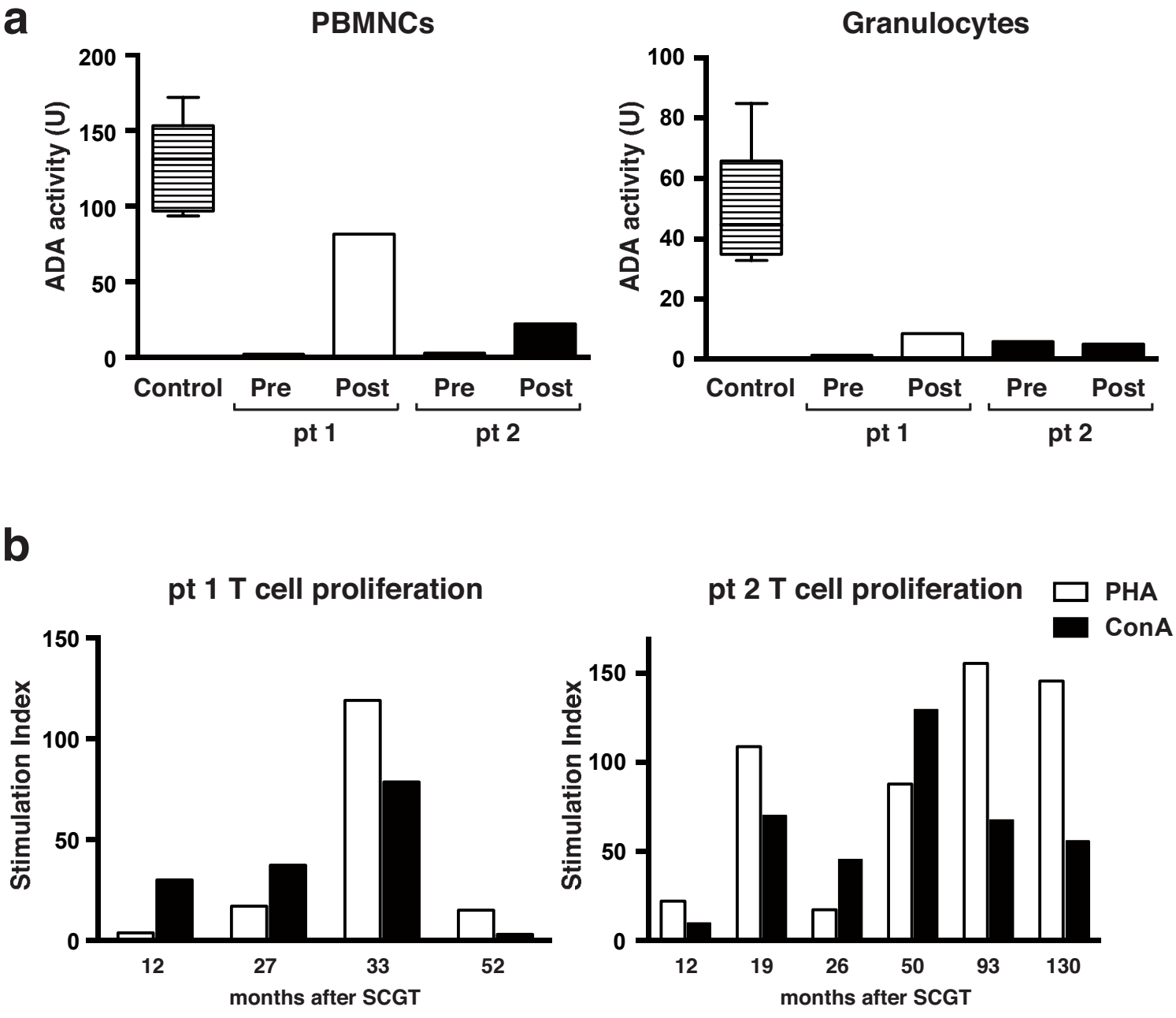

a
PBMNCs
Granulocytes
ADA activity (U)
200
150
100
50
0
100
80
60
40
20
0
Control
Pre
Post
Pre
Post
pt 1
pt 2
b
pt 1 T cell proliferation
pt 2 T cell proliferation
PHA
ConA
Stimulation Index
150
100
50
0
12
27
33
52
19
26
50
93
130
months after SCGT

Figure 6

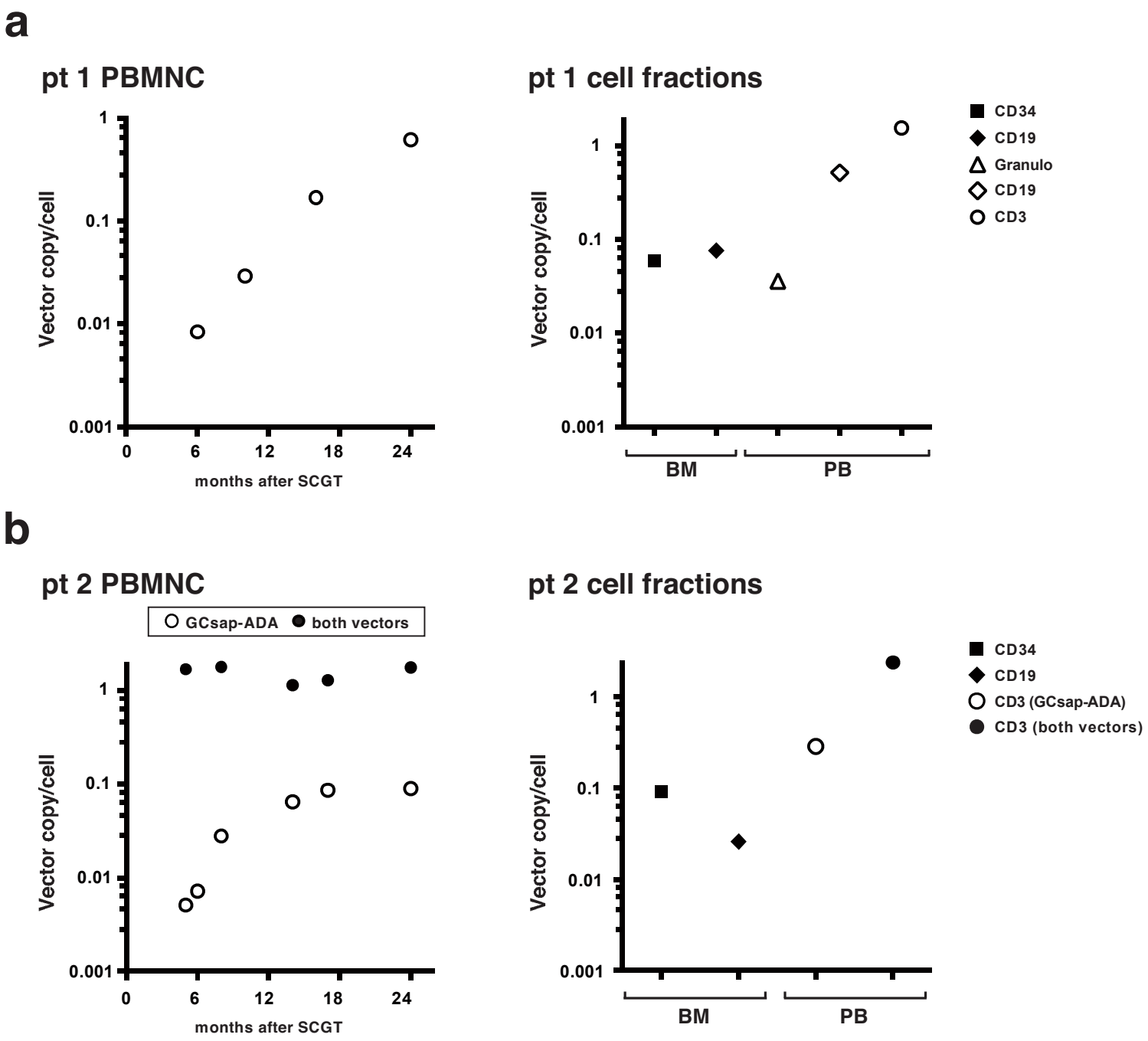
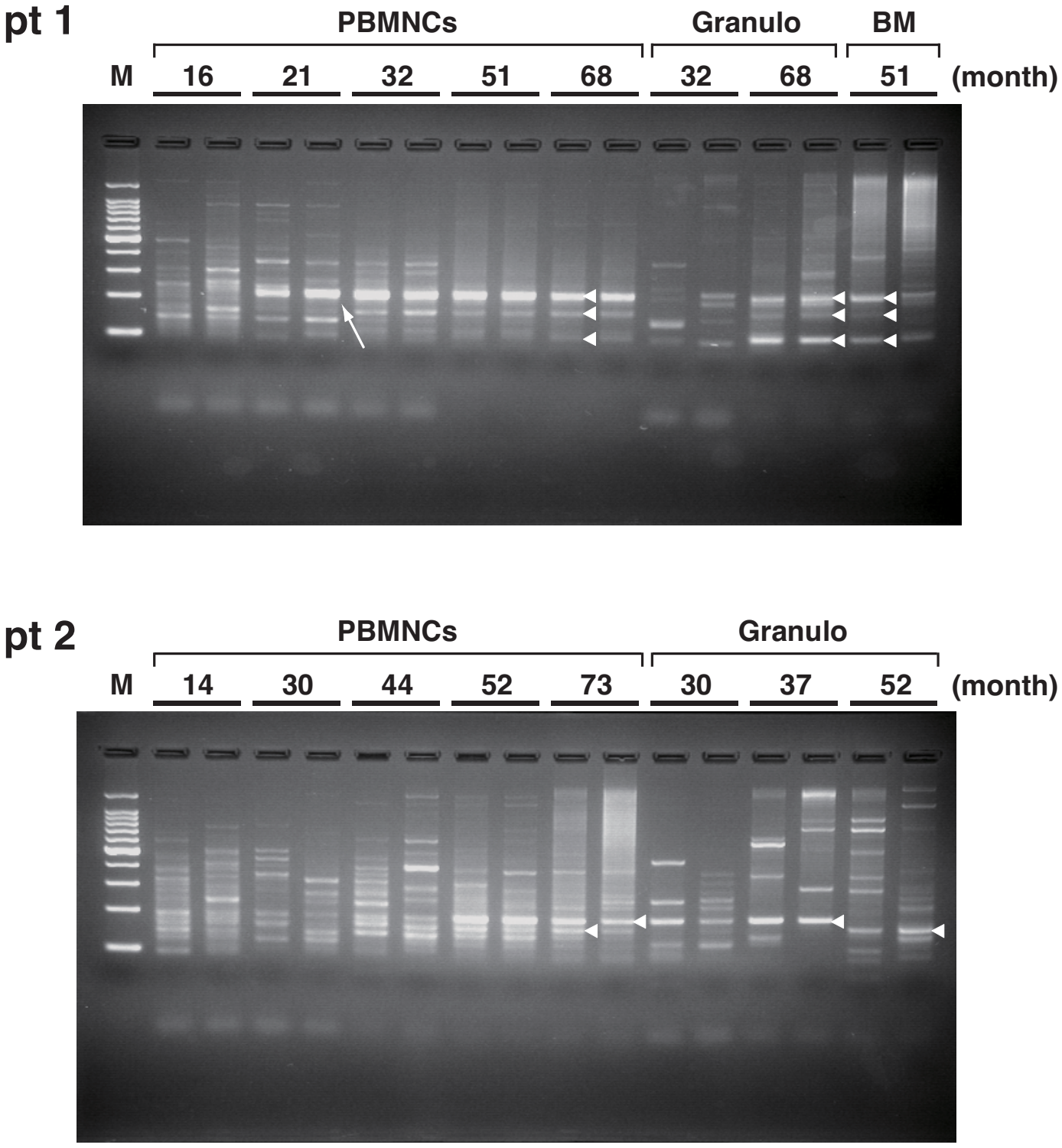

a
pt 1 PBMNC
Vector copy/cell
1
0.1
0.01
0.001
0
6
12
18
24
months after SCGT
pt 1 cell fractions
Vector copy/cell
BM
PB
CD34
CD19
Granulo
CD19
CD3
b
pt 2 PBMNC
GCsap-ADA
both vectors
Vector copy/cell
months after SCGT
pt 2 cell fractions
Vector copy/cell
BM
PB
CD34
CD19
CD3 (GCsap-ADA)
CD3 (both vectors)

Figure 7


pt 1
PBMNCs
Granulo
BM
M
16
21
32
51
68
32
68
51
(month)
pt 2
PBMNCs
Granulo
M
14
30
44
52
73
30
37
52
(month)