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## Mapping the pathogenesis of immune-mediated aplastic anemia: filling the gaps

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# Selective lymphodepletion underlies the efficacy of horse anti-thymocyte globulin-based immunosuppressive therapy in aplastic anemia

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## Key points

- ATGAM exposure varies substantially between AA patients, with higher exposure levels associating with earlier hematologic recovery
- Successful IST involves selective depletion of pathogenic T cells by ATGAM, and suppression of remaining CCR6<sup>+</sup> T and B cells by ciclosporin

## Abstract

Horse-derived anti-thymocyte globulin (ATGAM) in combination with long-term ciclosporin is the first-line treatment for most immune-mediated aplastic anemia (AA) patients. The exact impact of this immunosuppressive therapy (IST) on hematological recovery and the immune landscape, however, remains poorly understood. We report a longitudinal analysis of the pharmacodynamic effects of ATGAM-based IST in a cohort of 44 AA patients. With flow cytometry we quantified plasma levels of lymphocyte-binding ATGAM, which is believed to mediate the therapeutic effect. Population pharmacokinetic modeling revealed substantial between-patient variability in ATGAM exposure, with higher exposure levels associating to earlier hematologic recovery. ATGAM bound all lymphoid lineages and profoundly depleted T- and NK-cells at high plasma concentrations. Strikingly, ATGAM did not deplete B-cells but instead induced an increase of CD27<sup>+</sup> B-cells. Deep immunophenotyping on series of peripheral blood samples collected up to three years after the start of IST demonstrated that ATGAM induced rapid depletion of T-cells, including KLRG1<sup>+</sup> terminally differentiated CD8<sup>+</sup> T-cells and Th17-like CCR6<sup>+</sup>CD4<sup>+</sup> T-cells. Although naïve and pathogen-specific T-cells were also depleted, they recovered quickly, indicating preservation of protective immunity. Notably, CCR6<sup>++</sup> B-cells, implicated in AA pathogenesis, escaped ATGAM depletion but reduced gradually over time along with residual potentially pathogenic T-cells, including the CCR6<sup>+</sup>CD4<sup>+</sup> T-cells. This could explain the crucial contribution of long-term ciclosporin to successful IST. Collectively, our results identify ATGAM exposure as a factor influencing hematologic recovery, and indicate that the therapeutic effect of IST goes beyond total lymphodepletion but is rather the result of selective depletion and suppression of key lymphocyte subpopulations.



## Introduction

Immune-mediated aplastic anemia (AA) is a bone marrow failure syndrome caused by an autoimmune attack on the hematopoietic stem and progenitor cells (HSPCs)<sup>1</sup>. The resulting peripheral cytopenias can lead to severe bleeding and infections. Effective treatments are allogeneic hematopoietic stem cell transplantation (allo-HSCT) or immunosuppressive therapy (IST). IST is the first-line treatment for most patients due to older age or lack of a matched donor<sup>2</sup>.

Standard first-line IST for AA combines anti-thymocyte globulin (ATG) with long-term ciclosporin. ATG is a polyclonal antibody mixture that can be produced by immunizing horses or rabbits with human thymocytes. In AA, both horse-derived ATG (ATGAM®, Pfizer) and rabbit-derived ATG (Thymoglobulin®, Sanofi) have been applied. However, ATGAM is preferred since it results in superior response rates and overall survival<sup>3,4</sup>. Approximately 70% of patients show hematologic recovery after ATGAM-based IST, with a gradual improvement of peripheral blood counts over several months<sup>2,4,5</sup>.

The pharmacodynamics of ATGAM-based IST are poorly understood. Its therapeutic effect is attributed to the depletion of autoreactive immune cells implicated in AA pathogenesis. ATGAM, administered at 40 mg/kg/day for four consecutive days<sup>2</sup>, is thought to induce immediate lymphodepletion, possibly through complement-dependent lysis or activation-induced apoptosis<sup>6,7</sup>. Subsequently, long-term ciclosporin is believed to suppress residual and reconstituted lymphocytes through calcineurin and IL-2 blockade<sup>8,9</sup>. Since ATGAM is manufactured in horses, it contains antibodies against both non-human and human antigens. Therefore, only a small 'active' fraction can bind human immune cells and mediate its immunomodulatory effects. The precise specificities of this active ATGAM fraction are unknown and its activity differs from the more extensively studied rabbit-derived ATG<sup>7,10</sup>, however, ATGAM has been shown to bind a spectrum of proteins on the surface of immune cells<sup>11,12</sup>, and to reduce CD4<sup>+</sup>, CD8<sup>+</sup> and regulatory T-cells *in vivo*<sup>4,13</sup>. Similarly, the relationship between active ATGAM exposure and lymphodepletion is unclear, and the impact of ATGAM plus ciclosporin on the immune landscape has not been characterized in detail. Previously, we identified potentially pathogenic T- and B-cell subpopulations in AA patients<sup>14,15</sup>. These CCR6<sup>+</sup> B-cells, Th17-like CCR6<sup>+</sup>CD4<sup>+</sup> T-cells and KLRG1<sup>+</sup> terminally differentiated CD8<sup>+</sup> T-cells were enriched at diagnosis, colocalized in bone marrow, and significantly reduced six months post-ATGAM. This decrease could imply that IST selectively depletes specific lymphocyte subpopulations.

In this study, we aimed to delineate the effects of ATGAM exposure on hematologic recovery in 44 AA patients. For the first time, we quantified active ATGAM concentrations in patient

plasma using flow cytometry, and applied population pharmacokinetic (POPPK) modeling to estimate exposure. Furthermore, to elucidate the impact of IST with ATGAM plus ciclosporin on the immune landscape, we performed spectral flow cytometry on peripheral blood samples collected before and at multiple time points after the start of IST. Our results present active ATGAM exposure as a factor influencing hematologic recovery and reveal that successful IST involves selective removal and suppression of key lymphocyte subpopulations rather than a full immune 'reset'.

## Methods

### Patients and plasma samples

Plasma samples (n=381) were collected from 44 treatment-naïve AA patients before each ATGAM infusion and up to 82 days after the first infusion (median 9, range 4-13 samples/patient). PBMCs were collected from 3 of 44 patients at diagnosis and up to 3.5 years post-ATGAM (7-10 samples/patient), and 5 healthy donors. Patient characteristics are listed in Table 1. Patients <40 years were screened for Fanconi anemia to exclude congenital bone marrow failure. Patients received IST according to Dutch guidelines: four consecutive days of 40 mg/kg/day ATGAM and 5 mg/kg/day ciclosporin for at least six months<sup>16</sup>. Exceptions were UPN5 (two days ATGAM), UPN9 (four days ATGAM with two-day break after infusion two), and UPN46 (118 days ciclosporin). ATGAM was administered intravenously over 12 hours. 19 of 44 patients responded within six months after the start of IST, which was defined as becoming transfusion-independent and a neutrophil count of  $\geq 0.5 \cdot 10^9/L^2$ . Plasma or PBMCs were stored at -80°C or -196°C until bioanalysis, respectively. This study was approved by the Medical Ethical Committee of the Leiden University Medical Center (B21.003).

### ATGAM measurement and spectral flow cytometry

Flow cytometry was used to quantify active ATGAM concentrations in plasma, and to perform deep immunophenotyping in peripheral blood. For active ATGAM measurement, cryopreserved PBMCs from a single healthy donor were incubated with serial dilutions of patient plasma using a protocol based on a previously validated assay for quantifying lymphocyte-binding antibody concentrations<sup>17</sup>, and stained with a six-marker antibody panel including anti-horse IgG. Subsequently, cells were measured by flow cytometry, and active ATGAM plasma concentrations were determined. For deep phenotyping of lymphocytes, cryopreserved PBMC samples were stained with a 25-marker panel (Table S1), measured by spectral flow cytometry, and analyzed by dimensionality reduction<sup>18</sup>. Detailed description of all methods, including the quantification of total ATGAM in plasma by ELISAs, is provided in the Supplementary methods and Figures S1-3.

**Table 1.** Patient characteristics.

| Characteristic                                               | Patients (n=44)  |
|--------------------------------------------------------------|------------------|
| Age at diagnosis (median, range in years)                    | 54 (20-79)       |
| Sex (n)                                                      |                  |
| Male                                                         | 26               |
| Female                                                       | 18               |
| Weight at diagnosis (median, range in kg)                    | 80 (51-120)      |
| Body mass index at diagnosis (median, range)                 | 24.7 (17.6-34.3) |
| Height at diagnosis (median, range in cm)                    | 176 (156-198)    |
| Severity of disease at diagnosis (n)                         |                  |
| Non-severe AA                                                | 7                |
| Severe AA                                                    | 23               |
| Very severe AA                                               | 14               |
| Total lymphocyte count pre-ATGAM ( $\cdot 10^6/L$ )**        | 1375 (260-2890)  |
| CD4 <sup>+</sup> T-cell count pre-ATGAM ( $\cdot 10^6/L$ )** | 732 (21-1479)    |
| CD8 <sup>+</sup> T-cell count pre-ATGAM ( $\cdot 10^6/L$ )** | 363 (71-949)     |
| NK-cell count pre-ATGAM ( $\cdot 10^6/L$ )**                 | 77 (4-291)       |
| B-cell count pre-ATGAM ( $\cdot 10^6/L$ *)                   | 124 (17-631)     |
| Response to IST 6 months post-ATGAM (n)                      |                  |
| Non-response                                                 | 21               |
| Partial response                                             | 14               |
| Complete response                                            | 5                |
| Unknown (died <6 months after first ATGAM infusion)          | 4                |

\*Cell counts between days -2 to 0 pre-ATGAM infusion; \*\*Data missing for 2 patients.

### Exposure-response analyses

POPPK modelling was performed in NONMEM® to estimate ATGAM exposure as detailed in Supplementary methods, Tables S2-3 and Figure S4. Downstream exposure-response analyses used clinical data collected until day 180, because IST response is assessed 3-6 months post-ATGAM<sup>2</sup>. As a measure for hematologic recovery, we evaluated peripheral blood neutrophil and reticulocyte counts from routine measurements, generally taken at least weekly. Thrombocyte and hemoglobin levels were not analyzed to avoid potential bias caused by blood transfusions. To study lymphodepletion and immune reconstitution, we analyzed peripheral blood lymphocyte counts from routine measurements, typically collected once every two weeks. To correct for missing immune reconstitution data, non-linear mixed effect modeling was performed using SAEMIX<sup>19</sup>, see Supplementary methods and Figure S5. Data visualization and statistical analyses were performed in R v4.4.1 (Rstudio v2023.03) using Mann-Whitney *U* (continuous variables), Fisher's Exact (categorical variables), and Spearman's correlation tests. P-values  $\leq 0.05$  were considered significant.



## Results

### Active ATGAM binds all major lymphoid lineages

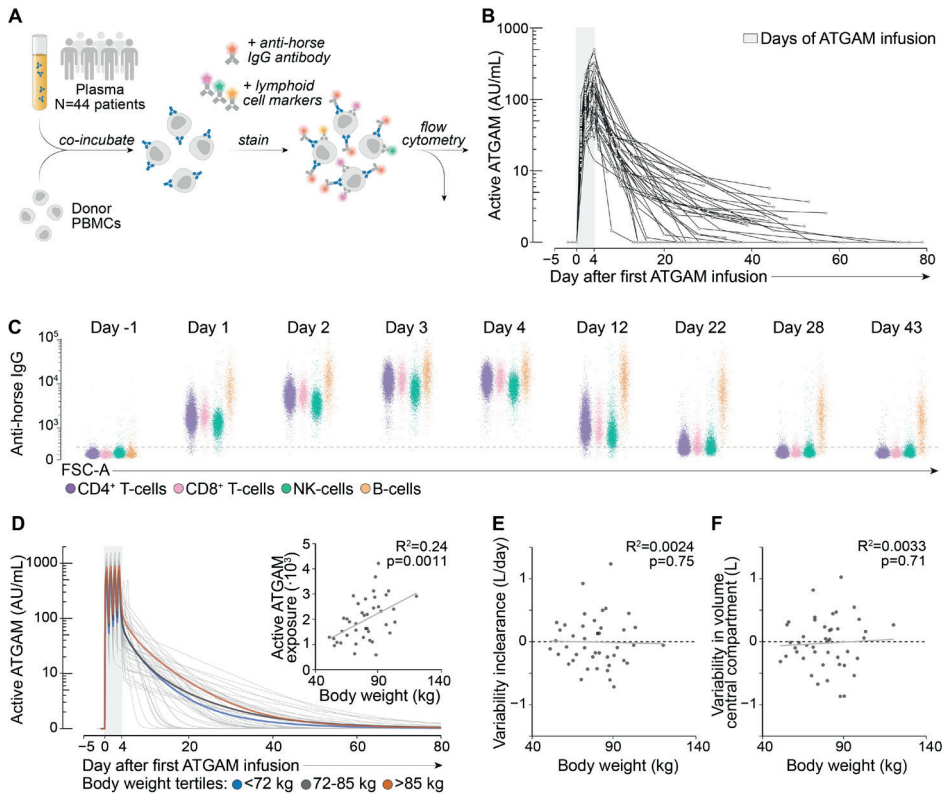
To study the effects of ATGAM, the concentration of ATGAM capable of binding PBMCs from an unrelated healthy donor (active ATGAM hereafter) was quantified in plasma from 44 adult AA patients using flow cytometry (Figure 1A). In patients that received the standard regimen of four consecutive days of 40 mg/kg/day ATGAM, active ATGAM concentrations increased over the four days of infusion and reached a median peak concentration of 131AU/mL on day 4 (Figure 1B). After the last infusion, concentrations rapidly decreased, and typically fell below the concentration of 10AU/mL by day 20. The pharmacokinetics of active ATGAM varied considerably between patients in both peak concentrations (range 31-497AU/mL on day 4) and clearance rates (Figure 1B). Similar variability was observed in total ATGAM concentrations (e.g. active ATGAM and ATGAM binding to other (non)-human targets combined) quantified in the same plasma samples using ELISA (Figure S6A). Active ATGAM in patient plasma was detected on donor T-, NK- and B-cells. However, pharmacokinetics differed as B-cell binding active ATGAM was detected longer than T- or NK-cell binding active ATGAM (Figure 1C; all patients in Figure S6B).

### Active ATGAM exposure is increased in AA patients with higher body weight

Given the variability in active ATGAM concentrations between patients, POPPK modeling was performed to estimate the full concentration-time curve of active ATGAM exposure. The POPPK of intravenously administered active ATGAM was best described by a two-compartment model with first-order linear elimination (structural model in Figure S7). The model showed adequate agreement between observed and model-estimated active ATGAM concentrations (Figure S8, individual patients in Figure S9). Exposure ( $AUC_{0-inf}$ ) was defined as the estimated area under the concentration-time curve (Table S4). Since this study is the first to report on active ATGAM, we also validated the accuracy of our approach by performing POPPK modeling for total ATGAM (Figure S10-11, Table S5) to simulate the dosing regimens described in the manufacturer's brochure<sup>20</sup>. This revealed strong agreement between model-estimated and reported total ATGAM concentrations for an external cohort of AA patients (Figure S12).

To identify factors driving between-patient variability in active ATGAM pharmacokinetics, the impact of clinical covariates was evaluated. No significant effects of sex, age and lymphocyte counts pre-ATGAM were observed (Figure S13). Since ATGAM is dosed based on body weight, we anticipated clearance to increase with body weight to obtain similar ATGAM exposures across patients. However, we found that patients with higher body weights had higher

active ATGAM concentrations over time, resulting in higher exposures (Figure 1D), as neither clearance nor volume of distribution were influenced by weight (Figure 1E-F).



**Figure 1. Active ATGAM binding specificities and concentration-time profiles in plasma from AA patients.**

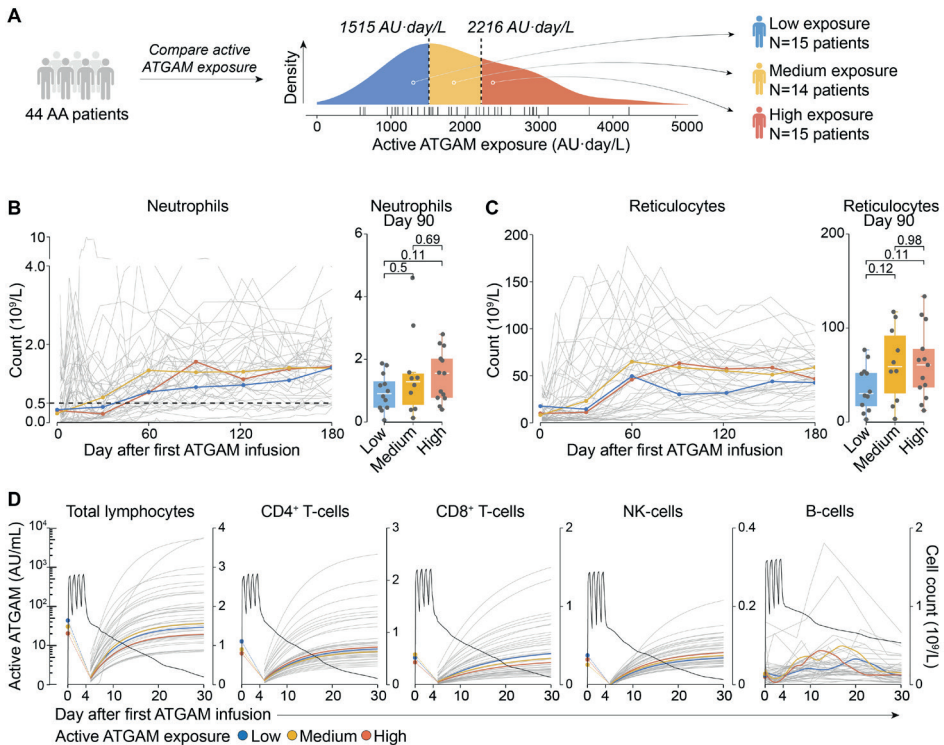
[A] Experimental approach. Donor PBMCs were incubated with plasma samples collected pre- and post-ATGAM, stained and analyzed by flow cytometry to determine the plasma levels of free active ATGAM capable of binding to human lymphocyte lineages. [B] Measured active ATGAM levels in patient plasma (in arbitrary units/mL;  $n=44$  patients) over time before, during and after ATGAM infusion on days 0-4. [C] Flow cytometric plots demonstrating the binding of active ATGAM in plasma from a representative patient (UPN33) to CD4<sup>+</sup> T-cells, CD8<sup>+</sup> T-cells, NK-cells or B-cells from a healthy donor, measured using an Alexa Fluor 488-conjugated anti-horse IgG antibody. [D] Model-estimated active ATGAM plasma concentrations over time before, during and after ATGAM infusion on days 0-4. Mean active ATGAM concentrations are presented for patients within the lowest (blue), medium (yellow) and highest (red) body weight quantiles, who received 4 consecutive days 40mg/kg/day ATGAM. Insert: Correlation between model-estimated active ATGAM exposure and body weight. [E-F] Covariate plots showing the relationship between body weight and the inter-individual variability in estimated active ATGAM clearance (E) or volume of distribution (F). Each dot represents one patient. Only patients that received the standard dosing regimen of 4 consecutive days 40mg/kg/day ATGAM are shown.

**Higher active ATGAM exposure associates with earlier hematologic recovery**

To investigate the effect of active ATGAM exposure on hematological recovery, patients were categorized into low, medium and high exposure groups based on the exposure tertiles of 1515 and 2216AU·day/L (Figure 2A). Neutrophil and reticulocyte counts served as a measure of bone marrow recovery. Two patients in the low-exposure group, one patient in the medium-exposure group and one in the high-exposure group were censored before day 180 due to death. Age, sex, disease severity, eltrombopag use and ciclosporin discontinuation did not significantly differ between the groups, and none of the patients started second-line treatment before day 180 (Table S6). In all three exposure groups, neutrophil and reticulocyte counts gradually increased post-ATGAM (Figure 2B-C). Reticulocyte counts peaked in a few patients within the first 120 days post-ATGAM. Notably, the high- and medium-exposure groups tended to have higher neutrophil and reticulocyte counts from day 60 onward, despite similar baseline counts compared to the low-exposure group (Figure 2B-C). This difference was most pronounced around day 90 (Figure 2B-C, box plots) and diminished 180 days post-ATGAM infusion, suggesting that increased active ATGAM exposure associates with earlier hematologic recovery but does not impact six-month response rates.

**ATGAM induces profound depletion of T- and NK-cells but not of B-cells**

As we showed that ATGAM binds all major lymphoid lineages, we studied the response of active ATGAM exposure on T-, NK- and B-cell counts in peripheral blood. To correct for missing immune reconstitution data, total lymphocyte, T-cell and NK-cell counts were estimated by non-linear mixed modeling. ATGAM induced a strong reduction of CD4<sup>+</sup> T-cells, CD8<sup>+</sup> T-cells and NK-cells, with no differences between the active ATGAM exposure groups (Figure 2D). Depletion of these lineages was short-lived and only observed immediately post-infusion, suggesting that T- and NK-cell depletion requires a minimal active ATGAM plasma concentration ( $\pm$ 100AU/mL, estimated from Figure 2D). Strikingly, B-cell counts did not show a consistent trend between patients post-ATGAM and therefore could not be modelled. Interestingly, B-cell counts even peaked post-ATGAM before returning to baseline around day 30, indicating that although ATGAM binds all lymphoid lineages, it exerts differential effects among cell types.



**Figure 2. Impact of active ATGAM exposure on peripheral blood counts.** [A] Histogram showing the distribution of active ATGAM exposure across the patient cohort. [B-C] Absolute neutrophil (B) and reticulocyte (C) counts over time up to 180 days after ATGAM infusion on days 0-4. Light gray lines present individual patients. Blue, yellow and red lines present group medians of low, medium and high active ATGAM exposure groups, on days 0, 30, 60, 91, 122, 152 and 180  $\pm$  7 days, respectively. Cell counts were unavailable for 2 (day 30), 5 (day 60), 9 (day 91), 9 (day 122), 7 (day 152) and 9 (day 180) patients divided across all exposure groups. Box plots represent the group median, whiskers represent 1.5 \* interquartile range. [D] Median model-estimated active ATGAM concentrations capable of binding to all lymphocytes, CD4<sup>+</sup> T-cells, CD8<sup>+</sup> T-cells, NK-cells or B-cell plotted against absolute total lymphocyte, CD4<sup>+</sup> T-cell, CD8<sup>+</sup> T-cell, NK-cell or B-cell counts, respectively. Light gray lines present cell counts of individual patients. Blue, yellow and red lines present median cell counts of low, medium and high active ATGAM exposure groups, respectively. Model-estimated lymphocyte, CD4<sup>+</sup> T-cell, CD8<sup>+</sup> T-cell, NK-cell counts are presented, whereas observed B-cell counts are shown.

### Detection of ATGAM on patient lymphocytes confirms B-cell binding *in vivo*

To study the paradox that ATGAM binds donor B-cells but does not induce B-cell depletion, we investigated the presence of ATGAM directly bound to patient PBMCs. Unique series of PBMC samples collected from three AA patients before and immediately after ATGAM infusion were studied. A 25-marker spectral flow cytometry panel including anti-horse IgG enabled the detection of ATGAM on the surface of in-depth phenotyped lymphocytes. To study the long-term effects of ATGAM plus ciclosporin, samples collected up to 3.5 years post-ATGAM were also analyzed (total of 7-10 samples/patient). Patients received 40 mg/kg/day ATGAM on four

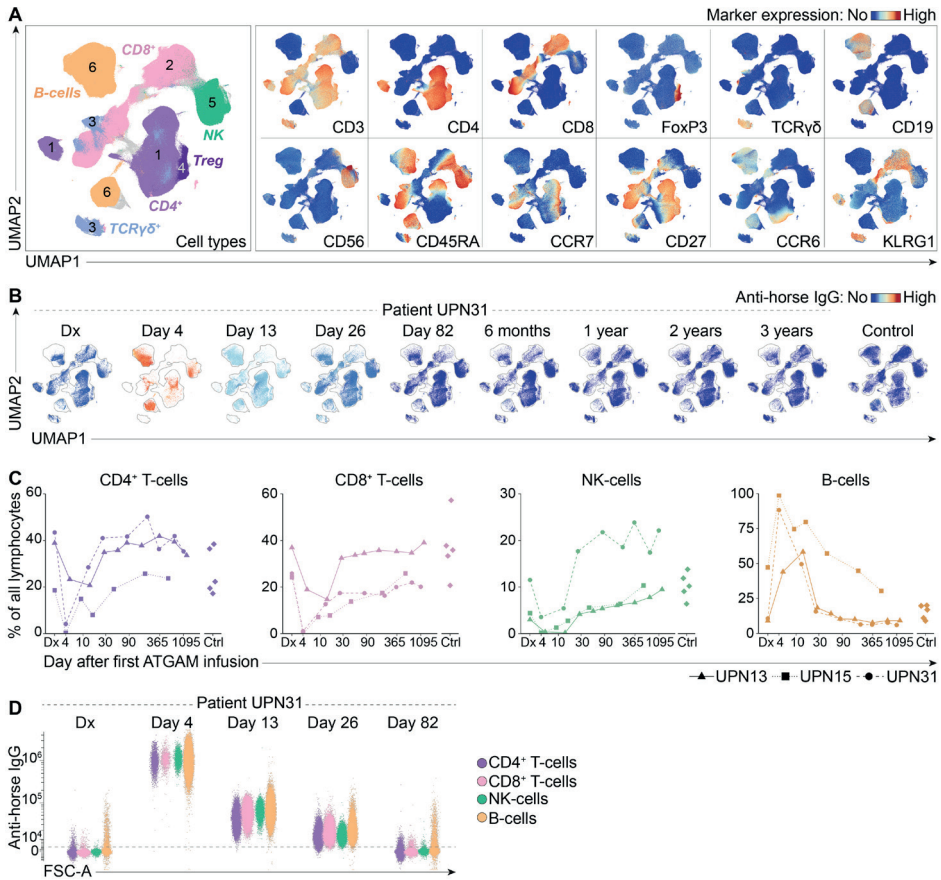


consecutive days, responded to IST (peripheral blood counts in Figure S14) and were CMV seropositive. Ciclosporin was discontinued after tapering two years post-ATGAM in patient UPN31, and three years post-ATGAM in UPN13 and UPN15. As a reference, PBMCs from five age-, sex- and CMV status-matched healthy donors were included.

To directly compare the immune landscape between time points, UMAPs were generated after downsampling  $0.1 \cdot 10^6$  single, live lymphocytes from each sample (Figure 3A). The following major lymphoid lineages were identified: 1) CD3<sup>+</sup>CD4<sup>+</sup> T-cells; 2) CD3<sup>+</sup>CD8<sup>+</sup> T-cells; 3) CD3<sup>+</sup>TCR $\gamma\delta$ <sup>+</sup> T-cells; 4) CD3<sup>+</sup>CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T-cells; 5) CD3<sup>+</sup>CD56<sup>+</sup> NK-cells and 6) CD19<sup>+</sup> B-cells. Visualization of all samples from a representative patient in the UMAP revealed a shift in the lymphoid compartment over time, with the most obvious changes observed immediately after the last ATGAM infusion on day 4, when T-cells and NK-cells were reduced but B-cells were still present (Figure 3B).

Quantification of lineage frequencies confirmed the presence of the major lymphoid lineages at AA diagnosis, with some heterogeneity between patients and no considerable differences compared to controls (Figure 3C, TCR $\gamma\delta$ <sup>+</sup> and regulatory T-cells in Figure S15A). Immediately after the last ATGAM infusion (day 4/5) the composition of the lymphoid compartment changed dramatically. In agreement with previous observations, percentages of CD4<sup>+</sup>, CD8<sup>+</sup>, TCR $\gamma\delta$ <sup>+</sup> and regulatory T-cells and NK-cells decreased in all patients, whereas B-cell frequencies increased. A trend towards normalization was observed from two weeks post-ATGAM onwards. Normalization of the lymphoid compartment continued up to six months post-ATGAM.

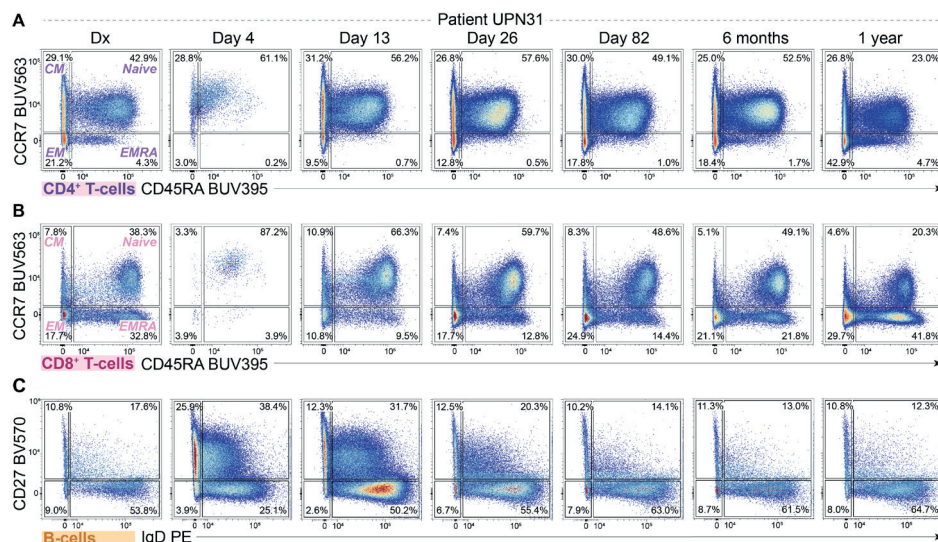
With anti-horse IgG, ATGAM was best detectable on day 4, and could be detected until day 26 on all circulating lymphocytes from the representative patient post-infusion (Figure 3B,D). The amount of ATGAM bound to T-cells or B-cells did not differ (Figure S15B). To rule out a potential influence of ATGAM Fc-binding to the Fc-receptor on B-cells, Fc-receptor blockade experiments were performed and no Fc-binding was found (Figure S16). ATGAM binding to patient lymphocytes gradually decreased within one month post-infusion. Lower concentrations of ATGAM again correlated with T- and NK-cell recovery from two weeks post-infusion onwards. These findings confirm that while ATGAM binds to all lymphoid lineages, it does not induce B-cell depletion to the same extent as observed in T- and NK-cells.



**Figure 3. Binding of ATGAM and the impact of ATGAM-based IST on lymphocytes from AA patients.** [A]  $3 \cdot 10^6$  single, live lymphocytes downsampled from all PBMC samples (UPN13, UPN15, UPN31 and five controls) visualized using a UMAP analysis. Colors indicate major lymphoid lineages or marker expression values. [B] UMAP plots showing the cellular composition of the lymphoid compartment in peripheral blood from UPN31 before and over time following ATGAM infusion on days 0-4. The signal of the Alexa Fluor 488-conjugated anti-horse IgG antibody is shown.  $0.1 \cdot 10^6$  single, live lymphocytes are shown per time point. [C] Frequencies of major lymphoid lineages, shown as a percentage of all single, live lymphocytes per time point. Each line represents an individual patient. [D] Flow cytometry plots visualizing the presence of ATGAM directly bound to T; NK- or B-cells from UPN31 over time, detected using an Alexa Fluor 488-conjugated anti-horse IgG antibody incorporated into the spectral flow cytometry panel.

### ATGAM profoundly depletes memory T-cells but induces an increase of CD27<sup>+</sup> B-cells

Next, we studied the immediate effects of ATGAM and long-term impact of ATGAM plus ciclosporin at the immune subpopulation level. In the representative patient, we observed a more profound decrease of memory T-cells compared to naïve T-cells post-ATGAM, with the strongest depletion of CD45RA<sup>+</sup>CCR7<sup>+</sup> terminally differentiated effector memory (EMRA) CD8<sup>+</sup> T-cells (Figure 4A-B, quantification: Figure S15C-D). In contrast, we observed a striking increase of memory CD27<sup>+</sup> B-cells immediately post-ATGAM (Figure 4C, S15E).

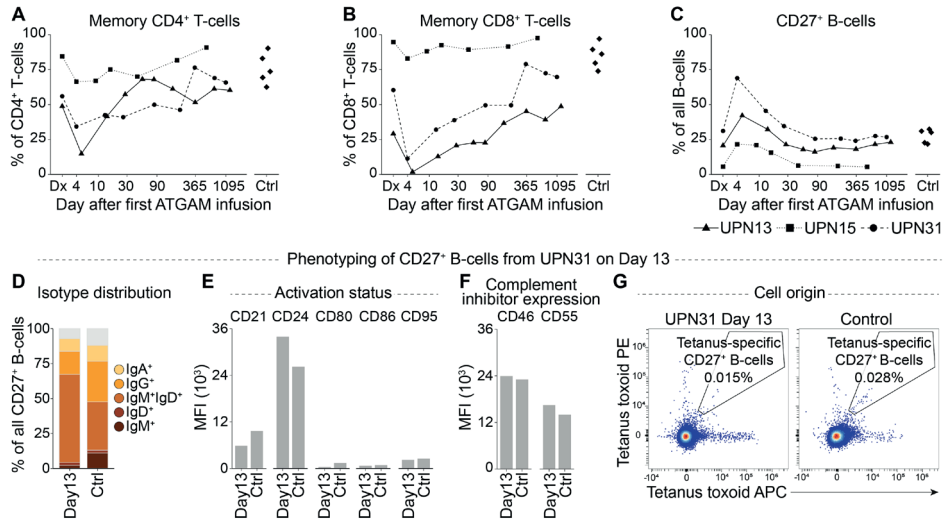


**Figure 4. Impact of ATGAM-based IST at the immune subpopulation level in a representative AA patient.** [A-C] Flow cytometry plots visualizing the composition of the [A] CD4<sup>+</sup> T-cell compartment, [B] CD8<sup>+</sup> T-cell compartment and [C] B-cell compartment from patient UPN31 before and over time following the start of ATGAM-based IST on day 0. CM: central memory, EM: effector memory, EMRA: terminally differentiated effector memory.

The profound depletion of memory T-cells and increase of memory CD27<sup>+</sup> B-cells was observable across all patients post-ATGAM (Figure 5A-C). Only in patient UPN15 memory T-cell frequencies remained high, which was attributable to the low number of analyzed T-cells, as this patient experienced the strongest T-cell depletion (Figure 3C). Within two weeks post-ATGAM, both memory T-cells and CD27<sup>+</sup> B-cells frequencies showed a trend towards normalization (Figure 5A-C). Notably, this normalization occurred independently of T-cell and B-cell proliferation, indicating that recovery was not driven by proliferation of remaining circulating cells (Figure S17).

To further characterize the increase of CD27<sup>+</sup> B-cells, we examined the identity of these cells in patient UPN31 on day 13 using a B-cell-oriented spectral flow cytometry panel. This revealed that the CD27<sup>+</sup> B-cells comprised of a heterogeneous population of both unswitched (IgM<sup>+</sup>IgD<sup>+</sup>) and switched (IgG<sup>+</sup>, IgA<sup>+</sup>) memory B-cells (Figure 5D). Compared to healthy controls, these cells preserved high levels of CD21 and CD24, compatible with a resting B-cell state (Figure 5E). Additionally, they did not differentially express the activation markers CD80, CD86 and CD95, and complement inhibitors CD46 and CD55 (Figure 5E-F), illustrating that the increase of CD27<sup>+</sup> B-cells is not the result of cell expansion or resistance to complement-mediated lysis. Consistent with these findings, the CD27<sup>+</sup> B-cells harbored

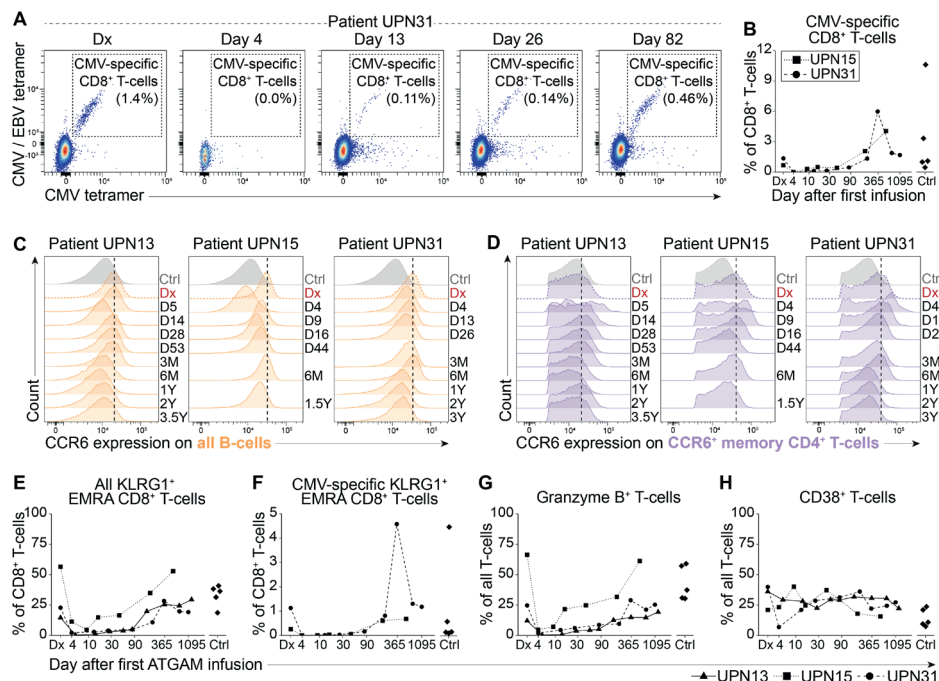
tetanus-specific cells, indicating their increase was driven by a recruitment of resting, pre-existing memory B-cells in circulation (Figure 5G).



**Figure 5. Dynamics of memory T-cells and CD27<sup>+</sup> B-cells, and identity of CD27<sup>+</sup> B-cells, post-ATGAM.** [A-C] Frequencies of memory CD4<sup>+</sup> T-cells, memory CD8<sup>+</sup> T-cells or CD27<sup>+</sup> B-cells over time, quantified in all three AA patients and controls. Memory T-cells include central memory, effector memory and terminally differentiated effector memory T-cells. [D] Isotype distribution of the CD27<sup>+</sup> B-cells from UPN31 on day 13 after the first ATGAM infusion, and the mean of two age-matched controls. [E] Expression of CD21, CD24, CD80, CD86 and CD95 on CD27<sup>+</sup> B-cells from patient UPN31 on day 13, and the mean of two age-matched controls. Median fluorescence intensities are shown. [F] Expression of complement inhibitors CD46 and CD55 on CD27<sup>+</sup> B-cells from patient UPN31 on day 13, and the mean of two age-matched healthy controls. Median fluorescence intensities are shown. [G] Flow cytometry plots showing the presence of tetanus toxoid-specific CD27<sup>+</sup> B-cells in patient UPN31 on day 13, and an age-matched healthy control. 0.1·10<sup>6</sup> CD27<sup>+</sup> B-cells are shown per plot.

### ATGAM-based IST depletes potentially pathogenic subpopulations while allowing the recovery of virus-specific lymphocytes

The rapid recovery of the T-cell compartment in the absence of proliferation, along with the persistence of pre-existing memory B-cells post-ATGAM, raises the question of how profoundly ATGAM depletes the T-cell compartment. Therefore, we investigated the origin of reconstituted T-cells by tracking the presence of CMV-specific CD8<sup>+</sup> T-cells using CMV peptide-HLA tetramers incorporated into our spectral flow cytometry panel. In one of three patients (UPN13), these analyses were not possible due to HLA-incompatibilities between the patient and the tetramers. In the representative patient (UPN31), we detected CMV-specific CD8<sup>+</sup> T-cells at diagnosis, which were no longer detectable immediately following ATGAM infusion, consistent with the observed T-cell depletion (day 4; Figure 6A). However, CMV-specific CD8<sup>+</sup> T-cells were detectable from day 13 onwards, and a similar trend was observed in UPN15 (Figure 6B).



**Figure 6. Effects of ATGAM on pre-existing and potentially pathogenic subpopulations.** [A] Flow cytometry plots showing the presence of CMV-specific CD8<sup>+</sup> T-cells within the CD8<sup>+</sup> T-cell compartment at diagnosis and after the first ATGAM infusion in patient UPN31. [B] Frequencies of CMV-specific CD8<sup>+</sup> T-cells, quantified as a frequency of all CD8<sup>+</sup> T-cells in patients UPN15 and UPN31, and all controls. [C-D] Histograms showing CCR6 expression on all CD19<sup>+</sup> B-cells (C) or CCR6<sup>+</sup> memory CD4<sup>+</sup> T-cells (D) in the three AA patients over time following ATGAM infusion and ciclosporin treatment. [E-H] Frequencies of all KLRG1<sup>+</sup> EMRA CD8<sup>+</sup> T-cells, CMV-specific KLRG1<sup>+</sup> EMRA CD8<sup>+</sup> T-cells, Granzyme B<sup>+</sup> cytotoxic T-cells and CD38<sup>+</sup> activated T-cells in all three AA patients over time following ATGAM infusion.

Given that our results indicate the persistence of pre-existing T- and B-cells post-ATGAM, we reasoned that the therapeutic effect of IST, comprising ATGAM and long-term ciclosporin, may not depend on a full immune 'reset'. Instead, it may rely on the selective depletion and suppression of pro-inflammatory CCR6<sup>+</sup> B-cells, CCR6<sup>+</sup> memory CD4<sup>+</sup> T-cells and KLRG1<sup>+</sup> EMRA CD8<sup>+</sup> T-cells, which we previously identified as part of a disease-specific immune cell network<sup>14</sup>. These subpopulations were enriched in AA patients at diagnosis, and co-localized in bone marrow regions harboring the active immune response<sup>14,15</sup>. In all three patients, we confirmed higher frequencies of CCR6<sup>+</sup> B-cells at diagnosis compared to controls (Figure 6C). CCR6 expression on B-cells remained high immediately post-ATGAM but gradually decreased with long-term ciclosporin use, with the strongest decrease in CCR6 expression >6 months post-ATGAM. CCR6 expression remained reduced after ciclosporin was discontinued (≥3 years post-ATGAM). A similar decrease of CCR6 expression on CCR6<sup>+</sup> memory CD4<sup>+</sup> T-cells was observed (Figure 6D). In contrast, KLRG1<sup>+</sup> EMRA CD8<sup>+</sup> T-cells were strongly depleted



immediately post-ATGAM (Figure 6E), and their recovery was delayed compared to other CD8<sup>+</sup> T-cells (Figure 3C). Further analysis showed that only a small fraction of these KLRG1<sup>+</sup> EMRA CD8<sup>+</sup> T-cells were CMV-specific, suggesting depletion also affected potentially autoreactive T-cells within this population (Figure 6F). Over time, KLRG1<sup>+</sup> EMRA CD8<sup>+</sup> T-cell frequencies returned to pre-ATGAM levels, indicating a temporary rather than a permanent depletion. A depletive effect was also observed for Granzyme B<sup>+</sup> cytotoxic T-cells (Figure 6G), while expression of the activation marker CD38 remained unaffected by ATGAM plus ciclosporin (Figure 6H). These findings underscore that IST results in an immediate but selective depletion of key T-cell populations by ATGAM, while other potentially pathogenic populations are reduced over several months, possibly through selective suppression by ciclosporin.

## Discussion

In this study, we investigated the pharmacodynamics of first-line IST with ATGAM plus long-term ciclosporin in AA patients. Our analysis is the first to focus on active ATGAM, the fraction capable of binding human lymphocytes and mediating the immunomodulatory effects that are believed to underlie its therapeutic efficacy. POPPK modeling revealed that active ATGAM exposure varies considerably among patients, and tends to correlate with hematologic recovery. At high plasma concentrations, ATGAM binds T- and NK-cells and drastically reduces their counts *in vivo*. Paradoxically, while ATGAM also binds B-cells, it does not induce their depletion but rather results in an increase of resting memory CD27<sup>+</sup> B-cells in circulation. Following ATGAM infusion, immune reconstitution occurs rapidly within two weeks, and is marked by the recovery of pre-existing cells alongside a selective suppression of specific pro-inflammatory subpopulations over time. Thus, the therapeutic effect of ATGAM-based IST goes beyond 'simple' lymphodepletion.

To date, ATGAM is administered at a fixed dose per kilogram bodyweight, although its optimal dose remains unclear since its exposure-response profile has not been characterized in detail<sup>2,21,22</sup>. This study provides unique insight into ATGAM pharmacokinetics and offers important considerations regarding dosing. Consistent with data on rabbit-derived ATG<sup>23,24</sup>, we demonstrate that linear weight-based dosing causes variability in active ATGAM exposure between patients. In line with a previous report<sup>25</sup> that suggested a benefit of higher ATGAM exposures on relapse rates, we found that higher active ATGAM exposures tend to associate with increased neutrophil and reticulocyte counts starting 60 days post-ATGAM infusion. Although these associations did not reach statistical significance, the observed trend could be suggestive of a possible relationship between active ATGAM exposure and the timing of hematologic recovery. These findings may complement previous studies that identified patient- and disease-specific characteristics predicting response to IST, suggesting that

hematologic recovery may be controlled by multiple factors<sup>26-32</sup>. The biological mechanism by which active ATGAM exposure may influence hematologic recovery remains unclear. In our study, active ATGAM exposure was not significantly associated with the extent or duration of T- or NK-cell depletion. Although we did not assess ciclosporin levels, their impact seems limited, given that ciclosporin dosing was concentration-controlled to a pre-specified target<sup>2</sup>. Interestingly, it has been reported that ATGAM can stimulate progenitor cells<sup>33</sup> and the release of factors regulating hematopoiesis<sup>13,34</sup>. Thus, higher ATGAM exposures could have a favorable effect through a stronger influence on the progenitors in the bone marrow.

By applying flow cytometry, we provide a detailed description of both the short- and long-term immunological effects of ATGAM plus ciclosporin in AA patients. Although it is known that ATGAM results in less profound lymphodepletion compared to other ATG preparations<sup>4</sup>, our findings show that immune reconstitution occurs early post-ATGAM while active ATGAM concentrations are still detectable. This indicates that a minimal level should be exceeded for adequate lymphodepletion, and that a better understanding of the exposure-response relationship of ATGAM is needed to optimize treatment across patients.

Importantly, our analyses revealed how successful IST may reshape the immune landscape to enable bone marrow recovery in AA patients. Our study therefore builds upon recent reports demonstrating the added value of high-dimensional single-cell techniques in deciphering the pathogenesis of AA<sup>14,15,35,36</sup>, and complements a comprehensive review of their application in AA<sup>37</sup>. Previously, using (imaging) mass cytometry on bone marrow samples from AA patients, we identified a disease-specific immune cell network of CCR6<sup>+</sup> B-cells, Th17-like<sup>32,38,39</sup> CCR6<sup>+</sup> memory CD4<sup>+</sup> T-cells and KLRG1<sup>+</sup> EMRA CD8<sup>+</sup> T-cells<sup>14,15</sup>. These immune cells were significantly enriched in AA patients at diagnosis compared to healthy donors, and showed a trend towards normalization six months after successful IST, implying a pathogenic role. In this study, we extend these findings by confirming their increased presence at diagnosis. Importantly, we further show how these cells are reduced with IST through an analysis of unique series of PBMC samples collected pre- and up to 3.5 years post-ATGAM. Our study thereby provides a more dynamic visualization of the reshaping of the immune landscape compared to previous reports that generally focused on samples collected pre- and six months post-ATGAM<sup>14,15,36,39</sup>. Although the analysis included only three patients, it allowed for a deeper investigation beyond the major immune lineages assessed in the full cohort of 44 AA patients, and sets the stage for more widely applied longitudinal analyses in future studies. We demonstrate that successful IST depletes potentially pathogenic populations via two distinct mechanisms: KLRG1<sup>+</sup> EMRA CD8<sup>+</sup> and CCR6<sup>+</sup> memory CD4<sup>+</sup> T-cells are depleted by ATGAM. In contrast, CCR6<sup>+</sup> B-cells and residual T-cells, including CCR6<sup>+</sup> memory CD4<sup>+</sup> T-cells, gradually decrease within several months during long-term ciclosporin use. These data indicate the persistence

of potentially pathogenic immune cell subpopulations post-ATGAM, and could explain why long-term ciclosporin is needed to achieve durable response rates.

Based on prior studies<sup>4,13</sup>, we expected ATGAM to bind T-cells and to cause rapid but short-lived T-cell depletion. Our data confirm this effect and further reveal important differences among T-cell subpopulations. Specifically, memory T-cells were more profoundly depleted than naïve T-cells, suggesting that ATGAM targets cytotoxic T-cells, while preserving part of the antigen-naïve T-cell repertoire that provides protection against future pathogen encounters. This effect is more pronounced among CD8<sup>+</sup> T-cells compared to CD4<sup>+</sup> T-cells, and remains detectable up to six months post-ATGAM, likely due to the inhibition of T-cell proliferation and differentiation by ciclosporin<sup>40</sup>. During this period, we observed reconstitution of CMV-specific CD8<sup>+</sup> T-cells, implying that ATGAM does not result in a full immune 'reset'. Although the recovery of pre-existing cells has been described with other ATG preparations<sup>41</sup>, it raises questions on how successful IST eliminates potential autoreactive T-cells while preserving protective immune memory. We consider three possible explanations. First, ATGAM may exert a stronger effect on autoreactive T-cells compared to other memory T-cells, possibly due to differences in cellular state. Similarly, it is possible that, in the absence of infections, remaining autoreactive T-cells post-ATGAM may be more susceptible to ciclosporin compared to pathogenic-specific T-cells due to continued antigenic triggering. Finally, it could be that ATGAM depletes memory T-cells in the blood and bone marrow, while not affecting tissue-resident T-cells. This would suggest that autoreactive T-cells are limited to the blood and bone marrow, and that targeting these compartments alone could suffice to enable bone marrow recovery.

Since ATGAM has strong effects on T-cells, it is noteworthy that our data confirm that ATGAM does not have the same effect on B-cells<sup>13</sup>. Instead of depletion, we report a shift in the B-cell compartment, characterized by an increase of memory CD27<sup>+</sup> B-cells immediately post-ATGAM infusion, which gradually normalizes within one month. These CD27<sup>+</sup> B-cells comprised a heterogeneous population of both non-switched and switched memory B-cells in a resting state. The CD27<sup>+</sup> B-cell population included pre-existing pathogen-specific cells as evidenced by the presence of tetanus toxoid-specific B-cells and was not proliferating. Therefore, we believe that the observed increase of CD27<sup>+</sup> B-cells may reflect redistribution of memory B-cells from the lymphoid organs into the circulation. This could be driven by an imbalance of chemotactic factors released following T-cell apoptosis. How B-cells survive ATGAM binding to their cell surface while T-cells are depleted should be clarified by future studies. Our data revealed no increased resistance of B-cells to complement-mediated lysis, nor an influence of Fc-binding. Given that ATGAM is a polyclonal antibody preparation generated against thymocytes, it is possible that ATGAM induces T-cell apoptosis by triggering T-cell activation (e.g. via anti-CD3 or TCR binding), without activating apoptotic pathways in B-cells.

To conclude, we unveil active ATGAM exposure as a clinically relevant factor influencing hematologic recovery in AA. We further demonstrate that successful IST with ATGAM plus long-term ciclosporin exerts differential effects among major lymphoid lineages, leading to selective depletion and suppression of CCR6<sup>+</sup> and KLRG1<sup>+</sup> T- and B-cell subpopulations implicated in AA pathogenesis. These findings confirm their potential pathogenicity, and establish these subpopulations as promising targets for targeted treatment.

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### **Authorship contributions**

E.S.P., C.R.P., S.M.G., E.H.M.v.E., J.M.G. and S.V. performed laboratory experiments. E.S.P. and D.J.A.R.M. performed pharmacokinetic modelling. E.S.P. analyzed the data. E.S.P., E.H.M.v.E., J.M.G., R.E.M.T., F.K., C.J.M.H., M.H.M.H., D.J.A.R.M. and J.M-L.T. interpreted the data. E.S.P. wrote the first draft of the manuscript. C.J.M.H. and J.M-L.T. recruited patients and collected samples. D.J.A.R.M. and J.M-L.T. conceptualized the study. M.H.M.H., D.J.A.R.M. and J.M-L.T. supervised the project. All authors critically reviewed the manuscript.

### **Conflicts of interest**

The authors declare no competing financial interests.

## Supplementary methods

### Quantification of total ATGAM concentrations

Total ATGAM was defined as all horse IgG antibodies in the ATGAM preparation<sup>42</sup>. Total ATGAM concentrations in patient plasma were quantified by sandwich ELISA. 96-well plates were coated overnight at 4°C with goat anti-horse IgG antibodies at a dilution of 1:2500 in PBS (Jackson ImmunoResearch, cat 108-005-003). Next, plates were blocked, washed using PBS with 0.005% Tween20, and incubated with diluted patient plasma samples for two hours at room temperature. Each sample was analyzed across eight serial dilutions. Subsequently, plates were washed five times using PBS with 0.005% Tween20 and incubated with a peroxidase-conjugated secondary goat anti-horse antibody at a dilution of 1:25,000. After a one hour incubation at room temperature, plates were washed five times using PBS with 0.005% Tween20 and stained with TMB substrate buffer for five minutes in the dark. Finally, staining was stopped and plates were measured at 450nm on a microplate photometer (ThermoFisher Multiskan FC). Total ATGAM concentrations in µg/mL in each plasma sample were calculated using the linear range of a standard curve, which was created by including serial dilutions of known ATGAM concentrations in each experiment.

### Quantification of active ATGAM concentrations

Active ATGAM was defined as the fraction of ATGAM that is capable of binding to human lymphocytes. To quantify active ATGAM concentrations in plasma, flow cytometry was performed based on a validated protocol for the quantification of alemtuzumab levels in patient plasma<sup>17</sup>, which since has been optimized for the measurement of rabbit-derived anti-thymocyte globulin plasma concentrations<sup>43</sup>. Recent work shows this assay resulted in high-quality pharmacokinetic data for a cohort of 121 acute lymphoblastic leukemia patients who received rabbit-derived anti-thymocyte globulin as part of the conditioning regimen pre-HSCT<sup>24</sup>. In our study, first, cryopreserved PBMCs isolated from a healthy donor were thawed and plated in 96-well plates. PBMCs from the same donor were used in all experiments to enable comparisons between experiments. Subsequently, cells were incubated with non-diluted and 3x, 9x, and 27x diluted patient plasma for 30 minutes at 4°C. Afterwards, cells were washed twice in PBS and incubated with an AlexaFluor 488-conjugated goat anti-horse antibody diluted at 1:100 in a volume of 10µL for 30 minutes at 4°C (Jackson ImmunoResearch, cat 108-545-003). Next, cells were washed twice in PBS and stained with a panel of six fluorochrome-conjugated antibodies in a volume of 10µL to distinguish all major immune lineages. The following antibodies were used: APC-H7 anti-human CD3 antibody diluted at 1:60 (BD Biosciences, cat 641406); PE-Cy7 anti-human CD4 antibody diluted at 1:3000 (Beckman Coulter, cat 6607101); Pacific Blue anti-human CD8 antibody diluted



at 1:2000 (BioLegend, cat 301026); PerCP anti-human CD14 antibody diluted at 1:50 (BD Biosciences, cat 563372); APC anti-human CD19 antibody diluted at 1:50 (BD Biosciences, cat 555415) and BV510 anti-human CD56 antibody at 1:50 (BD Biosciences, cat 563041). After a 30 minute incubation at 4°C, cells were washed and measured immediately on the BD LSRFortessa™ cell analyzer (BD Biosciences). Data were analyzed in FlowJo (BD Biosciences) or OMIQ (Dotmatics). The gating strategy presented in Figure S1 determined the geometric mean fluorescence intensity of AlexaFluor 488-stained total lymphocytes, CD4<sup>+</sup> T-cells, CD8<sup>+</sup> T-cells, B-cells, NK-cells and monocytes in each acquired sample. Concentrations of ATGAM capable of binding to all lymphocytes (active ATGAM), CD4<sup>+</sup> T-cells (CD4<sup>+</sup> T-cell-binding active ATGAM), CD8<sup>+</sup> T-cells (CD8<sup>+</sup> T-cell-binding active ATGAM), CD19<sup>+</sup> B-cells (B-cell-binding active ATGAM) and CD56<sup>+</sup> NK-cells (NK-cell-binding active ATGAM) were then calculated using the mean of two reference curves, which were created by exposing the PBMCs to known concentrations of ATGAM in each experiment. To ensure consistency between experiments, the reference curves were aliquoted and stored at -20°C to enable their use across experiments. Active ATGAM concentrations were calculated in arbitrary units (AU), and the concentration of 1mg/mL ATGAM was arbitrarily set as 1000 AU/mL. The lower limit of quantification (LLoQ) of the assay was 0.02 AU/mL. At high ATGAM concentrations of >200 AU/mL in patient plasma, accurate gating of NK-cells could not be performed. As a result, concentrations of NK-cell-binding active ATGAM were not determined on days 3 and/or 4 after the first ATGAM dose for a few patients. To confirm the robustness of the assay, we analyzed the duplicate measurement of undiluted patient plasma included in each experiment, and evaluated duplicate measurements of serial dilutions of ATGAM. This revealed strong correlations between technical duplicates ( $p < 0.001$ ), underscoring within-experiment reproducibility (Figure S2A). To confirm reproducibility between experiments, plasma samples collected from several AA patients pre- and post-ATGAM were measured in duplicate in independent experiments, which resulted in highly comparable results (Figure S2B).

### **Population pharmacokinetic modelling and estimation of ATGAM exposure**

To estimate the full concentration *versus* time profile of total and active ATGAM, non-linear mixed-effects modeling was performed using the acquired total or active ATGAM concentration-time data. Model development was performed in NONMEM version 7.4.4 (Icon Development Solutions, Ellicott City, MD). Pirana version 2.9.8 (Certara, Princeton, NJ) was used as a modeling platform, while R version 4.4.1 run in RStudio version 2023.03 was used for data visualization. Pharmacokinetic parameters were estimated using a first-order conditional estimation with interaction approach (FOCE+I). To identify the model with the best fit for the concentration-time data, both one- and two-compartment models,

incorporating linear, non-linear or parallel linear and non-linear elimination, were evaluated. Inter-individual variability (IIV) in parameter estimates was evaluated using an exponential error model, while residual unexplained variability was characterized by testing additive, proportional and combined error models. Inter-occasion variability was examined for all pharmacokinetic parameters to determine whether pharmacokinetics varied between different ATGAM infusions. A detailed overview of the steps taken during model development is shown in Tables S2 and S3.

To find the model that best described the concentration-time data, we compared each candidate model using the objective function value (OFV;  $-2 \log$  likelihood). A lower OFV indicated a better fit, and a model was considered to have a significantly improved fit if its OFV decreased by at least 6.63 ( $p \leq 0.01$ ; based on 1 degree of freedom, and assuming a chi-square distribution). Models were also assessed visually using goodness-of-fit (GOF) plots. These plots compared observed (measured) ATGAM concentrations against both individual predicted (IPRED) and population predicted (PRED) ATGAM concentrations to assess how well the model captured the measured concentration-time data. Other GOF plots compared conditional weighted residuals (CWRES) against time or PRED. The aim of these plots was to identify any systematic differences between the predicted and observed (measured) ATGAM concentrations. Other considerations in model selection included model pharmacokinetic parameter stability, inter-individual variability (IIV) and shrinkage, to ensure the model was well structured and not too complex for the available data.

To better understand the differences in the concentration-time profile of ATGAM between patients and to improve the predictive power of the model, we studied the influence of clinical covariates on the population pharmacokinetics of ATGAM. Potential covariates were selected based on physiological plausibility, and included age, sex and body weight. The effect of the selected covariates on ATGAM pharmacokinetics was initially evaluated visually, by examining how clearance (CL) or volume of distribution (V) were influenced by these covariates across all patients. Eta CL and V *versus* covariate plots were used. Given that ATGAM includes antibodies that can bind lymphocytes, we also assessed whether CL or V were influenced by differences in lymphocyte count pre-ATGAM between patients.

The final population pharmacokinetic models for total or active ATGAM were validated using prediction-corrected visual predictive checks (pcVPC)<sup>44</sup>. This approach relied on running the model 500 times to simulate new datasets, which were then compared to the observed (measured) ATGAM concentrations to confirm the performance of the model in predicting the ATGAM concentrations between patients. In addition, non-parametric bootstraps with 1000 simulations were performed to evaluate the precision of the final parameter estimates.

To estimate the full concentration *versus* time curve of the active ATGAM fractions capable of binding to CD4<sup>+</sup> T-cells, CD8<sup>+</sup> T-cells, B cells or NK cells, we ran the final population pharmacokinetic model (posthoc estimation) for active ATGAM using the concentration *versus* time data of each of these active ATGAM fractions.

To enable correlation analyses between ATGAM exposure and clinical parameters, we used the final population pharmacokinetic models that best described the concentration *versus* time data for total or active ATGAM to estimate individual pharmacokinetic parameters. These estimates were then used to calculate patients' cumulative exposure to total or active ATGAM, which was defined as the area under the concentration *versus* time curve ( $AUC_{0-\infty}$ ).

### Immune reconstitution data

To correct for missing immune reconstitution data, we performed non-linear mixed effect modeling using the SAEMIX package<sup>19</sup> in R version 4.4.1. We used exponential models to predict total lymphocyte, CD4<sup>+</sup> T-cell, CD8<sup>+</sup> T-cell and NK-cell counts on each day between days 4 and 40 after start of ATGAM infusion in each patient. These models considered both the general trend across all patients and inter-individual differences. Predictions were only made for patients who had at least one measurement available between days 4 and 40 following infusion (n=43 patients), and are presented in Figure S5. B-cell reconstitution was not modeled using SAEMIX since no consistent trends were observed in the B-cell count-time data shortly after ATGAM administration between patients.

### Spectral flow cytometry data acquisition and analysis

For deep immunophenotyping both before and at various time points after start of IST, spectral flow cytometry was performed. A 25-marker panel was designed to immunophenotype all major lymphoid lineages and to examine the binding of ATGAM to in-depth phenotyped immune cell subpopulations (Table S1). Cryopreserved PBMC samples from 3 AA patients, collected at AA diagnosis and up to 3.5 years after ATGAM infusion were studied (patients UPN13, UPN15 and UPN31; 7-10 samples per patient). All patients had very severe AA (VSAA), received four consecutive days 40 mg/kg/day ATGAM, responded to IST and were CMV seropositive at time of AA diagnosis. As a reference, cryopreserved PBMC samples from 5 age-, sex- and CMV status-matched healthy donors were studied. PBMC samples were thawed, plated into 96-well plates and rested at 37°C overnight. The next morning, cells were stained for viability with Zombie-NIR. Subsequently, cells were washed and stained with AlexaFluor 488-conjugated goat anti-horse IgG (Jackson ImmunoResearch) diluted at 1:100 in a volume of 10µL for 30 minutes at room temperature to detect ATGAM-bound cells. Next, cells were washed and stained for 7 of 25 markers of the panel extracellularly in a volume of 10µL.

This included BUV615-, PE- or APC-conjugated peptide-HLA tetramers, which were produced in-house and were added to evaluate the presence of CMV- and EBV-specific CD8<sup>+</sup> T-cells over time. After a 30 minute incubation at room temperature, cells were washed, fixed and stained intracellularly for the remaining 17 markers of the flow cytometry panel using the FoxP3/Transcription Factor Staining Buffer Set (Invitrogen; cat 00-5523-00) according to the manufacturer's instructions. As many markers as possible were stained intracellularly to avoid potential issues in detecting key cell surface markers, which could theoretically be masked for detection by the binding of ATGAM to these cell surface markers *in vivo*. Cells were then measured on the Aurora spectral flow cytometer (Cytek Biosciences). For each acquired flow cytometry data file, single, live lymphocytes were gated in OMIQ as shown in Figure S3. A median of  $0.3 \cdot 10^6$  (range  $0.02$ - $0.5 \cdot 10^6$ ) single, live lymphocytes were gated for each sample, resulting in a dataset of  $9.3 \cdot 10^6$  cells. To compare the cellular composition between samples,  $0.1 \cdot 10^6$  cells were selected from each sample by downsampling, and visualized by performing UMAP dimensionality reduction analyses in OMIQ based on 15 neighbors, a minimum distance of 0.3 and 400 epochs. Only for the day 5 sample from patient UPN13 less cells were available ( $0.02 \cdot 10^6$  cells), due lymphodepletion caused by ATGAM infusion. CD3, CD19 and anti-horse IgG stainings were not used generate the UMAP due to differences in marker expression intensities between samples. To distinguish all major lymphoid lineages, all single, live lymphocytes were clustered into 150 cell clusters using FlowSOM<sup>18</sup> in OMIQ, which were then merged into 6 clusters based on similarities in marker expression. Major lymphoid lineages were defined as follows: CD3<sup>+</sup>CD4<sup>+</sup> T-cells, CD3<sup>+</sup>CD8<sup>+</sup> T-cells, CD3<sup>+</sup>TCR $\gamma\delta$ <sup>+</sup> T-cells, FoxP3<sup>+</sup> regulatory T-cells, CD3<sup>+</sup>CD56<sup>+</sup> NK-cells and CD19<sup>+</sup> B-cells. Analyses at the subpopulation level were performed by manual gating in OMIQ. R version 4.4.1 ran in RStudio version 2023.03 was used for data visualization and analysis.

For detailed characterization of CD27<sup>+</sup> B-cells post-ATGAM, cryopreserved PBMCs collected from UPN31 on day 13 after the first ATGAM infusion were stained with a 16-marker spectral flow cytometry antibody panel directed against B-cell surface markers. This panel enabled analysis of B-cell isotype distribution, activation status and expression of complement inhibitors. The panel also incorporated PE- and APC-conjugated tetanus toxoid to allow for the detection of tetanus-specific B-cells. Cryopreserved PBMC samples from two sex- and age-matched healthy donors (also included in the larger spectral flow cytometry analysis) were included as a reference. Cells were fixed prior to acquisition on the Aurora spectral flow cytometer. Single, live B-cells were gated using the forward and side scatter, and by selecting CD19<sup>+</sup> B-cells while excluding all cells positive for the live dead stain.  $0.2 \cdot 10^6$  cells single, live B-cells were gated from the day 13 sample from UPN31, while  $0.5 \cdot 10^6$  and  $1.1 \cdot 10^6$  single, live B-cells were acquired for the controls.

## Supplementary tables

**Table S1.** Spectral flow cytometry panel for phenotyping of all lymphoid lineages.

| Marker              | Fluorochrome  | Clone    | Manufacturer              | Dilution | Extracellular /<br>intracellular staining |
|---------------------|---------------|----------|---------------------------|----------|-------------------------------------------|
| CD45RA              | BUV395        | HI100    | BD                        | 600      | Intracellular                             |
| CCR7                | BUV563        | 3D12     | BD                        | 100      | Extracellular                             |
| Tetramers CMV       | BUV615        | NA       | In-house                  | 75       | Extracellular                             |
| Tetramers EBV       | BUV615        | NA       | In-house                  | 75       | Extracellular                             |
| CD19                | BV421         | HIB19    | BioLegend                 | 160      | Intracellular                             |
| CD56                | BV510         | NCAM16.2 | BD                        | 30       | Intracellular                             |
| CD27                | BV570         | O323     | BioLegend                 | 30       | Extracellular                             |
| CD38                | BV605         | HIT2     | BD                        | 75       | Intracellular                             |
| CCR6                | BV711         | G034E3   | BioLegend                 | 40       | Extracellular                             |
| TCR $\gamma\delta$  | BV750         | 11F2     | BD                        | 75       | Extracellular                             |
| CD24                | BV786         | ML5      | BioLegend                 | 100      | Intracellular                             |
| Goat anti-horse IgG | AF488         | NA       | Jackson<br>Immunoresearch | 100      | Extracellular                             |
| CD3                 | RB545         | UCHT1    | BD                        | 1000     | Intracellular                             |
| FoxP3               | RB744         | 259D/C7  | BD                        | 75       | Intracellular                             |
| CD69                | RB780         | FN50     | BD                        | 2000     | Intracellular                             |
| IgD                 | PE            | IADB6    | Beckman Coulter           | 1000     | Extracellular                             |
| Tetramers EBV       | PE            | NA       | In-house                  | 100      | Extracellular                             |
| Granzyme B          | PE-CF594      | GB11     | BD                        | 1500     | Intracellular                             |
| CD28                | PE-Cy5        | CD28.2   | BD                        | 200      | Intracellular                             |
| CD8                 | PE-fire 700   | SK1      | BioLegend                 | 1000     | Intracellular                             |
| Ki-67               | PE-Cy7        | B56      | BD                        | 400      | Intracellular                             |
| CD4                 | PE-fire 810   | SK3      | BD                        | 800      | Intracellular                             |
| IgM                 | APC           | MHM-88   | BioLegend                 | 160      | Intracellular                             |
| Tetramers CMV       | APC           | NA       | In-house                  | 50       | Extracellular                             |
| KLRG1               | Spark NIR 685 | SA231A2  | BioLegend                 | 50       | Intracellular                             |
| HLA-DR              | AF700         | G46-6    | BD                        | 75       | Intracellular                             |
| Live/dead           | Zombie-NIR    | NA       | BioLegend                 | 500      | NA                                        |
| Perforin            | APC-fire 750  | B-D48    | BioLegend                 | 300      | Intracellular                             |

Table S2. Model building process for active ATGAM.

| Model number | Description                                                                                                                                                                       | Model                                                                                                                                                                                                                                               | Compared against | OFV    | ΔOFV   | Comments                                                                                              |
|--------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|--------|--------|-------------------------------------------------------------------------------------------------------|
| 1            | One-compartment with linear elimination                                                                                                                                           | CL = $\Theta 1 \times \text{EXP}(\text{IIV})$<br>V = $\Theta 2 \times \text{EXP}(\text{IIV})$                                                                                                                                                       | -                | 84.063 | -      | Additive error fixed to 0.0001 (rounding errors without fixation)                                     |
| 2            | One-compartment with parallel linear and non-linear elimination                                                                                                                   | CL = $\Theta 1 \times \text{EXP}(\text{IIV})$<br>V = $\Theta 2 \times \text{EXP}(\text{IIV})$<br>Vmax = $\Theta 3 \times \text{EXP}(\text{IIV})$<br>Km = $\Theta 4 \times \text{EXP}(\text{IIV})$                                                   | 1                | 84.063 | 0      | Additive error fixed to 0.0001                                                                        |
| 3            | Two-compartment with linear elimination                                                                                                                                           | CL = $\Theta 1 \times \text{EXP}(\text{IIV})$<br>Vc = $\Theta 2 \times \text{EXP}(\text{IIV})$<br>Q = $\Theta 3 \times \text{EXP}(\text{IIV})$<br>Vp = $\Theta 4$                                                                                   | 2                | 57.611 | -26.45 | Additive error fixed to 0.0001                                                                        |
| 4            | Two-compartment with linear elimination + IIV V2                                                                                                                                  | CL = $\Theta 1 \times \text{EXP}(\text{IIV})$<br>Vc = $\Theta 2 \times \text{EXP}(\text{IIV})$<br>Q = $\Theta 3 \times \text{EXP}(\text{IIV})$<br>Vp = $\Theta 4 \times \text{EXP}(\text{IIV})$                                                     | 3                | -12.41 | -70.02 | Additive error fixed to 0.0001<br>Worse VPC (see Figure S4)                                           |
| 4            | Two-compartment with linear elimination + OMEGA BLOCK between IIV CL and V1                                                                                                       | CL = $\Theta 1 \times \text{EXP}(\text{IIV})$<br>Vc = $\Theta 2 \times \text{EXP}(\text{IIV})$<br>Q = $\Theta 3 \times \text{EXP}(\text{IIV})$<br>Vp = $\Theta 4$                                                                                   | 3                | 44.027 | -13.58 | Additive error fixed to 0.0001                                                                        |
| 5            | Two-compartment with linear elimination + OMEGA BLOCK between IIV CL and V1 + different proportional error $\geq 60\text{AU/mL}$ and $< 60\text{AU/mL}$                           | CL = $\Theta 1 \times \text{EXP}(\text{IIV})$<br>Vc = $\Theta 2 \times \text{EXP}(\text{IIV})$<br>Q = $\Theta 3 \times \text{EXP}(\text{IIV})$<br>Vp = $\Theta 4$                                                                                   | 4                | 34.705 | -9.322 | <b>Final model</b><br>Additive error fixed to 0.0001<br>IIV CL: 43.1%<br>IIV Vc: 52%<br>IIV Q: 143.2% |
| 6            | Two-compartment with linear elimination + OMEGA BLOCK between IIV CL and V1 + different proportional error $\geq 60\text{AU/mL}$ and $< 60\text{AU/mL}$ + bodyweight as covariate | CL = $\Theta 1 \times \text{EXP}(\text{IIV}) \times (\text{WT}/\text{MWT})^{\Theta 5}$<br>Vc = $\Theta 2 \times \text{EXP}(\text{IIV}) \times (\text{WT}/\text{MWT})^{\Theta 6}$<br>Q = $\Theta 3 \times \text{EXP}(\text{IIV})$<br>Vp = $\Theta 4$ | 5                | 35.505 | 0.8    | Additive error fixed to 0.0001<br>IIV CL: 45.2%<br>IIV Vc: 50.8%<br>IIV Q: 145.9%                     |



| Model number | Description                                                                                                                                                                                       | Model                                                                                                                                                                                                     | Compared against | OFV            | $\Delta$ OFV   | Comments                                                                          |
|--------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|----------------|----------------|-----------------------------------------------------------------------------------|
| 7            | Two-compartment with linear elimination + OMEGA BLOCK between IIV CL and V1 + different proportional error $\geq 60\text{AU/mL}$ and $< 60\text{AU/mL}$ + lymphocyte count pre-ATGAM as covariate | $CL = \Theta 1 \times \text{EXP(IIV)}$<br>$CL = (1 + (\text{LYMPH}/\text{MLYMPH})) \times \Theta 5$<br>$Vc = \Theta 2 \times \text{EXP(IIV)}$<br>$Q = \Theta 3 \times \text{EXP(IIV)}$<br>$Vp = \Theta 4$ | 5                | 36.813         | 2.108          | Additive error fixed to 0.0001<br>IIV CL: 42.9%<br>IIV Vc: 50.7%<br>IIV Q: 144.2% |
| 8            | Two-compartment with parallel linear and non-linear elimination                                                                                                                                   | $CL = \Theta 1 \times \text{EXP(IIV)}$<br>$Vc = \Theta 2 \times \text{EXP(IIV)}$<br>$Vp = \Theta 3$<br>$V_{\text{max}} = \Theta 4 \times \text{EXP(IIV)}$<br>$K_m = \Theta 5 \times \text{EXP(IIV)}$      | -                | Model unstable | Model unstable | Additive error fixed to 0.0001                                                    |

CL indicates clearance; IIV: inter-individual variability; Km: Michaelis-Menten constant; LYMPH: lymphocyte count pre-ATGAM; MLYMPH: median lymphocyte count pre-ATGAM; MWT: median population weight; OFV: objective function value; Q: intercompartmental clearance; V: volume of distribution; Vc: volume of distribution central compartment; Vp: volume of distribution peripheral compartment; Vmax: maximum elimination rate; WT: bodyweight.

**Table S3.** Model building process for total ATGAM.

| Model number | Description                                                                            | Model                                                                                                                                                                                                            | Compared against | OFV     | $\Delta$ OFV | Comments                                                                                                |
|--------------|----------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|---------|--------------|---------------------------------------------------------------------------------------------------------|
| 1            | One-compartment with linear elimination                                                | CL = $\Theta 1 \times \text{EXP(IIV)}$<br>V = $\Theta 2 \times \text{EXP(IIV)}$                                                                                                                                  | -                | 2026.3  | -            | Additive error fixed to 0.0001 (rounding errors without fixation)                                       |
| 2            | One-compartment with parallel linear and non-linear elimination                        | CL = $\Theta 1 \times \text{EXP(IIV)}$<br>V = $\Theta 2 \times \text{EXP(IIV)}$<br>Vmax = $\Theta 3 \times \text{EXP(IIV)}$<br>Km = $\Theta 4 \times \text{EXP(IIV)}$                                            | 1                | 2026.4  | 0.06         | Additive error fixed to 0.0001. Model instable.                                                         |
| 3            | Two-compartment with linear elimination                                                | CL = $\Theta 1 \times \text{EXP(IIV)}$<br>Vc = $\Theta 2 \times \text{EXP(IIV)}$<br>Q = $\Theta 3 \times \text{EXP(IIV)}$<br>Vp = $\Theta 4$                                                                     | 1                | 1937.99 | -88.32       | Additive error fixed to 0.0001<br>Model unstable                                                        |
| 4            | Two-compartment with linear elimination + Vp fixed                                     | CL = $\Theta 1 \times \text{EXP(IIV)}$<br>Vc = $\Theta 2 \times \text{EXP(IIV)}$<br>Vp = $\Theta 4$ FIX                                                                                                          | 3                | 1940.7  | 2.72         | <b>Final model</b><br>Additive error fixed to 0.0001<br>IIV CL: 44.0%<br>IIV Vc: 57.2%<br>IIV Q: 290.0% |
| 5            | Two-compartment with linear elimination + Vp fixed + OMEGA BLOCK between IIV CL and V1 | CL = $\Theta 1 \times \text{EXP(IIV)}$<br>Vc = $\Theta 2 \times \text{EXP(IIV)}$<br>Q = $\Theta 3 \times \text{EXP(IIV)}$<br>Vp = $\Theta 4$ FIX                                                                 | 4                | 1935.9  | -4.79        | Additive error fixed to 0.0001<br>IIV CL: 44.2%<br>IIV Vc: 58.1%<br>IIV Q: 303.3%                       |
| 6            | Two-compartment with linear elimination + Vp fixed + bodyweight as covariate           | CL = $\Theta 1 \times \text{EXP(IIV)} \times (\text{WT/MWT})^{\Theta 5}$<br>Vc = $\Theta 2 \times \text{EXP(IIV)} \times (\text{WT/MWT})^{\Theta 6}$<br>Q = $\Theta 3 \times \text{EXP(IIV)}$<br>Vp = $\Theta 4$ | 4                | 1947.2  | 6.48         | Additive error fixed to 0.0001<br>IIV CL: 50.5%<br>IIV Vc: 59.9%<br>IIV Q: 295.8%                       |

| Model number | Description                                                     | Model                                                                                                                                                         | Compared against | OFV            | $\Delta$ OFV   | Comments                       |
|--------------|-----------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|----------------|----------------|--------------------------------|
| 8            | Two-compartment with parallel linear and non-linear elimination | $CL = \Theta 1 \times EXP(IIV)$<br>$Vc = \Theta 2 \times EXP(IIV)$<br>$Vp = \Theta 3$<br>$Vmax = \Theta 4 \times EXP(IIV)$<br>$Km = \Theta 5 \times EXP(IIV)$ | -                | Model unstable | Model unstable | Additive error fixed to 0.0001 |

CL indicates clearance; IIV: inter-individual variability; Km: Michaelis-Menten constant; MWT: median population weight; OFV: objective function value; Q: intercompartmental clearance; V: volume of distribution; Vc: volume of distribution central compartment; Vp: volume of distribution peripheral compartment; Vmax: maximum elimination rate; WT: bodyweight.

Table S4. Model estimates for active ATGAM pharmacokinetic parameters.

| Parameter                                                      | Median (range)        | Mean   | RSE (%) | Shrinkage | 1000 bootstrap run results |                 |
|----------------------------------------------------------------|-----------------------|--------|---------|-----------|----------------------------|-----------------|
|                                                                |                       |        |         |           | Median                     | 95% CI          |
| Clearance (CL in L/day)                                        | 6.5 (3.4-24.0)        | 6.97   | 11%     |           | 7.02                       | 5.07 - 9.80     |
| Volume of distribution of central compartment ( $V_c$ in L)    | 1.5 (0.6-4.2)         | 1.51   | 17%     |           | 1.51                       | 0.88 - 2.86     |
| Intercompartmental clearance ( $Q$ in L/day)                   | 2.3 (0.5-65.2)        | 3.2    | 38%     |           | 3.32                       | 1.32 - 9.18     |
| Volume of distribution of peripheral compartment ( $V_p$ in L) | 11.3                  | 11.2   | 12%     |           | 11.56                      | 6.51 - 22.94    |
| Inter-individual variability (IIV)                             |                       |        |         |           |                            |                 |
| Clearance (IIV CL, %)                                          |                       | 43.1   | 13%     | 9%        | 41.98                      | 31.01 - 53.62   |
| $CL \sim V_c$ (%)                                              |                       | -      | -       | -         | 39.26                      | 15.72 - 56.14   |
| Volume of distribution of central compartment (IIV $V_c$ , %)  |                       | 52     | 16%     | 22%       | 49.48                      | 28.36 - 68.12   |
| Intercompartmental clearance (IIV $Q$ , %)                     |                       | 143.2  | 17%     | 8%        | 141.75                     | 102.51 - 194.50 |
| Random residual variability                                    |                       |        |         |           |                            |                 |
| Proportional error (CV%)                                       |                       | 0.3    | 12%     |           | 0.30                       | 0.23 - 0.57     |
| Additional error (AU/mL)                                       |                       | 0.0001 | fixed   |           | 0.0001                     | 0.0001 - 0.0001 |
| Half-life distribution phase (days)                            | 0.09 (0.02-0.25)      |        |         |           |                            |                 |
| Half-life elimination phase (days)                             | 4.5 (0.5-16.1)        |        |         |           |                            |                 |
| Peak concentration ( $C_{max}$ in AU/mL)                       | 668.1 (208.6-1595.9)  |        |         |           |                            |                 |
| Total exposure ( $AUC_{0-inf}$ )                               | 1801.2 (583.9-4218.7) |        |         |           |                            |                 |

**Table S5.** Model estimates for total ATGAM pharmacokinetic parameters.

| Parameter                                                      | Median (range)           | Mean   | RSE (%) | Shrinkage | 1000 bootstrap run results |                 |
|----------------------------------------------------------------|--------------------------|--------|---------|-----------|----------------------------|-----------------|
|                                                                |                          |        |         |           | Median                     | 95% CI          |
| Clearance (CL in L/day)                                        | 0.4 (0.2-1.4)            | 0.426  | 8%      |           | 0.42                       | 0.36 - 0.50     |
| Volume of distribution of central compartment ( $V_c$ in L)    | 3.3 (0.9-8.7)            | 3.22   | 8%      |           | 3.17                       | 2.65 - 3.67     |
| Intercompartmental clearance ( $Q$ in L/day)                   | 0.1 (0.0006-2.7)         | 0.032  | 12%     |           | 0.03                       | 0.01 - 0.15     |
| Volume of distribution of peripheral compartment ( $V_p$ in L) | 0.9                      | 0.949  | fixed   |           | 0.95                       | 0.95 - 0.95     |
| Inter-individual variability (IIV)                             |                          |        |         |           |                            |                 |
| Clearance (IIV CL, %)                                          |                          | 44     | 14%     | 10%       | 43.10                      | 28.65 - 55.37   |
| Volume of distribution of central compartment (IIV $V_c$ , %)  |                          | 57.2   | 10%     | 4%        | 57.10                      | 46.13 - 69.76   |
| Intercompartmental clearance (IIV $Q$ , %)                     |                          | 290    | 17%     | 41%       | 276.86                     | 137.16 - 402.10 |
| Random residual variability                                    |                          |        |         |           |                            |                 |
| Proportional error (CV%)                                       |                          | 0.355  | 10%     |           | 0.35                       | 0.28 - 0.42     |
| Additional error (ug/mL)                                       |                          | 0.0001 | fixed   |           | 0.0001                     | 0.0001 - 0.0001 |
| Half-life distribution phase (days)                            | 4.3 (0.2-8.9)            |        |         |           |                            |                 |
| Half-life elimination phase (days)                             | 16.8 (1.8-1015.9)        |        |         |           |                            |                 |
| Peak concentration ( $C_{max}$ in $\mu\text{g/mL}$ )           | 2500.7 (1175.4-7649.0)   |        |         |           |                            |                 |
| Total exposure ( $\text{AUC}_{0-\text{inf}}$ )                 | 28160.5 (9761.4-90999.0) |        |         |           |                            |                 |

**Table S6.** Patient characteristics by active ATGAM exposure groups.

| Characteristic                                           | Low<br>active ATGAM<br>exposure<br>(n=15) | Medium<br>active ATGAM<br>exposure<br>(n=14) | High<br>active ATGAM<br>exposure<br>(n=15)                         | P-value |
|----------------------------------------------------------|-------------------------------------------|----------------------------------------------|--------------------------------------------------------------------|---------|
| Age at diagnosis (median in years)                       | 40                                        | 60                                           | 57                                                                 | 0.21    |
| Sex (n)                                                  |                                           |                                              |                                                                    | 0.32    |
| Male                                                     | 7                                         | 8                                            | 11                                                                 |         |
| Female                                                   | 8                                         | 6                                            | 4                                                                  |         |
| Disease severity at diagnosis (n)                        |                                           |                                              |                                                                    | 0.91    |
| Non-severe AA                                            | 2                                         | 3                                            | 2                                                                  |         |
| Severe AA                                                | 7                                         | 7                                            | 9                                                                  |         |
| Very severe AA                                           | 6                                         | 4                                            | 4                                                                  |         |
| Weight at diagnosis (median in kg)                       | 71                                        | 75                                           | 88                                                                 | 0.003   |
| Height (median in cm)                                    | 170                                       | 173                                          | 180                                                                | 0.003   |
| Body mass index at diagnosis (median)                    | 22.4                                      | 24.5                                         | 26.0                                                               | 0.33    |
| Neutrophils pre-ATGAM (median in $10^9/L$ )              | 0.33                                      | 0.24                                         | 0.31                                                               | >0.9    |
| Reticulocytes pre-ATGAM (median in $10^9/L$ )            | 17.5                                      | 8.4                                          | 10                                                                 | 0.6     |
| Lymphocytes pre-ATGAM (median in $10^6/L$ ) <sup>a</sup> | 1625                                      | 1485                                         | 1315                                                               | 0.75    |
| Eltrombopag added to IST (n)                             | 5                                         | 3                                            | 8                                                                  | 0.11    |
| Eltrombopag added with start ATGAM                       | 3                                         | 2                                            | 2                                                                  |         |
| Eltrombopag added 20-180 days post-ATGAM                 | 2:<br>Day 96<br>Day 100                   | 1:<br>Day 92                                 | 7:<br>Day 69<br>Day 86<br>Day 138<br>Day 160<br>Day 163<br>Day 165 |         |
| Other second-line treatment<br>≤180 days post-ATGAM      | 0                                         | 0                                            | 0                                                                  | 1.0     |
| Ciclosporin discontinued<br>≤180 days post-ATGAM         | 0                                         | 0                                            | 1                                                                  | 0.40    |
| Deaths ≤180 days post-ATGAM                              | 2                                         | 1                                            | 1                                                                  | 0.78    |

<sup>a</sup>Data missing for 2 AA patients (1 in low and 1 in high exposure groups). Statistical differences between continuous variables calculated by Kruskal Wallis test. Statistical differences between categorical variables calculated by Fisher's Exact test. Statistical differences in addition of eltrombopag to IST, ciclosporin discontinuation and death calculated by log-rank (survival) test.



## Final active ATGAM pharmacokinetic model code

\$PROBLEM PK

\$INPUT ID TIME DV TAD ATGMR C AMT RATE MDV EVID WEIGHT HEIGHT LYMPHO CD4  
CD8 NK B MONO dose\_code ATGMgKg DaysATG ATGMgKgDay DayATGStart AGE GENDER  
RESPONSE\_3M RESPONSE\_6M BMI

\$DATA PK\_ATGAM\_data\_ACTIVE.csv IGNORE=@

\$SUBROUTINES ADVAN6 TOL=3

\$MODEL

COMP=(CENTRAL DEFOBS DEFDOSE)

COMP=(PERIPH)

COMP= AUC

\$PK

CL = THETA(1) \* EXP(ETA(1))

V1 = THETA(2) \* EXP(ETA(2))

Q = THETA(3) \* EXP(ETA(3))

V2 = THETA(4)

S1 = V1

K10=CL/V1

K12=Q/V1

K21=Q/V2

\$DES

C1=A(1)/V1

DADT(1)=-K10\*A(1)-K12\*A(1)+K21\*A(2)

DADT(2)=-K21\*A(2)+K12\*A(1)

DADT(3)= C1

\$ERROR

IPRED = F

IF(DV.GE.60) W = SQRT(THETA(5)\*\*2\*IPRED\*\*2 + THETA(6)\*\*2)

IF(DV.LT.60) W = SQRT(THETA(7)\*\*2\*IPRED\*\*2 + THETA(6)\*\*2)

Y = IPRED + W\*EPS(1)

IRES = DV-IPRED

IWRES = IRES/W

AUC = A(3)

AUCCL = AMT/CL

```

$THETA
(0, 6.97) ; CL
(0, 1.51) ; V1
(0, 3.2) ; Q
(0, 11.2) ; V2
(0, 0.3) ; Prop.RE (sd)
(0.0001) FIX ; Add.RE (sd)
(0, 0.579) ; Prop.RE (sd)
$OMEGA BLOCK(2)
0.186 ; IIV CL
0.18 0.27 ; IIV V1
$OMEGA
2.05 ; IIV Q

$SIGMA
1 FIX ; Proportional error PK

$EST METHOD=1 INTER MAXEVAL=2000 NOABORT SIG=2 PRINT=1 POSTHOC
$COV

; Xpose
$TABLE CL V1 V2 Q ID TIME DV MDV EVID PRED IPRED IWRES CWRES ETA1 ETA2 ETA3
LYMPHO CD4 CD8 B NK MONO AGE GENDER WEIGHT HEIGHT BMI AUCCL AUC Response_3M
Response_6M ONEHEADER NOPRINT FILE=sdtab002
$TABLE CL V1 ID TIME DV MDV EVID PRED IPRED IWRES CWRES ETA1 ETA2 ETA3 LYMPHO
CD4 CD8 B NK MONO AGE GENDER WEIGHT HEIGHT AUCCL AUC Response_3M Response_6M
ONEHEADER NOPRINT FIRSTONLY FILE=patab002

```

## Final total ATGAM pharmacokinetic model code

\$PROBLEM PK

\$INPUT ID TIME DV TAD ATGNR C AMT RATE MDV EVID WEIGHT HEIGHT LYMPH CD4  
CD8 NK B MONO dose\_code ATGMgKg DaysATG ATGMgKgDay DayATGStart AGE GENDER  
RESPONSE\_3M RESPONSE\_6M

\$DATA PK\_ATGAM\_data\_TOTAL.csv IGNORE=@

\$SUBROUTINES ADVAN6 TOL=3

\$MODEL

COMP=(CENTRAL DEFOBS DEFDOSE)

COMP=(PERIPH)

COMP= AUC

\$PK

TVCL = THETA(1)

CL = TVCL \* EXP(ETA(1))

TVV1 = THETA(2)

V1 = TVV1 \* EXP(ETA(2))

TVQ = THETA(3)

Q = TVQ \* EXP(ETA(3))

TVV2 = THETA(4)

V2 = TVV2

S1 = V1

K10=CL/V1

K12=Q/V1

K21=Q/V2

\$DES

C1=A(1)/V1

DADT(1)=-K10\*A(1)-K12\*A(1)+K21\*A(2)

DADT(2)=-K21\*A(2)+K12\*A(1)

DADT(3)= C1

```

$ERROR
IPRED = F
  W = SQRT(THETA(5)**2*IPRED**2 + THETA(6)**2)
  Y = IPRED + W*EPS(1)
IRES = DV-IPRED
IWRES = IRES/W
AUC = A(3)
AUCCL = AMT/CL

$THETA
(0, 0.424) ; CL
(0, 3.23) ; V1
(0, 0.0299) ; Q
(0.949) FIX ; V2
(0, 0.353) ; Prop.RE (sd)
(0.00001) FIX ; Add.RE (sd)

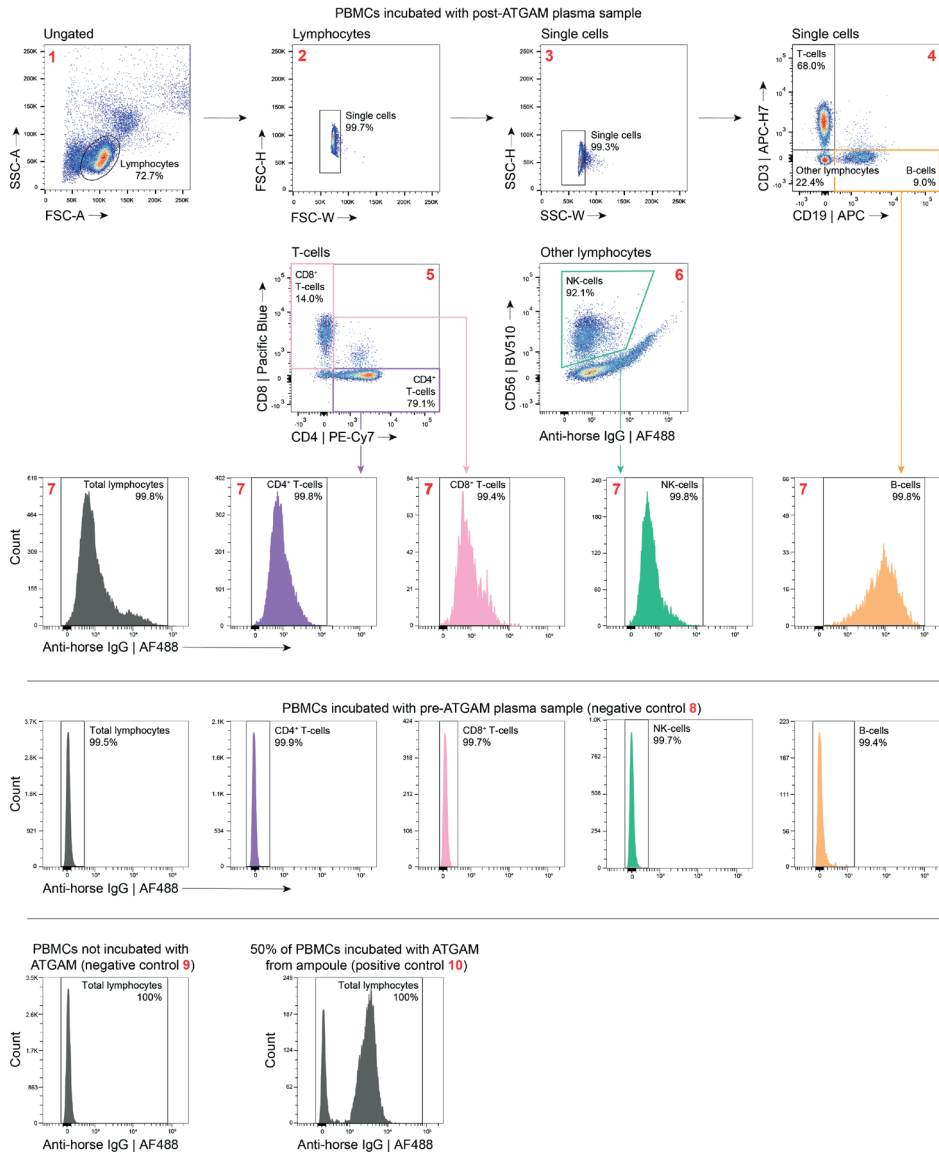
$OMEGA
0.194 ; IIV CL
0.318 ; IIV V1
8.74 ; IIV Q

$SIGMA
1 FIX ; Proportional error PK

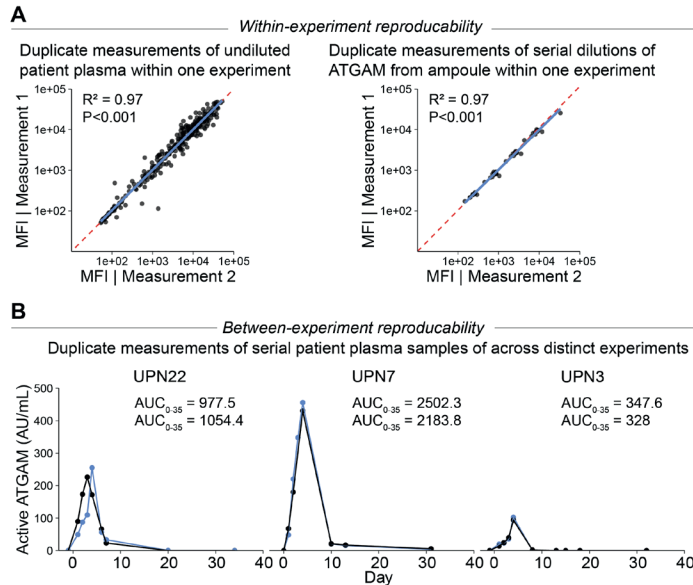
$EST METHOD=1 INTER MAXEVAL=2000 NOABORT SIG=2 PRINT=1 POSTHOC
$COV

; Xpose
$TABLE CL V1 V2 Q ID TIME DV MDV EVID PRED IPRED IWRES CWRES ETA1 ETA2 ETA3
LYMPH CD4 CD8 B NK MONO AGE GENDER WEIGHT HEIGHT AUCCL AUC Response_3M
Response_6M ONEHEADER NOPRINT FILE=sdtab005
$TABLE CL V1 V2 Q ETA1 ETA2 ETA3 LYMPH CD4 CD8 B NK MONO AGE GENDER WEIGHT
HEIGHT ONEHEADER NOPRINT FIRSTONLY FILE=patab005

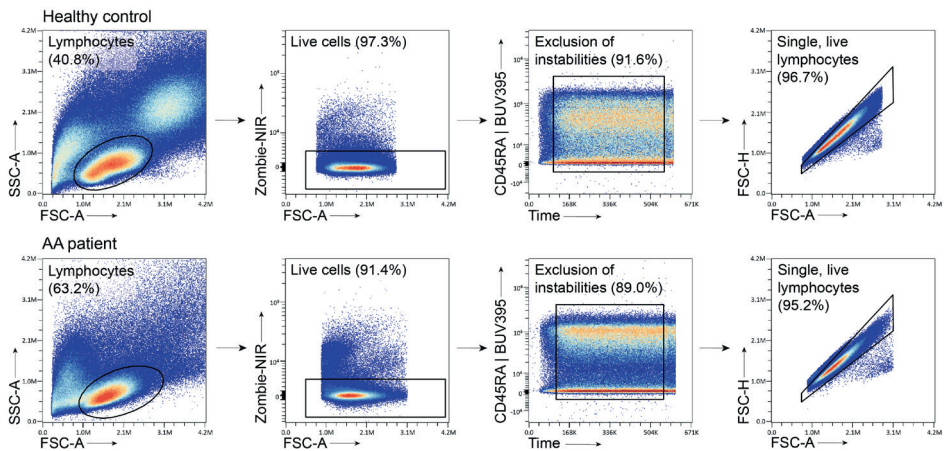
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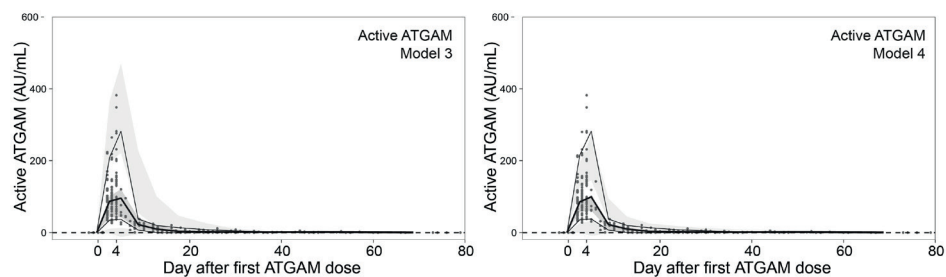
**Figure S1. Gating strategy for quantification of active ATGAM plasma concentrations.** Lymphocytes were gated using the forward and side scatter (panel 1). Doublets were excluded, and single lymphocytes were selected for further analysis (panels 2-3). Within the single lymphocytes, CD3<sup>+</sup> T-cells, CD19<sup>+</sup> B-cells or CD3<sup>+</sup>CD19<sup>-</sup> other lymphocytes were identified (panel 4). CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells were identified within the T-cell gate (panel 5). CD56<sup>+</sup> NK-cells were identified within the 'other lymphocyte' gate (panel 6). The geometric mean fluorescence intensity for the Alexa Fluor 488 anti-horse IgG antibody was determined for each gated population (panels 7). This was used to calculate the active ATGAM concentrations in the patient plasma sample using a reference curve obtained by exposing PBMCs to known concentrations of ATGAM. The gating strategy is shown for PBMCs incubated with a representative post-ATGAM patient plasma sample. Negative controls were included in each experiment and included PBMCs incubated with pre-ATGAM patient plasma samples (panels 8) and PBMCs not exposed to ATGAM (panel 9). Positive controls were PBMCs exposed to known concentrations of ATGAM (panel 10).



**Figure S2. Reproducibility and robustness of the active ATGAM assay.** [A] Within-experiment reproducibility, shown by correlation plot of duplicate measurements of undiluted patient plasma within a single experiment (left), or duplicate measurements of serial dilutions of ATGAM (right). [B] Between-experiment reproducibility, shown by duplicate measurements of active ATGAM concentrations in patient plasma in independent experiments.

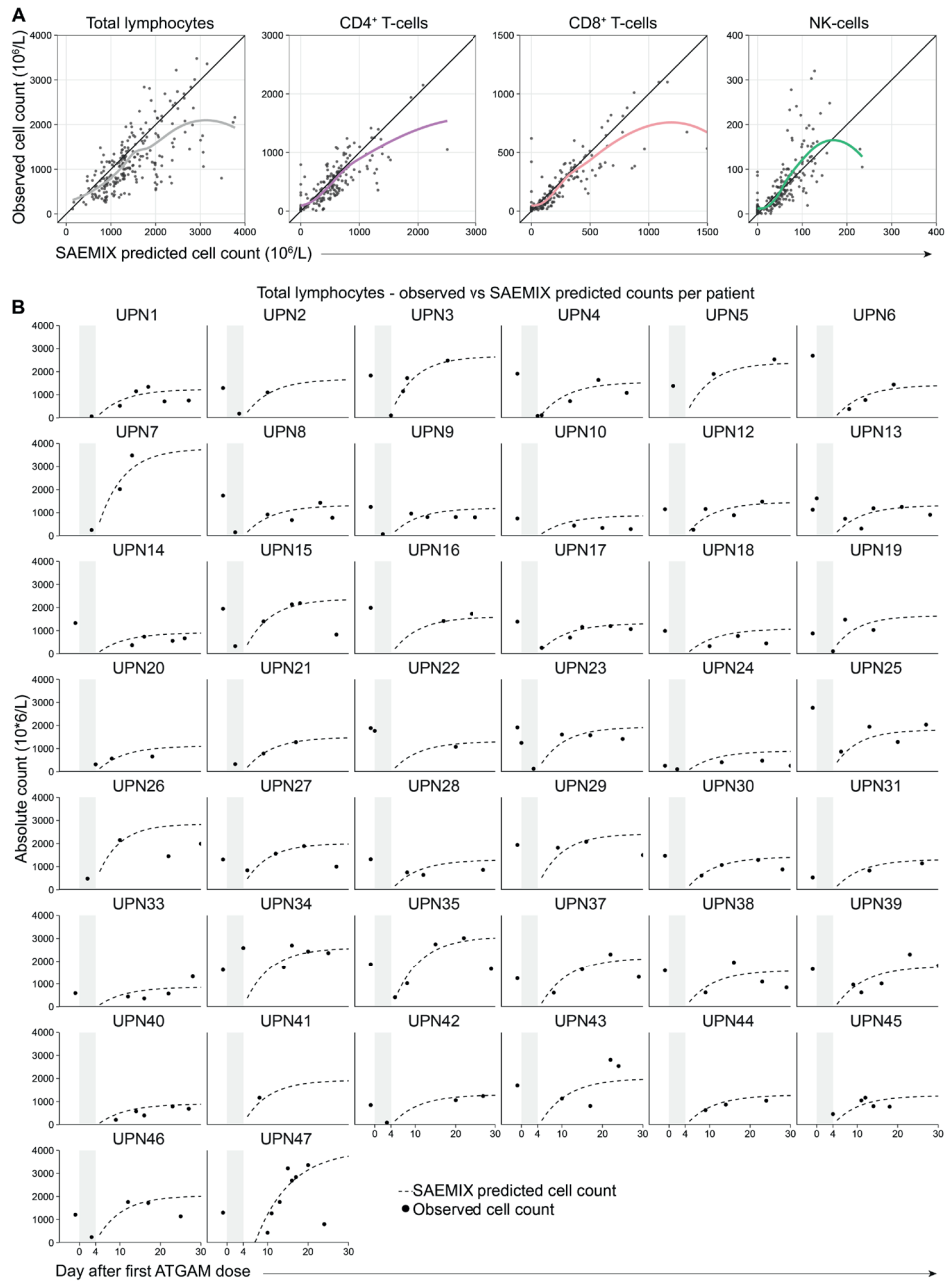


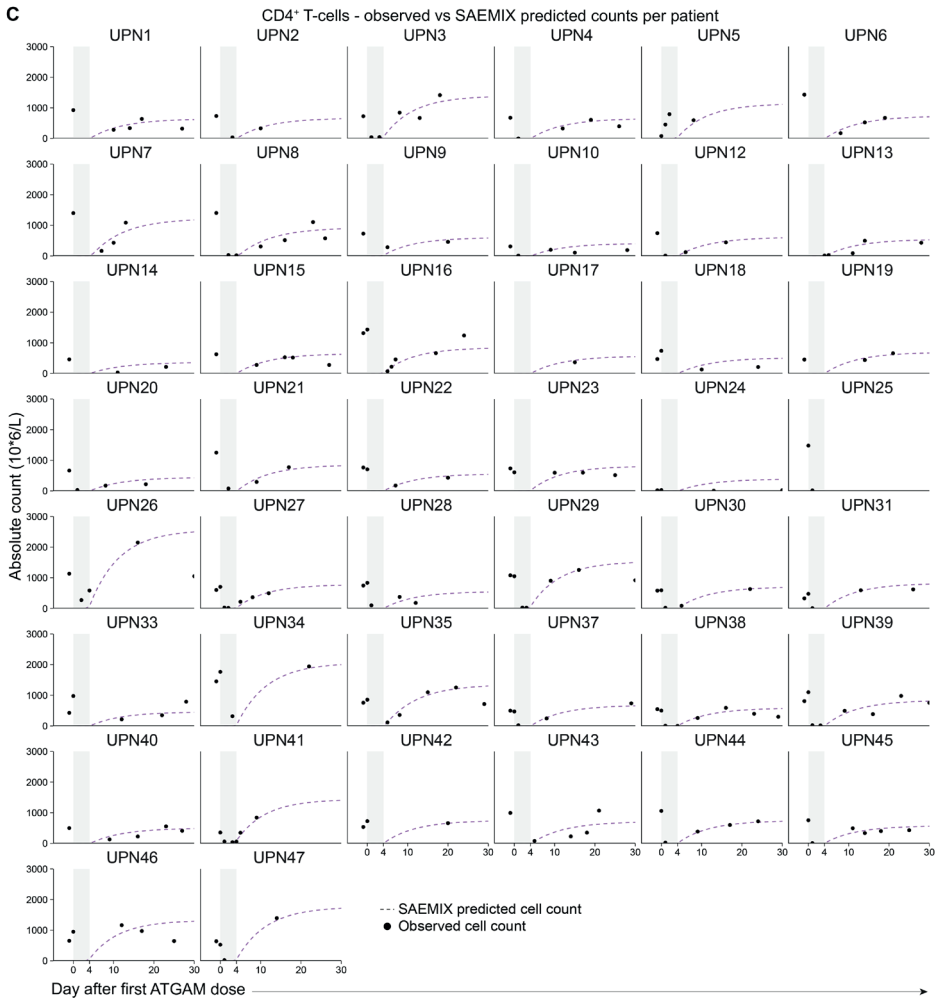
**Figure S3. Gating strategy for selection of single, live lymphocytes from spectral flow cytometry data.** Lymphocytes were gated on OMIQ based on the forward and side scatter (panel 1). Next, live cells were identified by selecting cells negative for Zombie-NIR (panel 2), and acquisition instabilities were removed by excluding events recorded at the beginning or end of each measurement (panel 3). Finally, doublets were excluded using the forward scatter height (FSC-H) and forward scatter area (FSC-A) parameters (panel 4). Data for a representative healthy control and aplastic anemia patient are shown. Single, live lymphocytes gated in panel 4 were selected for downstream analyses.

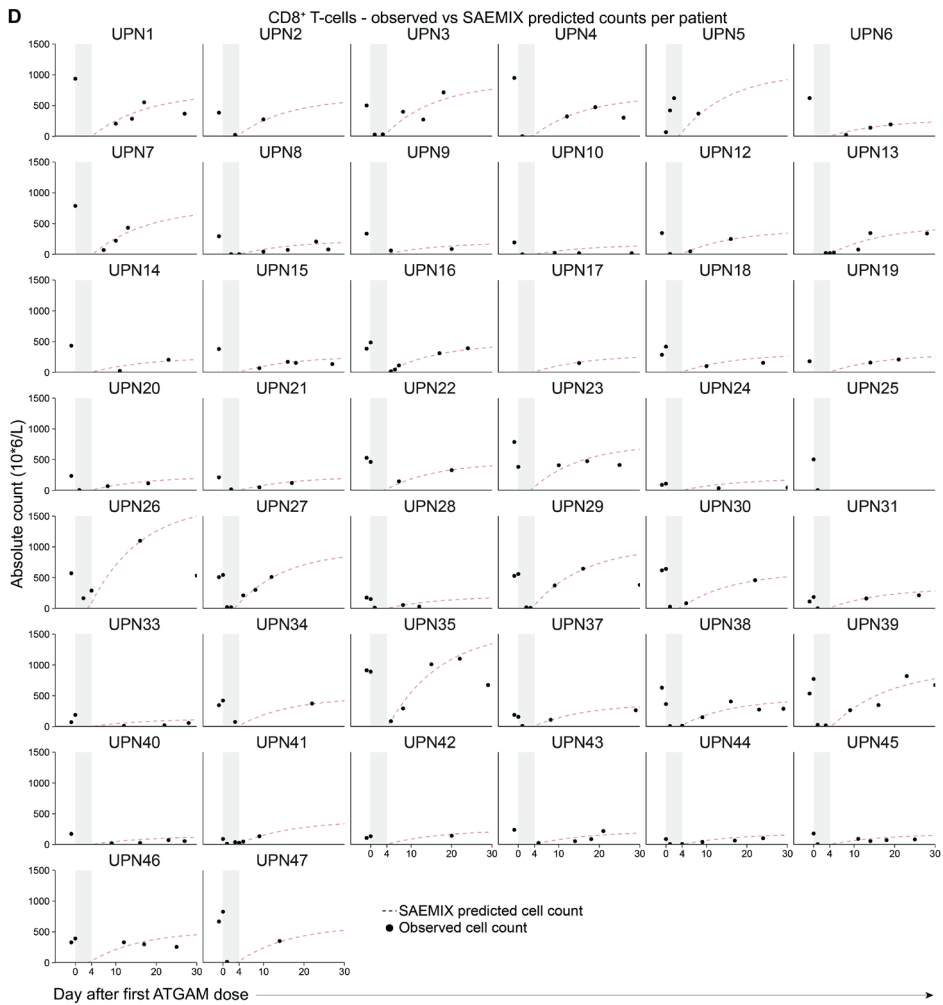


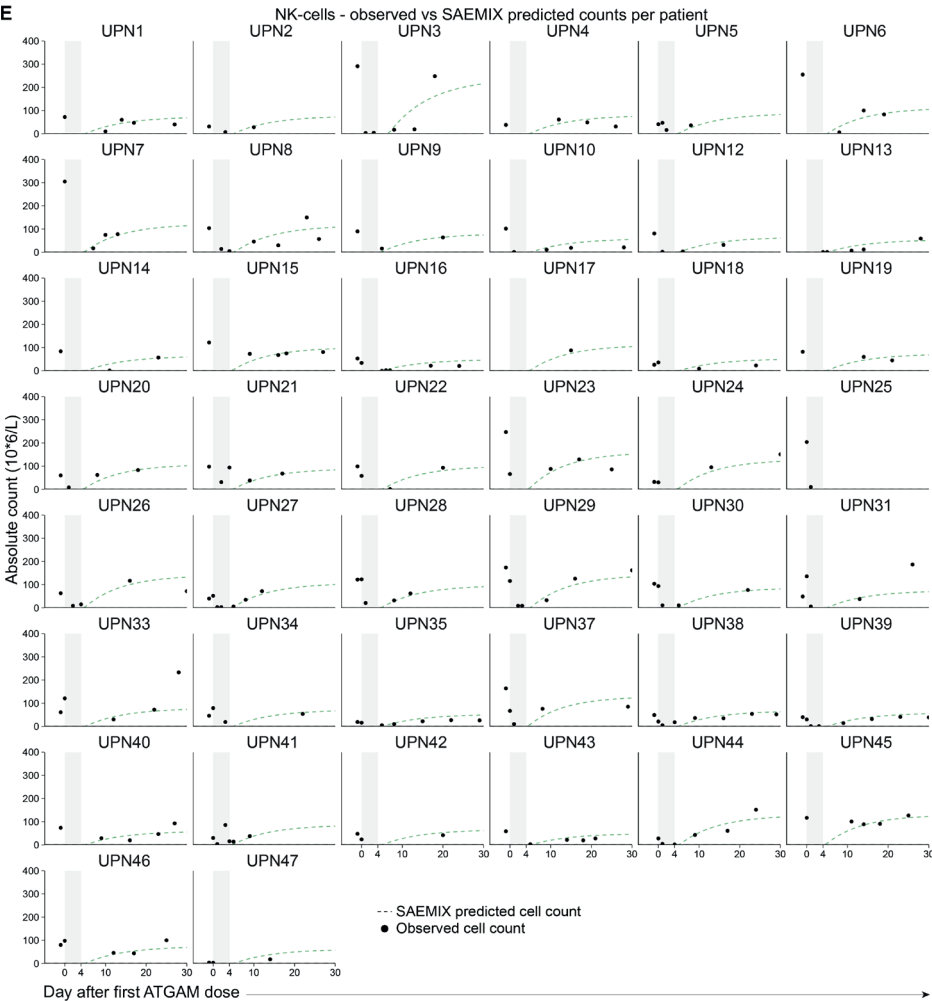
**Figure S4. Visual predictive checks for population pharmacokinetic model 3 and model 4 for active ATGAM.** Prediction-corrected visual predictive check (VPC) for the final model for active ATGAM. Black lines represent the predicted active ATGAM concentrations at the 95th, 50th, and 5th percentiles. Shaded light gray areas indicate the 95% confidence intervals for the predicted 5th and 95th percentiles, while the dark gray shaded area represents the 95% confidence interval for the predicted 50th percentile. Observed active ATGAM concentrations in all 44 AA patients are shown by black dots.



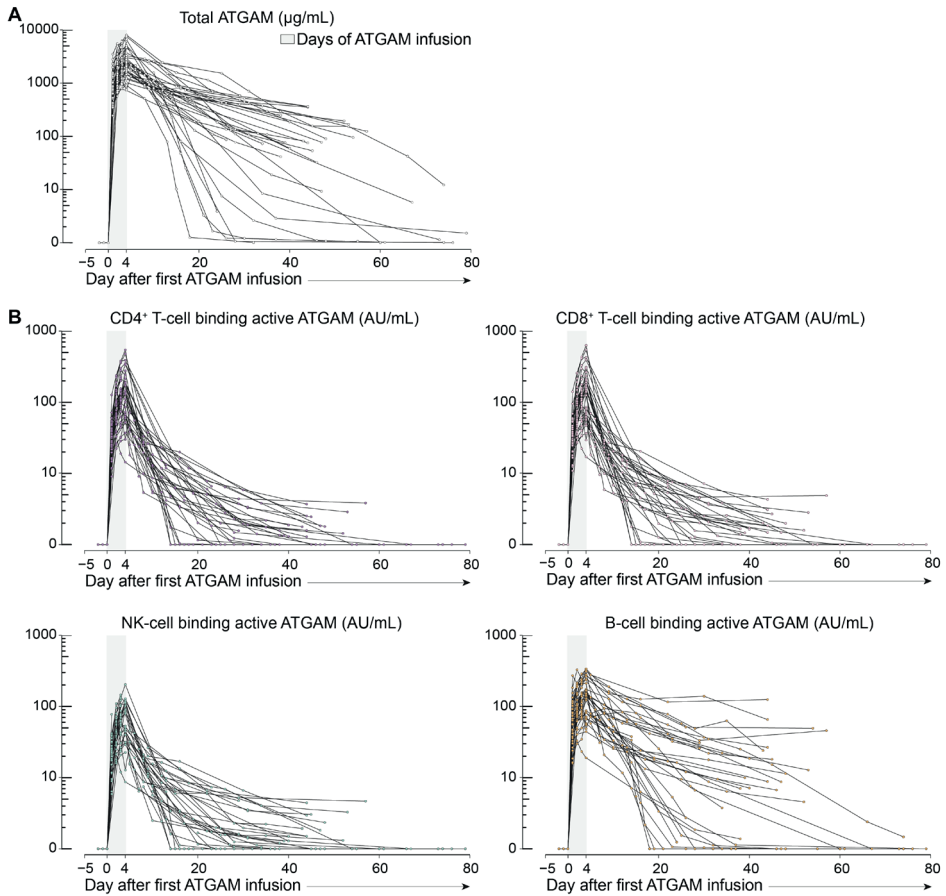




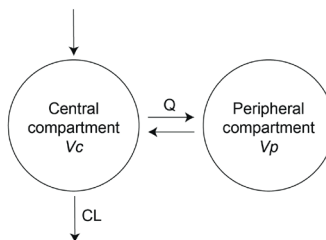




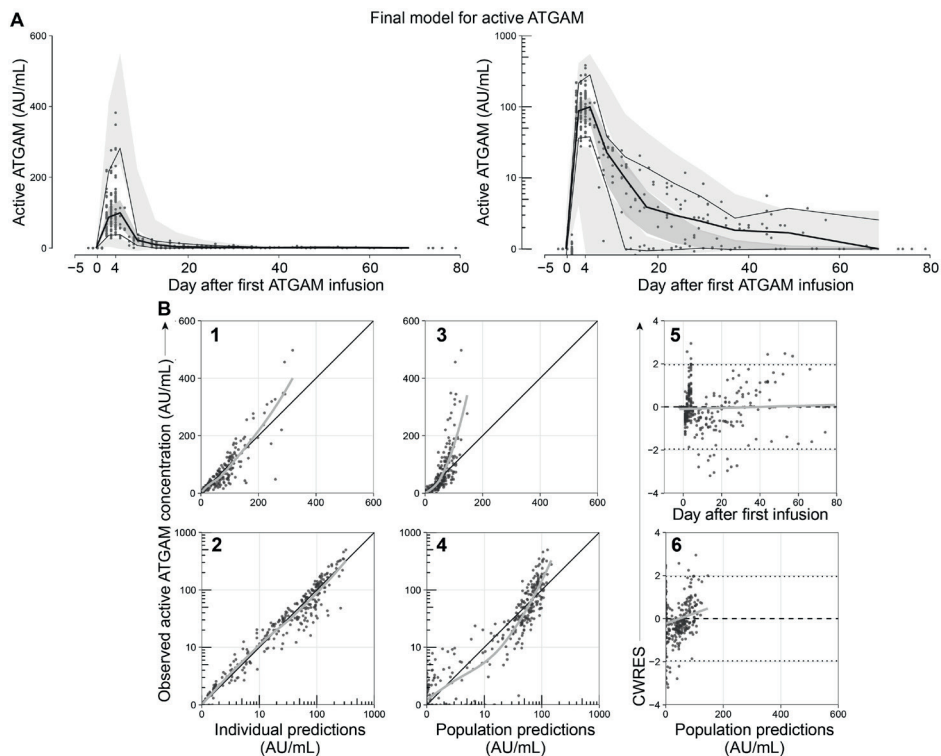
**Figure S5. Observed versus predicted immune reconstitution data.** [A] Observed versus SAEMIX-predicted total lymphocyte, CD4<sup>+</sup> T-cell, CD8<sup>+</sup> T-cell and NK-cell counts shown for all patients. The trend between observed and predicted counts are shown using loess regression. [B-D] Observed versus SAEMIX-predicted total lymphocyte [B], CD4<sup>+</sup> T-cell [C], CD8<sup>+</sup> T-cell [D] and NK-cell [E] counts per patient. Dots in present observed lymphocyte counts; dotted lines present predictions.



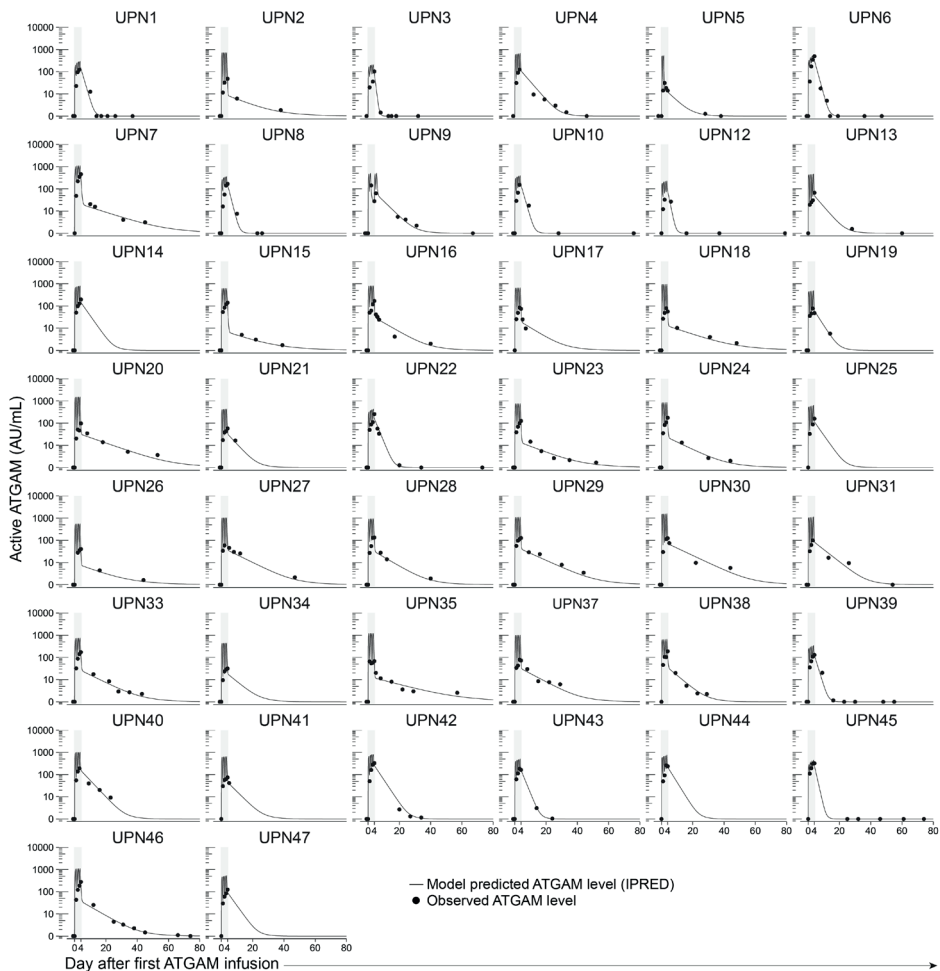
**Figure S6. Concentration-time profile of total ATGAM and lineage-binding active ATGAM in patient plasma.** [A] Measured total ATGAM, or [B] measured CD4<sup>+</sup> T-cell-binding active ATGAM, CD8<sup>+</sup> T-cell-binding active ATGAM, CD19<sup>+</sup> B-cell-binding active ATGAM, and NK-cell binding active ATGAM concentrations in patient plasma (in µg/mL or arbitrary units/mL; n=44 patients) over time before, during and after ATGAM infusion on days 0-4, shown on a logarithmic scale.



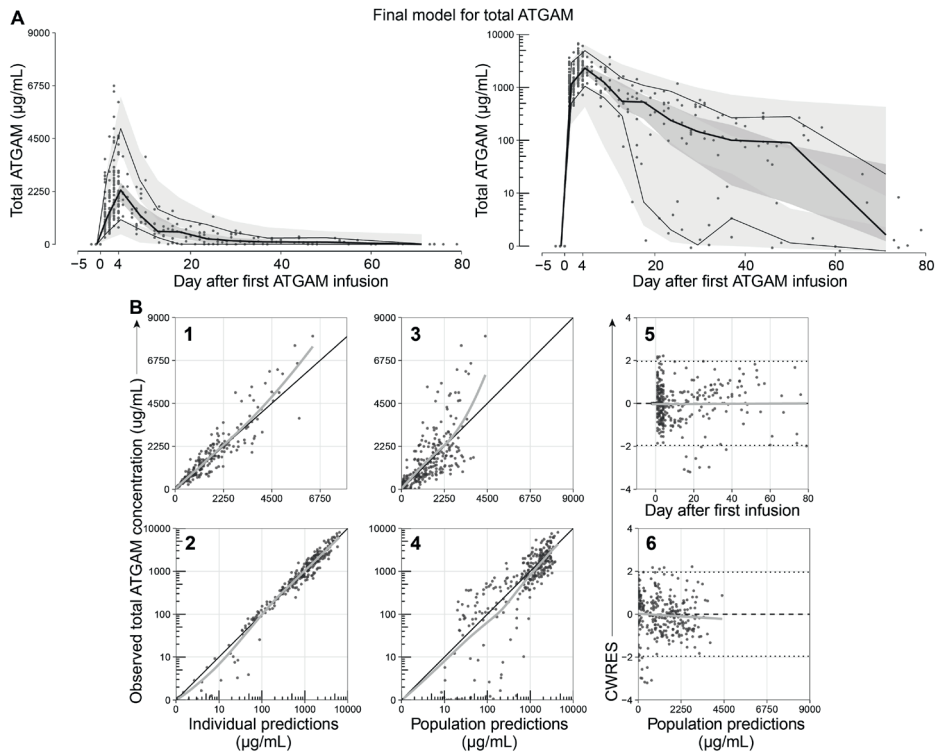
**Figure S7. Population pharmacokinetic model overview.** A two-compartment population pharmacokinetic model best described the total and active ATGAM concentration-time data. CL: linear clearance, Q: intercompartmental clearance between central compartment with volume  $V_c$ , and peripheral compartment with volume  $V_p$ .



**Figure S8. Prediction-corrected visual predictive check and goodness of fit plots for the final pharmacokinetic model for active ATGAM.** [A] Prediction-corrected visual predictive check (VPC) for the final model for active ATGAM. Black lines represent the predicted active ATGAM concentrations at the 95th, 50th, and 5th percentiles. Shaded light gray areas indicate the 95% confidence intervals for the predicted 5th and 95th percentiles, while the dark gray shaded area represents the 95% confidence interval for the predicted 50th percentile. Observed active ATGAM concentrations in all 44 AA patients are shown by black dots. VPC is shown on a linear (left) or a logarithmic (right) scale. [B] Goodness of fit plots for the final model for active ATGAM. Observed versus individual predicted (IPRED) active ATGAM concentrations on a linear (1) and logarithmic (2) scale; observed versus population predicted (PRED) active ATGAM concentrations on a linear (3) and logarithmic (4) scale; conditional weighted residuals (CWRES) versus time after ATGAM infusion on days 0-4 (5); conditional weighted residuals (CWRES) versus population predicted active ATGAM concentrations (6). Dotted lines in (5) and (6) present the  $\pm 1.96$  ranges of CWRES (95% confidence interval), which should include 95% of the CWRES values.

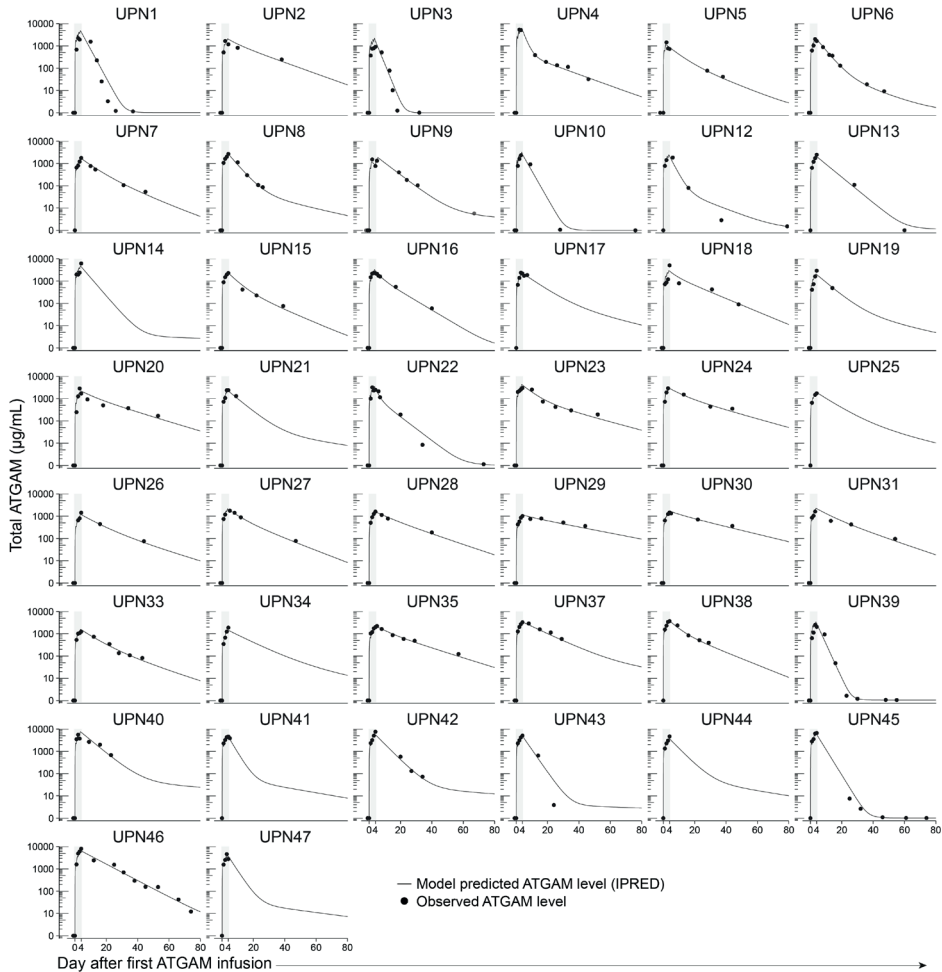


**Figure S9. Observed versus model-predicted active ATGAM concentrations per patient.** Observed active ATGAM concentrations are shown by black dots, while modelled active ATGAM concentrations in time are shown using black lines. Data are presented on a logarithmic axis.

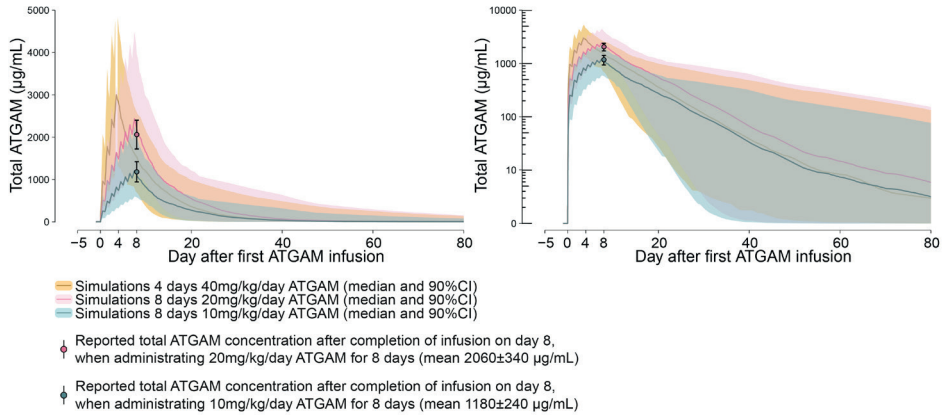


**Figure S10. Prediction-corrected visual predictive check and goodness of fit plots for the final pharmacokinetic model for total ATGAM.** [A] Prediction-corrected visual predictive check (VPC) for the final model for total ATGAM. Black lines represent the predicted total ATGAM concentrations at the 95th, 50th, and 5th percentiles. Shaded light gray areas indicate the 95% confidence intervals for the predicted 5th and 95th percentiles, while the dark gray shaded area represents the 95% confidence interval for the predicted 50th percentile. Observed total ATGAM concentrations in all 44 AA patients are shown by black dots. VPC is shown on a linear (left) or a logarithmic (right) scale. [B] Goodness of fit plots for the final model for total ATGAM. Observed versus individual predicted (IPRED) total ATGAM concentrations on a linear (1) and logarithmic (2) scale; observed versus population predicted (PRED) total ATGAM concentrations on a linear (3) and logarithmic (4) scale; conditional weighted residuals (CWRES) versus time after ATGAM infusion on days 0-4 (5); conditional weighted residuals (CWRES) versus population predicted total ATGAM concentrations (6). Dotted lines in (5) and (6) present the  $\pm 1.96$  ranges of CWRES (95% confidence interval), which should include 95% of the CWRES values.

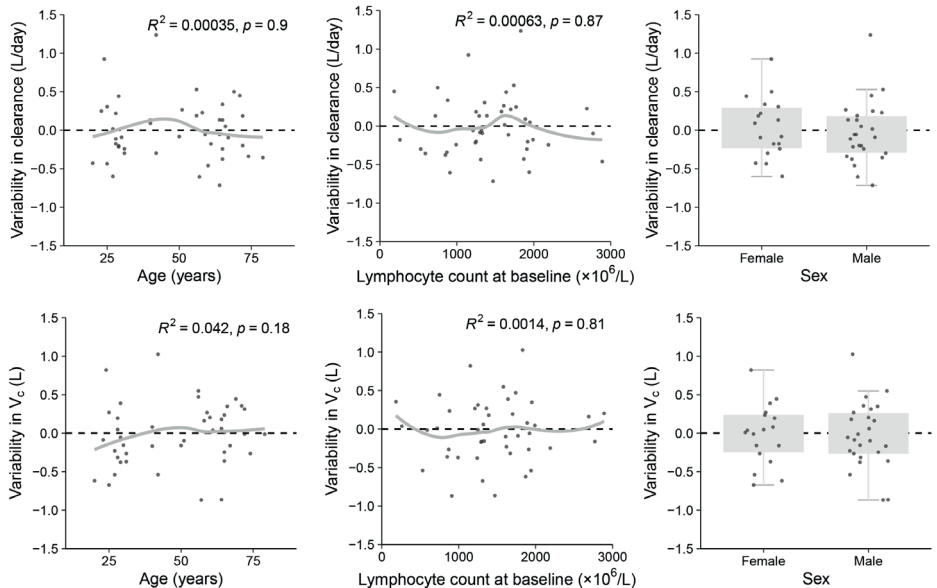




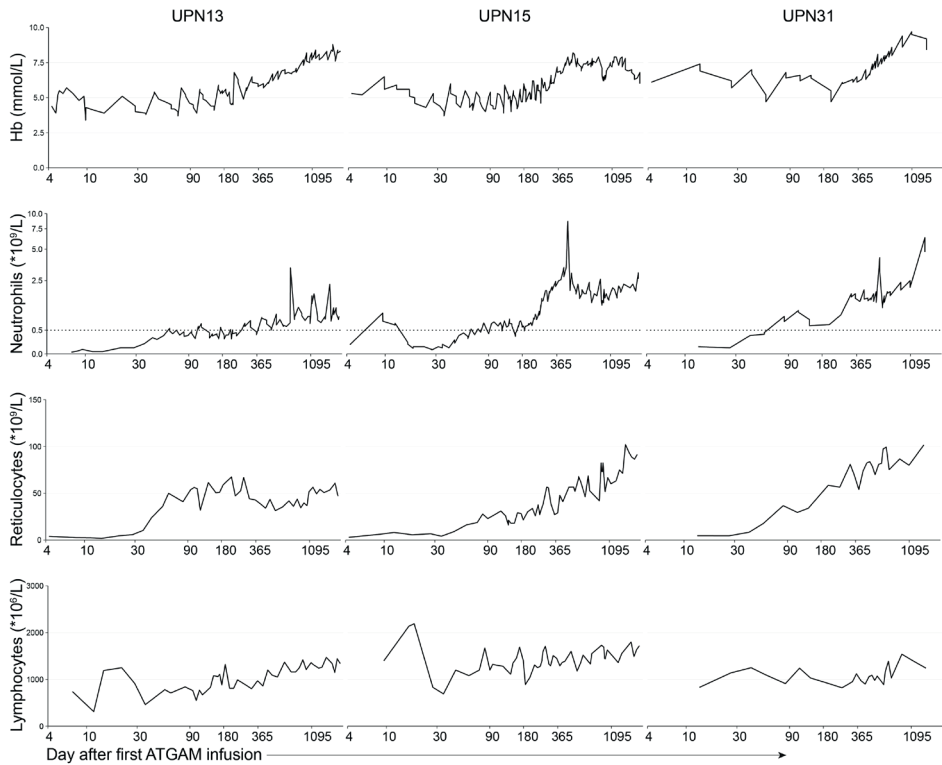
**Figure S11. Observed versus model-predicted total ATGAM concentrations per patient.** Observed total ATGAM concentrations are shown by black dots, while modelled total ATGAM concentrations in time are shown using black lines. Concentrations are presented on a logarithmic axis.



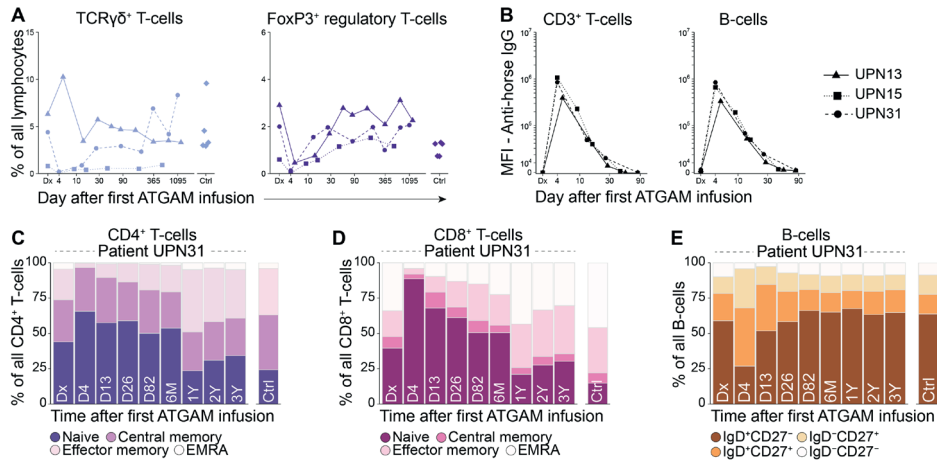
**Figure S12. Model simulations for alternative ATGAM dosing schemes.** The final pharmacokinetic model for total ATGAM was used to simulate the concentration-time profile of total ATGAM in time after the administration of 8 consecutive days 10mg/kg/day ATGAM, 8 consecutive days 20mg/kg/day ATGAM or 4 consecutive days 40mg/kg/day ATGAM. Lines and shaded areas represent the median and 90% confidence interval obtained from 100 simulations. Dots and error bars represent the mean and range of reported ATGAM peak concentrations following the completion of infusion on day 8 when ATGAM was administered at 10mg/kg/day ATGAM or 20mg/kg/day ATGAM for 8 consecutive days (reported concentrations are described in the manufacturer's brochure by Pfizer).



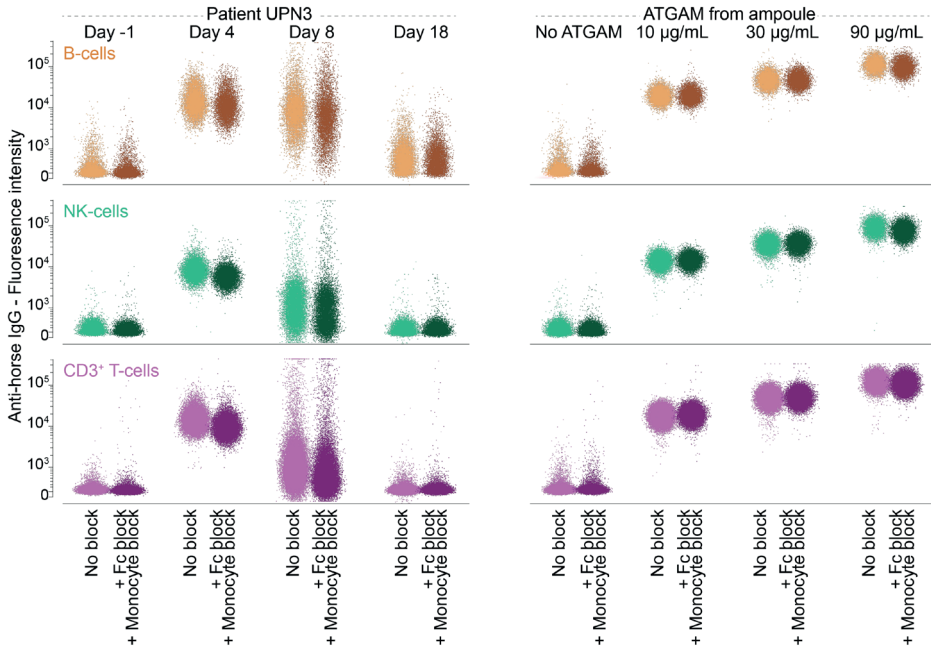
**Figure S13. Impact of demographical and clinical covariates on inter-individual variability in pharmacokinetic parameters.** Impact of age, lymphocytes pre-ATGAM and sex on inter-individual variability in clearance (row 1) and volume of distribution of the central compartment (row 2) of active ATGAM. Each dot represents a patient.



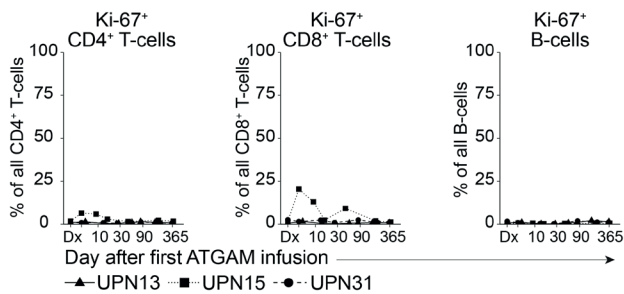
**Figure S14. Peripheral blood counts in time after start of ATGAM-based IST.** Hemoglobin (Hb), neutrophil, reticulocyte and total lymphocyte counts in patients UPN13, UPN15 and UPN31 over time, up to 3.5 years after start of ATGAM-based IST.



**Figure S15. Lymphocyte compartment pre- and over time post-ATGAM.** [A] Frequencies of major lymphoid lineages, shown as a percentage of all single, live lymphocytes per time point. Each line represents an individual patient. [B] Presence of ATGAM on the cell surface of T-cells (right) and B-cells (left), quantified over time using the mean fluorescence intensity of the Alexa Fluor 488-conjugated anti-horse IgG antibody. Each line represents an individual patient. [C-E] Bar plots quantifying the frequencies of subpopulations identified within the CD4<sup>+</sup> T-cell, CD8<sup>+</sup> T-cell and B-cell compartments from the representative patient UPN31. Flow cytometry plots are shown in Figure 4.



**Figure S16. Impact of Fc-receptor blockade on ATGAM binding to lymphoid lineages.** PBMCs from a healthy donor were pre-incubated without or with Fc-receptor block (Invitrogen, cat 14-9161-73) and monocyte block (Biolegend, cat 426103) for 30 minutes. Subsequently, cells were washed, co-incubated with undiluted plasma samples collected pre- and post-ATGAM infusion from patient UPN3 (right), or known ATGAM concentrations (right), and stained as described in the methods section (see 'Quantification of active ATGAM concentrations').



**Figure S17. Proliferating CD4<sup>+</sup> T-cells, CD8<sup>+</sup> T-cells and B-cells after start of ATGAM-based IST.** Ki-67<sup>+</sup> cells were gated within the CD4<sup>+</sup> T-cell, CD8<sup>+</sup> T-cell and B-cell compartments. Data are shown for patients UPN13, UPN15 and UPN31.

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