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Rabbit antithymocyte globulin (Thymoglobulin®) impairs the thymic output of both conventional and regulatory CD4+ T cells after allogeneic hematopoietic stem cell transplantation in adult patients

Running title: ATG and thymic function

Il-Kang Na[1,2,6]*, Friedrich Wittenbecher[1]*, Mikalai Dziubianau[3], Anne Herholz[4], Angela Mensen[2], Désirée Kunkel[5], Olga Blau[1], Igor Blau[1], Eckhard Thiel[1], Lutz Uharek[1], Carmen Scheibenbogen[2], Kathrin Rieger[1]*, and Andreas Thiel[3]*

[1]Department of Hematology, Oncology and Tumor Immunology, Charité, Berlin, Germany; [2]Institute of Medical Immunology, Charité CVK, Berlin, Germany; [3]Regenerative Immunology and Aging, Berlin-Brandenburg Center for Regerative Therapies (BCRT), Charité CVK, Berlin, Germany; [4]Department of Medicine, Division of Gastroenterology, Infectiology and Rheumatology; [5]BCRT Flow Cytometry Lab, Berlin-Brandenburg Center for Regerative Therapies (BCRT), Charité CVK, Berlin, Germany, and [6]Experimental and Clinical Research Center (ECRC), Berlin, Germany

*IKN and FW as well as KR and AT contributed equally to this manuscript.

Correspondence

Il-Kang Na, Charité - Campus Virchow-Klinikum, Hämatologie, Onkologie und Tumorimmunologie, Augustenburger Platz 113353 Berlin.
Phone: international +49.30.450553111.
Fax: international +49.30.450553914. E-mail: il-kang.na@charite.de

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Abstract

Background. Rabbit antithymocyte globulin-Genzyme™ is used to prevent graft-versus-host disease after allogeneic hematopoietic stem cell transplantation. Common disadvantages of treatment are infectious complications. The effect of rabbit antithymocyte globulin-Genzyme™ on thymic function has not been well-studied.

Design and methods. Multicolor flow cytometry was used to analyze the kinetics of conventional and regulatory T cells in rabbit antithymocyte globulin-Genzyme™ treated (n=12) versus non-treated (n=8) adult patients during the first six months after allogeneic hematopoietic stem cell transplantation.

Results. Rabbit antithymocyte globulin-Genzyme™ treated patients exhibited almost undetectable levels of recent thymic emigrants (CD45RA+CD31+) of both conventional and regulatory CD4 T cells throughout the six months after allogeneic hematopoietic stem cell transplantation whereas CD4+CD45RA- memory T cells were less affected, but also significantly lower compared to non-rabbit antithymocyte globulin-Genzyme™ treated patients. *In vitro,* rabbit antithymocyte globulin-Genzyme™ induced apoptosis and cytolysis of human thymocytes, and its cytotoxic effects were higher compared to rabbit antithymocyte globulin-Fresenius™.

Conclusions. Rabbit antithymocyte globulin-Genzyme™ in combination with conditioning regimen heavily impairs thymic recovery of both conventional and regulatory CD4+ T cells. The sustained depletion of conventional and regulatory CD4+ T cells bears a high risk of both infections and graft-versus-host disease. Our data warrant thymus protective therapies for rabbit antithymocyte globulin-Genzyme™ treated patients and comparative trials to other rabbit antithymocyte globulin preparations or lymphocyte depleting compounds.

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Introduction

The thymus is required for *de novo* generation of naïve T cells with a broad T cell receptor repertoire (1). Although thymic function in humans decreases after puberty, the adult thymus still contributes substantially to the restoration of immune competence after myeloablative therapy (2). Factors that inhibit thymic function after allogeneic hematopoietic stem cell transplantation (alloHSCT) include thymic damage by the conditioning regimen (3, 4) as well as GvHD, where cell damage is mediated by donor alloreactive T cells (5, 6). The consequence of delayed thymic recovery is profound T cell deficiency and repertoire restriction after alloHSCT (1, 7). In consequence, the delay in thymic recovery is associated with a higher incidence of infections (8) and disease relapse (9), furthermore it hinders success of immunotherapeutic strategies.

CD31 (PECAM-1) has been demonstrated to characterize the T cell receptor excision circles (TREC)-rich CD4 recent thymic emigrants (RTE) (10, 11), and is already adopted into clinical practice as an excellent marker for thymic function in solid organ transplantation (12) and after autologous HSCT (13, 14). Junge *et al.* (2007) could show a correlation between CD31+ CD4 T cells and the recurrence of TREC during aging of healthy individuals and in six lymphopenic children after alloHSCT (15).

Rabbit antithymocyte globulin-Genzyme™ (ATG-G, Thymoglobulin®, Genzyme™ Transplant, Cambridge, MA, USA) therapy is commonly used for *in vivo* T cell depletion in the context of alloHSCT to prevent graft-versus-host disease (GvHD) (16). Different rabbit ATG preparations are currently available of which ATG-G and ATG-Fresenius™ (ATG-F, Fresenius™ Biotech GmbH, Munich, Germany) are frequently applied. ATG-F is manufactured by immunization of rabbits with a Jurkat cell line whereas ATG-G is manufactured by immunization of rabbits with human thymocytes. In this study ATG-G was used, which contains a diverse spectrum of antibody specificities depleting various T cell subsets (17). Clinical data regarding the effect of ATG-G on thymopoiesis after alloHSCT are scarce, and the effect of ATG-G on thymic output of regulatory T cells (Treg) in particular after alloHSCT has not been studied yet.

The aim of this study was to assess the impact of ATG-G on the reconstitution of recent thymic emigrants (RTEs), naïve and memory T cell subsets of both conventional (Tcon) and regulatory (Treg) CD4 T cells in adult alloHSCT patients. Suggesting a direct effect of ATG-G on the thymus, we also tested the ATG-G toxicity on human thymocytes *in vitro* and compared to ATG-F as well as to the toxicity on peripheral blood mononuclear cells (PBMC).

Design and Methods

Patients and treatment

Blood samples for the kinetic study were obtained from 20 adult patients, who received myeloablative conditioning (MAC, n=12), reduced intensity conditioning (RIC, n=7) or non-myeloablative conditioning (nonMAC, n=1, patient diagnosed with CLL) according to standard protocols. Patient characteristics are listed in **Table 1**. All hematopoietic grafts were obtained by apheresis after stem cell mobilization with granulocyte-colony stimulating factor (G-CSF) and were provided from HLA-matched related (MRD, n=7) and unrelated (MUD, n=13) healthy donors. GvHD prophylaxis consisted of Cyclosporin A in combination with either methotrexate (n=15) or mycophenolate mofetil (n=5). ATG-G (2 mg/kg/d) was additionally administered in case of MUD transplantation on days -3, -2 and -1 before transplantation according to standard procedure protocols in 12 patients. Patient samples were obtained once before transplantation and at days 15, 30, 60, 90 and 180 after transplantation. Analyses were performed with fresh blood samples in order to avoid possible influences caused by freeze-thaw cycles. Human thymocytes were obtained from fresh thymic tissue of patients, who underwent cardiac surgery. Studies were approved by the local ethics committee of the Charité Berlin (approval no. EA4/128/09 and no. EA1/233/09) and patients gave informed consent.

Antibody staining and Flow cytometry

PBMCs were isolated by density gradient centrifugation (Ficoll-Hypaque; GE Healthcare) from fresh heparinized blood samples in order to avoid possible influences caused by freeze-thaw cycles. PBMCs were distributed into different tubes. In one tube PBMCs were incubated for 15 minutes at room

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temperature with the following conjugated monoclonal antibodies for later assessment of central and effector memory CD4 T cells as well as recent thymic emigrant CD4 T cells: anti-CD3 APC/Cy7 (clone UCHT1; BioLegend), anti-CD4 Alexa Fluor 700 (clone RPA-T4; BioLegend), anti-CD8 V500 (clone RPA-T8; BD Biosciences), anti-CD45RA PerCP/Cy5.5 (clone HI100; BioLegend),anti-CD45RO Pacific Blue (clone UCHL1; BioLegend), anti-CD197 (CCR7) Alexa Fluor 488 (clone TG8/CCR7; BioLegend), anti-CD62L APC (clone DREG-56; BioLegend), CD27 PE/Cy7 (clone O323; eBioscience) and anti-CD31 PE (clone WM59, BioLegend). PBMCs were washed and resuspended for flow-cytometric analysis. In a separate tube PBMCs were incubated for 15 minutes at room temperature with the following conjugated monoclonal antibodies for later assessment of RTEs of conventional and regulatory CD4 T cells: anti-CD3 APC/Cy7 (clone UCHT1; BioLegend), anti-CD4 Alexa Fluor 700 (clone RPA-T4; BioLegend), anti-CD8 V500 (clone RPA-T8; BD Horizon), anti-CD25 PE/Cy7 (clone 2A3; BD Horizon), anti-CD31 APC (clone AC128; Miltenyi), anti-CD45RA eFlour605 (clone HI100; eBioscience) and CD45RO Pacific Blue (clone UCHL1; BioLegend). After washing and resuspension PBMCs from the second tube were treated with fixation buffer and permeabilization buffer (eBioscience) and then incubated with anti-FOXP3 PE (clone PCH101; eBioscience) for 30 minutes at room temperature. PBMCs were then washed and resuspended for flow cytometric analysis. Phenotypic analysis of the samples was carried out using a BD™ LSRII (Becton Dickinson, Palo Alto, CA, USA) supported by FlowJo 9.3 software (TreeStar, Ashland, OR, USA).

For *in vitro* experiments, thymocytes and PBMCs were washed with PBS after stimulation and resuspended in PBS/ Flebogamma solution for blocking on ice for 20min. Cells were stained for 15min at room temperature with Propidium Iodide Staining solution and AnnexinV according to the manufacturer´s instructions (BD Pharmingen). After washing thymocytes and PBMCs were resuspended in AnnexinV binding buffer and analyzed by flow cytometry using a BD™ Fortessa flow cytometer (Becton Dickinson, Palo Alto, CA, USA) supported by FlowJo 9.3 software (TreeStar, Ashland, OR, USA).

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Thymocyte and PBMC cell culture

Fresh thymic tissue was obtained and manually prepared in sterile buffer solution (PBS with 2mM EDTA and 2%BSA) for tissue dissociation with the gentleMACS Dissociator (Miltenyi Biotech, Bergisch Gladbach, Germany) from patients, who had been partly thymectomized in the context of heart surgery. After dissociation, single cell suspensions were obtained by passing through 70μm and 40μm cell strainers with washing in between. Cells were then counted and resuspended in stimulation medium (RPMI-1640, 1%P/S, 10%FCS, 1%NEAA and 0.05mM beta-Mercaptoethanol). PBMCs were isolated by density gradient centrifugation (Ficoll-Hypaque; GE Healthcare) from fresh heparinized blood samples from healthy donors and resuspended in stimulation medium (RPMI-1640, 1% P/S, 10%FCS). Thymocytes and PBMCs where then supplemented with either ATG-F (Fresenius™ Biotech GmbH, Munich, Germany) or ATG-G (Thymoglobulin®, Genzyme™ Transplant, Cambridge, MA, USA) at following concentrations: 2.5μg/ml, 25μg/ml and 100μg/ml. No treatment was given to controls. Cells were redistributed in 96-well plates with $2x10^6$ cells in 500μl stimulation medium per two wells and incubated for 20h at 37°C. Cells were then co-stained with propidium iodide solution and AnnexinV and immediately analyzed by flow cytometry, in order to determine the level of necrosis and apoptosis.

Complement dependent cytotoxicity assay

Freshly isolated human thymocytes or PBMCs were resuspended at a density of $5x10^6$ cells/ml in culture medium containing 2.5μg/ml, 25μg/ml or 100 μg/ml ATG-F or ATG-G respectively. 100 μl of each suspension was added to a round bottom microtiter plate and incubated for 60 min at 4°. After addition of 100 μl freshly prepared human serum the cells were incubated for 60 min at 37°C. Finally the samples were stained with propidium iodide solution and immediately analyzed by flow cytometry.

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Statistics and software

Calculations were performed in GraphPad Prism v5.0 (GraphPad Software, La Jolla, CA). Group comparisons were made with the Mann-Whitney-U test.

Results

ATG-G treatment impairs CD4 but not CD8 T cell recovery

Within the CD3 T cell compartment we analyzed the recovery of conventional CD4 T cells (Tcon, **Figure 1A**) and regulatory CD4 T cells (Treg, **Figure 1B**). In the ATG-G group the total peripheral CD4+ Tcon and Treg cell counts were diminished by roughly 95% at day +15, and a significantly delayed recovery from day 30 to day 180 was observed compared to the patient cohort not treated with ATG-G. Mean CD4+ Tcon cell counts did not exceed 250 cells/μl at any time point after transplantation if treated with ATG-G, while mean Treg cell counts did not exceed 15 cells/μl. In contrast, there was no significant difference for the CD8 T cell recovery between the ATG-G treated versus non-treated cohort at any of the defined time points (**Figure 1C**).

T cell subset dependent recovery post alloHSCT

At next, we differentiated naïve (CD45RO-CD45RA+) and memory (CD45RO+CD45RA-) CD4+ Tcon and Treg. The effect of ATG-G was most pronounced in the naïve subsets of both Tcon (**Figure 2A**) and Treg (**Figure 2B**). The naïve cell counts were significantly lower in the ATG-G group at all time points after transplantation as compared to the non-ATG-G group.

Memory CD4 Tcon (**Figure 2C**) and Treg (**Figure 2D**) were also more severely depleted in the ATG-G group than in the non-ATG-G group, but showed an overall more rapid recovery starting from day +30 after transplantation than the respective naïve subsets. Memory T cells were further differentiated into central memory CD4 T cells (CM) and effector memory CD4 T cells (EM). CM CD4 T cells showed an almost complete depletion on day 15 in ATG-G treated patients. Their recovery was delayed until day 180 compared to the non-ATG-G group (**Figure 2E**). Levels of EM

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CD4 T cells were only significantly lower on day 15, and otherwise comparable in both groups (**Figure 2F**).

Impaired thymic generation of both naïve conventional and naïve FOXP3+ regulatory CD4+ T cells

Since the naïve cell subset in particular was depleted, we delineated the presence of RTE, in order to determine the thymic function and output of thymic T cells. We analyzed the recovery of the RTE CD4+ T cells based on the CD31 expression. Representative gates of Tcon RTE and Treg RTE are shown in **Figure 3A and 3B** respectively. In the ATG-G treated group a nearly complete lack of RTE of both Tcon and Treg cells was observed from day 15 until day 180. The Tcon RTE cell count initially decreased by 99.5% and its recovery did not start until day 180 (**Figure 3C**). Similarly, Treg RTE cells were strongly depleted and remained stable at very low levels throughout day 180 in the ATG-G group (**Figure 3D**). In contrast, no significant decrease was observed in the non-ATG-G group.

Differential effects of ATG-G and ATG-F on human thymocytes and PBMCs in vitro

We hypothesized, that ATG-G might also have a strong cytotoxic effect on human thymocytes as ATG-G treated patients revealed significantly lower RTE counts and ATG-G is manufactured by immunization of rabbits with human thymocytes. We compared ATG-G with ATG-F, as ATG-F is manufactured by immunization of rabbits with Jurkat cells and not thymocytes. The *in vitro* cytotoxic effect of both ATG-preparations on cultured human thymocytes (**Figure 4A**) and PBMCs (**Figure 4B**) was studied at concentrations of 2.5μg/ml, 25μg/ml and 100μg/ml. In order to determine levels of necrosis and apoptosis, we co-stained for PI and AnnexinV. An untreated population was used to define the basal level of apoptotic and dead cells. The cytotoxic effect of ATG was generally higher for the thymocytes compared to the PBMCs and most prominent in the PI+/AnnexinV+ cells, characterizing the late apoptotic and necrotic cells shown in **Figure 4**. ATG-G treatment of thymocytes with 25 μg/ml ($p<0.05$) and PBMCs with 100 μg/ml

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($p<0.01$) led to significantly more PI+/ AnnexinV+ cells than ATG-F treatment. The difference between the two types of ATG for the PI-/AnnexinV+ and PI+/AnnexinV- cells was not significant at any concentration for thymocytes and PBMCs (data not shown).

In order to investigate complement-mediated lysis as another important mechanism, we performed a complement-dependent cytotoxicity assay in accordance to Popow et al. (18) showing a dose-dependent specific lysis of thymocytes (**Figure 5A**) as well as PBMCs (**Figure 5B**) by both ATG types. ATG-G caused more complement-mediated lysis of thymocytes and PBMCs than ATG-F at the concentrations 25 and 100μg/ml, respectively.

Discussion

In our study, we analyzed the effect of ATG-G on thymic function and reconstitution of the T cell compartment, early after alloHSCT. Our initial findings demonstrated a significantly delayed recovery of total CD4 T cell counts in ATG-G treated patients, whereas total CD8 T cell counts were similar in ATG-G treated and non-treated patients. A number of studies found a more rapid recovery of CD8 than CD4 T cells in alloHSCT patients (19, 20), and various factors as for example higher age and toxicity of the conditioning regimen have been associated with delayed CD4 T cell recovery (21-23). A functional thymus has been shown to be necessary for the reconstitution of naïve CD4 T cells, whereas thymic-independent pathways seem to exist to regenerate naïve CD8 T cells (24). Our study suggests, that ATG-G treatment is an additional factor that is associated with severe and prolonged thymic dysfunction after alloHSCT.

ATG-G treated patients exhibited a reconstitution failure of RTE of both Tcon and Treg in peripheral blood, with hardly any recovery until day +180 after alloHSCT. Regarding Tcon our results support previous findings of Sairafi *et al.*, who reported on reduced TREC levels in ATG-G treated patients in a retrospective study (25). Regarding Treg we found an impairment of thymic generation of Treg, which has not been shown yet. Matsuoka *et al.* (26) have demonstrated abnormal Treg homeostasis after alloHSCT in non-ATG-G treated patients with limited thymic generation of Treg and disturbed

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proliferative and apoptotic patterns. In this present study we could show, that ATG treatment is aggravating the imbalance by further limiting the thymic output. Normal Treg homeostasis may be crucial especially after alloHSCT, as Tregs regulate responses to allogeneic target antigens (27) and therefore are important in controlling GvHD (28-30).

Results from *in vitro* studies (31, 32) and solid organ transplantation (33) suggested that ATG-G is capable of promoting Treg expansion thus contributing to GvHD prophylaxis. On the other hand, impaired thymic generation of Treg, as found in our study, may favor GvHD. Total body irradiation (TBI) is also known to negatively influence thymocyte viability (6). A possible potentiation of irradiation and ATG-G effects cannot be excluded and might have additionally influenced RTE recovery in this study (**Table 1**).

In regard to the duration of ATG-G effects, Waller et al. have performed pharmacokinetic analyses showing a rapid clearance of active ATG-G (half-life t (1/2) six days) to sub-therapeutic levels (<1μg/mL) by a median of 17 ± 9 days post transplantation at comparable treatment conditions (34). They postulated that slow and prolonged T cell recovery was therefore not due to effects of ATG-G, assuming ATG-G activity only if present in therapeutic levels. However, in view of our results for Tcon RTE and Treg RTE recovery we suggest, that ATG-G also targets the thymus with long-term consequences for the thymic T cell recovery beyond week three after transplantation. ATG-G is prepared by immunizing rabbits with cells derived from the thymus organ, so that ATG-G should contain antibodies potentially targeting all cells, which are harbored within the thymus. The complex architecture of the human thymus is difficult to mimic in an experimental setup and it is thus not possible to determine *in vitro* concentrations of ATG that realistically reflect those concentrations of ATG that would be found within the thymus *in vivo* in the clinical situation of alloHSCT. Additionally, human thymic tissue is difficult to obtain, as it is hardly bioptically accessible. We thus decided to test different concentrations of ATG-G in a simplified experimental approach with fresh cultured human thymocytes. While induction of apoptosis by ATG-G in PBMCs has been shown previously (35), our results show an

apoptotic effect of ATG-G also on cultured human thymocytes. Furthermore, thymocytes appear to be more susceptible to ATG-G induced cell death than PBMCs. As ATG-F is produced by immunization of rabbits against a Jurkat cell line and not against human thymocytes, we also compared the effect of ATG-F and ATG-G on thymocytes. We could show a significantly stronger pro-apoptotic effect (late apoptosis) of ATG-G on thymocytes than ATG-F at 25μg/ml. In a recent study Popow et al. could show complement dependent lysis to be an important mechanism for ATG mediated cytotoxicity (18). Our results suggest that complement dependent lysis is of equal importance in thymocyte toxicity of ATG-G.

Our *in vitro* data appear consequential in view of the different production methods of the two ATG-preparations and support the assumption, that ATG-G directly affects the thymus. In this respect it would be interesting to perform comparative clinical studies with other T-cell depleting therapeutic approaches such as alemtuzumab or ATG-F, all differing in their biologic activity. However, there are only few studies comparing the different *in vivo* T cell depletion treatments in alloHSCT patients, for example by Soiffer et al. investigating ATG-containing and alemtuzumab-containing regimens (36) or by Basara et al. analyzing ATG-G and ATG-F in regard to GvHD and relapse risk (37). In view of our results thymic function should be examined in detail in future comparative studies, as this might help with the choice of T cell depleting treatment for different clinical indications.

While recovery of naïve T cells was almost completely suppressed within in the first six months in ATG-G treated patients, memory T cell counts continuously increased, but were delayed compared to patients without ATG-G therapy. Looking at the memory compartment, our results suggest a preferential effect of ATG-G on CM CD4 T cells compared to EM CD4 T cells in the setting of alloHSCT. To our knowledge this has so far only been shown in solid organ transplantation (33, 38). It has been suggested that CD4 memory T cells can cause a GvL-effect without causing GvHD in mice (39), a phenomenon that has more recently been ascribed to EM CD4 T cells in

mouse models (40, 41). From clinical studies on the other hand it is known that ATG-G contributes to prevention of GvHD without causing higher relapse rates in humans. The shift in the CD4 memory compartment after ATG-G treatment in favor of EM CD4 T cells in our study might thus be interpreted as an indication for one of the mechanisms underlying this mode of action of ATG-G.

Taken together, our findings suggest that ATG-G, which is raised against human thymocytes, may directly affect thymic cells and result in functional deficits. However, due to the rather small sample number, these data need to be confirmed in larger studies. In this respect, we suggest further comparative trials of different T-cell depleting approaches. Another possible consequence from our study is that thymus-protective and/or – supportive therapies as keratinocyte growth factor, Interleukin 7, growth hormone and others, which are already discussed as treatment approaches to limit thymic damages by conditioning regimens (irradiation / chemotherapy) or GvHD, should be considered especially when ATG-G is used as GvHD prophylaxis.

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Authorship and Disclosures

IKN designed experiments, analyzed results and wrote the paper, FW designed and performed experiments, analyzed results and wrote the paper, MD designed and performed flow cytometry experiments, AH performed flow cytometry experiments and analyzed results from flow cytometry experiments, AM designed and performed the *in vitro* experiments with human thymocytes and PBMCs and analyzed the results accordingly, DK contributed to experimental design of flow cytometry experiments and supported these experiments, OB, IB, ET and LU provided conceptual insight and helped interpreting clinical results, CS provided important conceptual insight and

contributed to analysing results, KR designed experiments, provided clinical data and interpreted results, AT provided important conceptual insight, contributed to analyzing results and helped in writing the paper.

The authors have no conflicts of interest or financial ties to disclose.

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Table

	Group ATG-G	%	***Group non-ATG-G***	%
	n=12 (d+180 n=11)		n=8 (d+180 n=3)	
Age, median (range)	50 (27-68)		57 (34-66)	
Male	8	**66**	3	**38**
Disease				
CLL			1	**13**
ALL	2	**17**		
AML	10	**83**	7	**87**
Donor				
MRD			7	**87**
MUD	12	**100**	1	**13**
Pretransplant conditioning				
MAC	9	**75**	4	**50**
Cy/12 Gy TBI	*8*	*66*	*2*	*25*
Fludara/8 Gy TBI	*1*	*8*		
Bu/Cy			*1*	*13*
MitoFLAG			*1*	*13*
RIC	3	**25**	3	**38**
Fludara/Treo	*2*	*17*	*2*	*25*
FLAMSA	*1*	*8*	*1*	*13*
nonMAC			1	**13**
Fludara/2 Gy TBI			*1*	*13*
CMV Status				

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R1/D1	4	**34**	4	**50**
R1/D0	3	**25**	1	**13**
R0/D1	1	**8**	1	**13**
R0/D0	4	**34**	2	**25**
Acute GvHD grade after transplant				
0	3	**25**	2	**25**
I-II	7	**58**	5	**63**
III-IV	2	**17**	1	**13**
CMV-Reactivation	7	**58**	3	**38**
EBV-Reactivation	8	**66**	2	**25**

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Table 1. Patient characteristics

Abbreviations: AML, acute myeloid leukaemia; ALL, acute lymphatic leukaemia; Bu, busulfane; CLL, chronic lymphatic leukaemia; Cy, cyclophosphamide; FLAMSA, Fludarabin, Cytosin-Arabinosid and Amsacrin; Fludara, fludarabine; GvHD, graft-versus-host disease; Gy, Gray; MAC, myeloblative conditioning; MitoFLAG, Mitoxantrone, Fludarabine, Cytosin-Arabinosid and granulocyte-colony stimulating factor; nonMAC, non-myeloablative conditioning; RIC, reduced intensity conditioning; TBI, total body irradiation; Treo, Treosulfane.

Legend to Figures

Figure 1. CD4 T lymphopenia in ATG-G treated patients.

Graphs illustrate (A) conventional CD4 (Tcon; CD3+CD4+CD25lowFOXP3- lymphocytes), (B) regulatory CD4 (Treg; CD3+CD4+CD25highFOXP3+ lymphocytes) and (C) CD8 (CD3+CD8+ lymphocytes) T cell subsets (mean values of absolute numbers of cells/µl ± with standard error of mean (SEM)) before (pre) and after transplantation. Differences in cell counts between ATG-G and non-ATG-G treated patients were statistically evaluated using the Mann Whitney test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

Figure 2. Reconstitution of CD4 T cell naïve and memory compartment

Graphs illustrate (A) naïve conventional CD4 T cells (naïve Tcon; CD25lowFOXP3-CD45RA+), (B) naïve regulatory CD4 T cells (naïve Treg; CD25highFOXP3+CD45RA+), (C) memory Tcon (CD25lowFOXP3-CD45RO+CD45RA-) and (D) memory Treg (CD25highFOXP3+CD45RO+CD45RA-). Graphs (E) and (F) illustrate central memory (CM, CD45RO+CD62L+CCR7+) and effector memory (EM, CD45RO+CD62L-CCR7-) subsets of total CD4 T cells respectively. Data were collected before (pre) and after transplantation. Mean values of absolute numbers of cells/µl ± standard error of mean (SEM) are given. Differences in

cell counts between ATG-G and non-ATG-G treated patients were statistically evaluated using the Mann Whitney test. * P < 0.05; ** P < 0.01; *** P < 0.005.

Figure 3. Reduced thymic generation of Tcon and Treg in ATG-G treated patients.

Representative gate of (A) naïve conventional CD4 T cells (naïve Tcon; CD25lowFOXP3-CD45RA+) and (B) naïve regulatory CD4 T cells (naïve Treg; CD25highFOXP3+CD45RA+) for identification of CD31+ recent thymic emigrants (RTE). Graphs illustrate mean values of absolute numbers of cells/µl ± standard error of mean (SEM) before (pre) and after transplantation of (C) TconRTE and (D) TregRTE. Differences in cell counts between ATG-G and non-ATG-G treated patients were statistically evaluated using the Mann Whitney test. * P < 0.05; ** P < 0.01; *** P < 0.005.

Figure 4. Differential effect of ATG-G and ATG-F on human thymocytes and PBMCs.

Percentage of PI+/AnnexinV+ (A) thymocytes and (B) PBMCs (mean values with standard error of mean (SEM)) after treatment with either ATG-G (dark grey) or ATG-F (light grey) for 20 hours at different concentrations. Representative dot plots for thymocytes and PBMCs are shown (upper images). Differences in percentages of PI+/AnnexinV+ cells were statistically evaluated using the Mann Whitney test. * P < 0.05; ** P < 0.01; *** P < 0.005. Different ATG concentrations were tested in three independent experiments

Figure 5. Dose-dependent complement-mediated lysis of thymocytes and PBMC by ATG-G and ATG-F.

Percentage of PI+ (A) thymocytes and (B) PBMC (mean values with standard error of mean (SEM), n=3) after complement-mediated specific lysis after treatment with either ATG-G (filled circles) or ATG-F (open triangles) at different concentrations. Percntage of PI+ cells were tested in two independent experiments.

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Figure 1

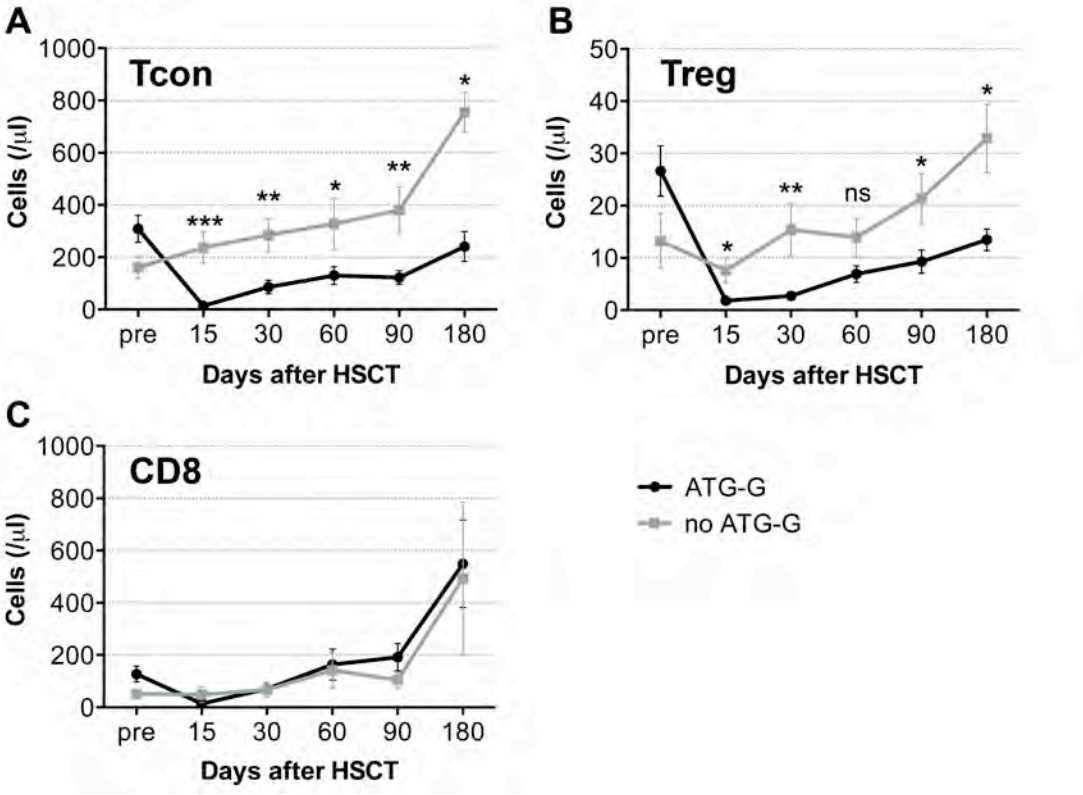

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Figure 2

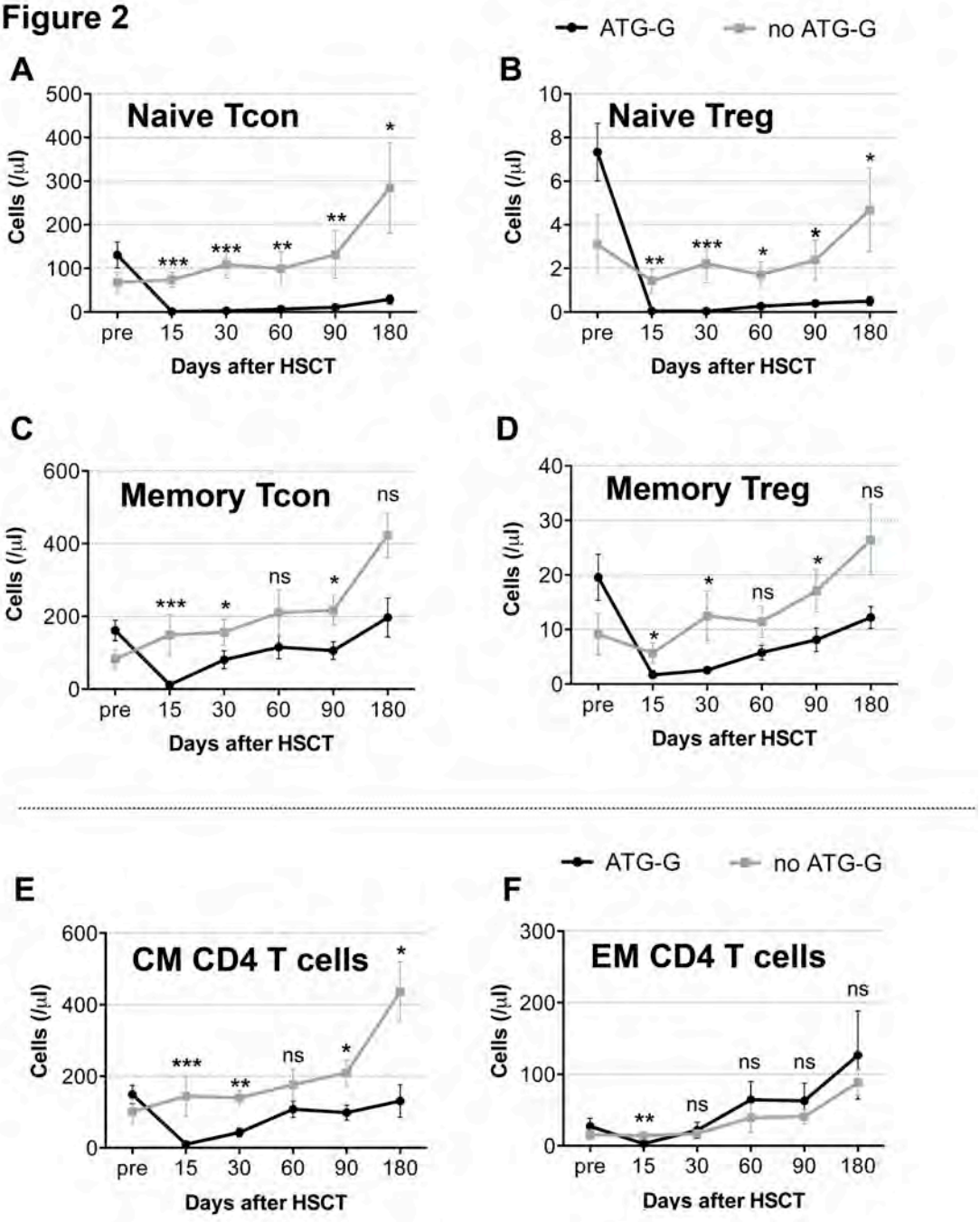

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Figure 3

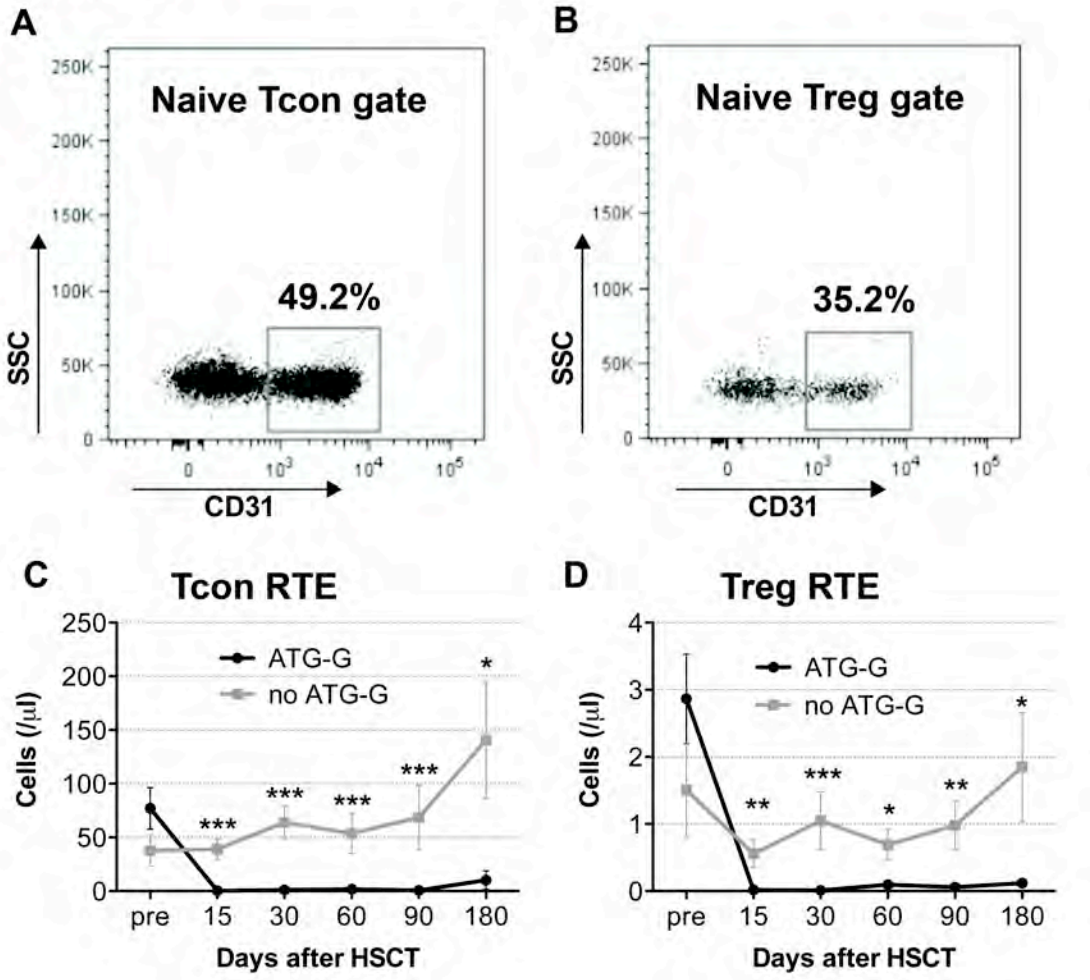

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Figure 4

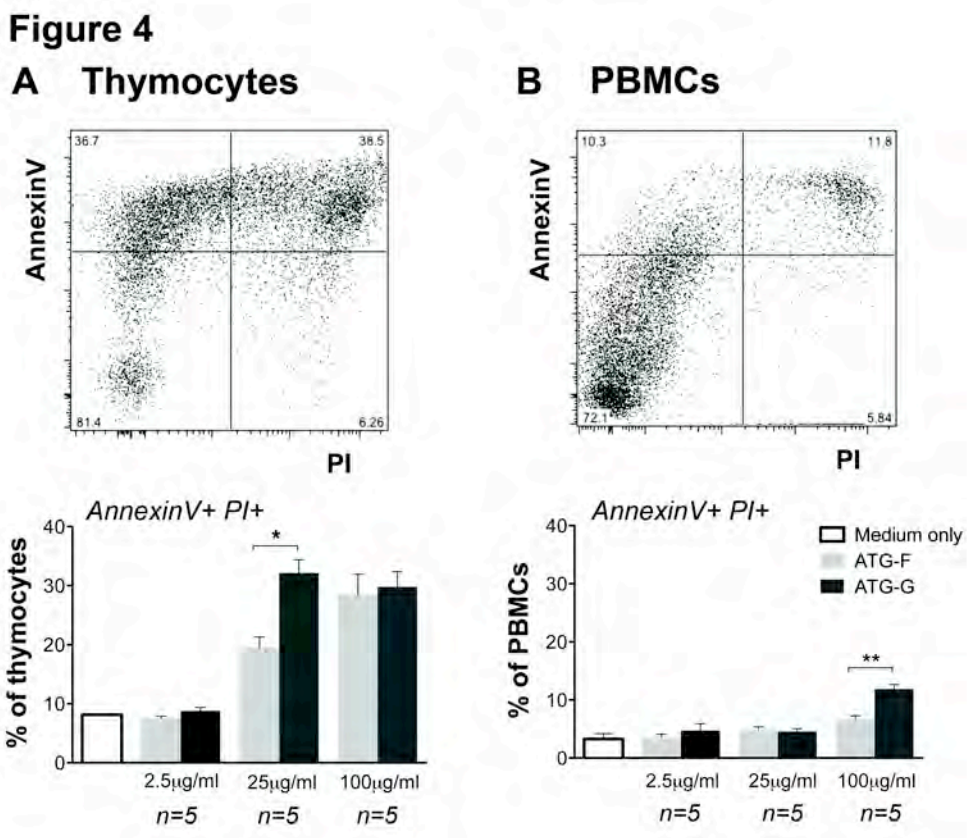

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Figure 5