Leukemia Research Reports 2 (2013) 82–85

Contents lists available at ScienceDirect

Leukemia Research Reports

journal homepage: www.elsevier.com/locate/lrr

Brief communication

Rabbit antithymocyte globulin induces rapid expansion of effector memory CD8 T cells without accelerating acute graft versus host disease ☆

Friedrich Wittenbecher [a], Kathrin Rieger [a], Mikalai Dziubianau [d], Anne Herholz [b], Angela Mensen [e], Igor Wolfgang Blau [a], Lutz Uharek [a], Bernd Dörken [a,f], Andreas Thiel [c], Il-Kang Na [a,e,f,*]

[a] Department of Hematology, Oncology and Tumor Immunology, Charité, Berlin, Germany
[b] Department of Medicine, Division of Gastroenterology, Infectiology and Rheumatology, Charité, Berlin, Germany
[c] Regenerative Immunology and Aging, Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Charité CVK, Berlin, Germany
[d] Renal and Transplant Research Unit, Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Charité CVK, Berlin, Germany
[e] Institute of Medical Immunology, Charité CVK, Berlin, Germany
[f] Experimental and Clinical Research Center (ECRC), Berlin, Germany

ARTICLE INFO

Article history:
Received 8 June 2013
Received in revised form
24 July 2013
Accepted 15 September 2013
Available online 27 September 2013

Keywords:
HSCT
antithymocyte globulin
CD8 T cell
effector memory
naive

ABSTRACT

Rabbit antithymocyte globulin (Thymoglobulin®) is commonly used as graft-versus-host disease (GvHD) prophylaxis. Since we found similar total CD8 T cell numbers in patients with and without Thymoglobulin® therapy within the first six months after allogeneic hematopoietic stem cell transplantation, we have analyzed the reconstitution of the CD8 T cell compartment in detail. After T cell-depletion, higher and more sustained proliferative capacity of memory CD8 T cells resulted in their rapid expansion, whereas the fraction of naive CD8 T cells decreased. Importantly, this shift towards effector memory CD8 T cells did not accelerate the incidence of GvHD.

1. Introduction

One of the major complications associated with allogeneic hematopoietic stem cell transplantation (alloHSCT) is the high incidence of acute graft-versus-host disease (GvHD) caused by donor T-lymphocytes. Depletion of donor T-lymphocytes is a well-established therapeutic option for GvHD-prophylaxis. Recently, we showed a sustained depletion of conventional and regulatory CD4 T cells in patients treated with ATG-Genzyme™ (Thymoglobulin®, ATG-G) [1], a polyclonal antibody preparation containing a broad spectrum of antibodies directed against T cells [2] and potentially thymic stromal cells. Interestingly, total CD8 T cell counts were similar in ATG-G treated and untreated patients[1]. In principle, T cell recovery after transplantation can take place through thymus dependent regeneration as well as by proliferation of existing T cells [3].

Here, we addressed whether the rapid CD8 T cell recovery after ATG-G depletion resulted from accelerated CD8 T cell regeneration or increased peripheral CD8 T cell proliferation and in how far this influenced GvHD incidence. Therefore, we studied the distribution of different CD8 T cell subsets and their proliferative capacity in ATG-G treated patients of the same patient cohort.

2. Material and methods

A detailed description of methods and patient cohorts can be found in Na et al. [1] (approval by the Charité – Berlin local ethics committee no. EA4/128/09). Briefly, we compared CD8 T cell reconstitution in 12 alloHSCT patients receiving ATG-G according to standard procedure protocols (ATG-G group, for $d+180$: $n=11$) with 8 alloHSCT patients not receiving ATG-G (noATG-G group, for $d+180$: $n=3$). Patient characteristics and clinical outcome are

* Corresponding author at: Charité – Campus Virchow-Klinikum, Hämatologie, Onkologie und Tumorimmunologie Augustenburger Platz 1 13353 Berlin, Germany. Tel.: +49 30450553111; fax: +49 30450553914.
E-mail address: il-kang.na@charite.de (I.-K. Na).

2213-0489/$ - see front matter © 2013 The Authors. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.lrr.2013.09.001

Table 1
Patient characteristics and clinical outcome.

	Patient no.	*Age*[a](years)	*Diagnosis*	*Donor*	*Conditioning regimen*	*Deceased*[b]	*Leukocyte engraftment*[c]	*Grade of aGVHD*
ATG-G group	1	50	AML	MUD	Cy/TBI		*d*+15	I
2 *mg/kg/day on days* −3,−2,−1 *before transplantation*	2	27	AML	MUD	Cy/TBI		*d*+18	III
	3	28	ALL	MUD	Cy/TBI		*d*+15	I
	4	48	AML	MUD	Cy/TBI	x	d+12	I
	5	57	ALL	MUD	Cy/TBI		*d*+13	III
	6	58	AML	MUD	Fludara/Treo		*d*+16	I
	7	50	AML	MUD	Cy/TBI		*d*+14	None
	8	69	AML	MUD	FLAMSA		*d*+13	II
	9	60	AML	MUD	Fludara/Treo		*d*+16	I
	10	47	AML	MUD	Cy/TBI		*d*+13	None
	11	57	AML	MUD	Fludara/8 Gy TBI		*d*+17	None
	12	48	AML	MUD	Cy/TBI		*d*+13	I
noATG-G group	13	58	AML	MRD	Fludara/Treo		*d*+15	II
	14	41	AML	MRD	FLAMSA	x	*d*+8	None
	15	34	AML	MRD	Cy/TBI	x	*d*+17	None
	16	57	AML	MRD	Cy/TBI	x	*d*+15	II
	17	40	AML	MUD	Bu/Cy	x	*d*+30	III
	18	61	AML	MRD	MitoFLAG	x	*d*+17	I
	19	57	AML	MRD	Fludara/Treo		*d*+19	II
	20	67	CLL	MRD	Fludara/2 Gy TBI		*d*+17	II

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphatic leukemia; Bu, busulfane; CLL, chronic lymphatic leukemia; Cy, cyclophosphamide; FLAMSA, Fludarabin, Cytosin-Arabinosid and Amsacrin; Fludara, fludarabine; aGvHD, acute graft-versus-host disease; Gy, Gray; MitoFLAG, Mitoxantrone, Fludarabine, Cytosin-Arabinosid and granulocyte-colony stimulating factor; MMF, mycophenolate mofetil; MTX, methotrexate; TBI, total body irradiation; Treo, treosulfane.

[a] Age at transplantation.
[b] Deceased between days +90 and +180 after transplantation.
[c] Time to leukocyte engraftment in days after transplantation.

presented in Table 1. Indications for transplantation were hematologic malignancies in all cases (AML $n=17$, ALL $n=2$, CLL $n=1$). Patients received ATG-G additionally to standard conditioning regimens if transplanted with grafts from matched-unrelated donors (MUD) as opposed to matched related donors (MRD) in the noATG-G group. One patient receiving an MUD graft did not receive ATG-G.

Multicolor flow cytometry (Becton Dickinson LSRII supported by FlowJo 9.3 software, TreeStar) was used to identify different CD8 T cell subsets after standard staining procedures of peripheral blood mononuclear cells (PBMC) from fresh heparinized blood samples. The following antibodies were used to characterize the CD8 T cell subsets: 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), and anti-CD197 (CCR7) Alexa Fluor 488 (clone TG8/CCR7; BioLegend). For evaluation of Ki67 expression stainings were performed using 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-CD45RA eFlour605 (clone HI100; eBioscience) and anti-Ki67 FITC (clone B56, BD Pharmingen). CD3 T cell counts were obtained by staining whole blood samples with CD3-Cy5 (clone UCTH-1, DRFZ, Berlin) and using BD TruCount™ controls (BD Biosciences). For group comparisons the Mann-Whitney-U test was used (GraphPad Software, La Jolla, CA).

3. Results

Analysis of the CD3 T cell compartment revealed comparable cell counts in both groups except day 15 after transplantation with significantly lower absolute counts in the ATG-G group (Fig. 1A; 46.29 cells/μl ± 11.61 (SEM) versus 365.1 cells/μl ± 102.8 (SEM), $p<0.005$). The share of $CD8^+$ T cells was significantly higher in the ATG-G group at all time points post transplantation except *d*+180 (Fig. 1B). On day 15 we observed high rates of CD8 T cell proliferation only in the ATG-G group, as shown by Ki67 staining (Fig. 1C,D; 49.0% ± 6.8% in ATG-G group versus 11.0% ± 2.6% in noATG-G group on *d*+15, $p<0.01$). We then compared proliferative activity of $CD45RA^+$ and $CD45RA^-$ CD8 T cells within the ATG-G group. Proliferation rates of $CD45RA^-$ CD8 T cell subsets were higher at all time points after transplantation as compared to $CD45RA^+$ CD8 T cell subsets with significant differences on days 60, 90 and 180 (Fig. 1E,F). At day 15 $CD45RA^+$ CD8 T cells also showed a strikingly high proportion of $Ki67^+$ cells (35.1% ± 7.7%). However, at day 30 this share dropped (3.2% ± 0.9%) and remained low.

Next, we used a gating strategy originally suggested by Sallusto et al. [4] to assess naive ($CD45RA^+CCR7^+$), central memory (CM, $CD45RA^-CCR7^+$), effector memory (EM, $CD45RA^-CCR7^-$) and effector ($CD45RA^+CCR7^-$) CD8 T cells (Fig. 2A). We observed significantly higher frequencies of EM CD8 T cells in the ATG-G group at all time points except *d*+180 (Fig. 2B). In contrast, frequencies of naive CD8 T cells were significantly lower in ATG-G treated patients compared to patients without ATG-G treatment at all time points post transplantation (Fig. 2C). The frequencies of CM and effector CD8 T cells were comparable in both groups (Fig. 2D, E).

4. Discussion

Investigating the impact of ATG-G as a T cell depleting strategy [2], as expected we observed lower CD3 T cell counts in the ATG-G group. Considering the half-life of ATG-G (clearance to subtherapeutic levels by a median of 17 ± 9 days [5]), unsurprisingly there was only a significant difference of CD3 T cell counts early after alloHSCT. The observed persistent increase of CD8 T cell frequencies is likely to be mainly due to decreased CD4 T cell counts as previously described [1].

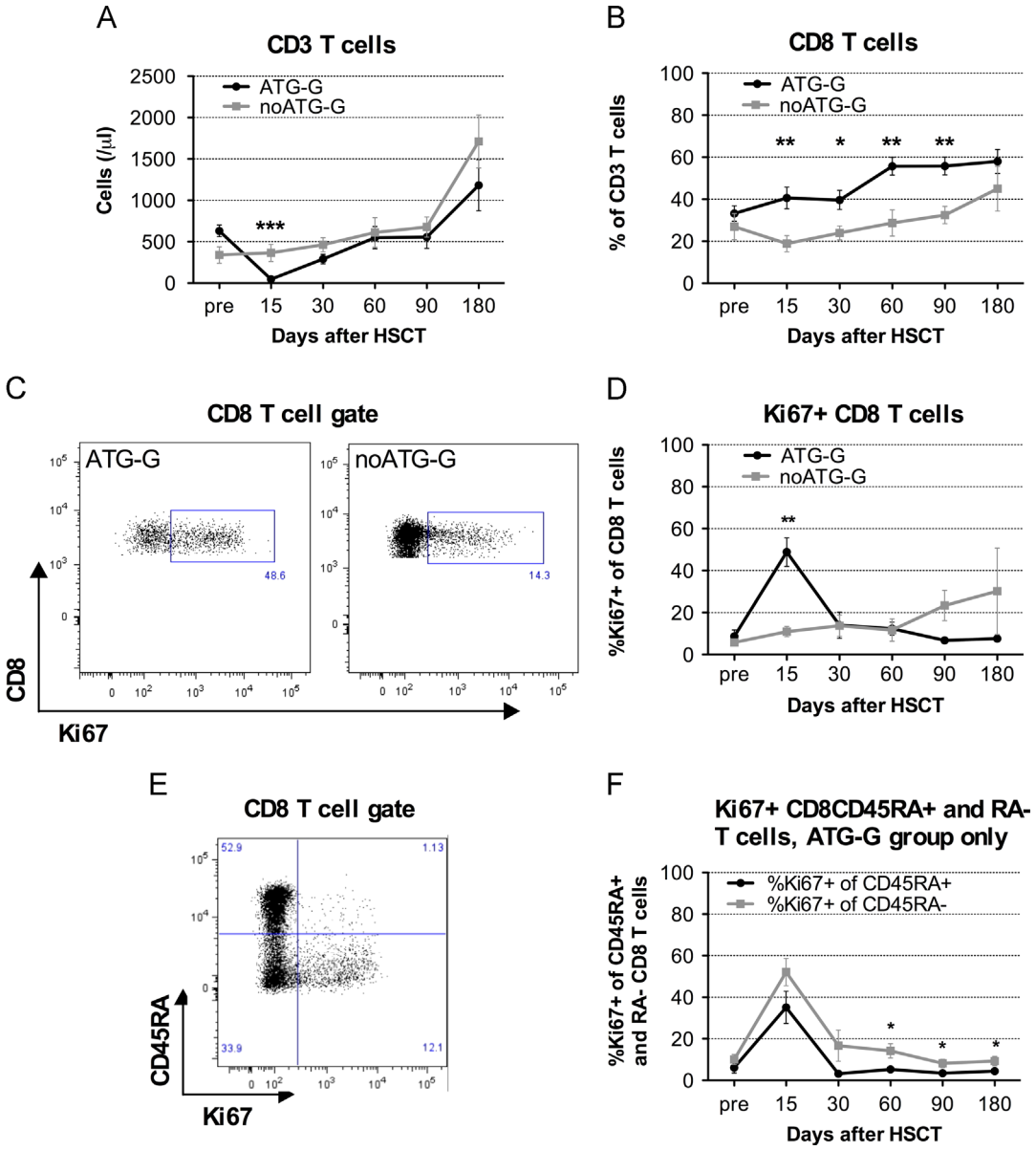

Fig. 1. Proliferative capacity of CD8 T cells after alloHSCT. Mean values of (A) CD3 T cell counts (cells/µl ± standard error of mean (SEM)) and (B) CD8 T cell frequencies (% ± SEM) are shown. Proliferation of total (C, D) and CD45RA$^+$ and RA- (E, F; ATG-G group only) CD8 T cells was measured by Ki67 analysis. For statistical evaluation the Mann Whitney U test was used. *$p < 0.05$; **$p < 0.01$; ***$p < 0.005$.

Analysis of the CD8 T cell compartment in detail revealed a shift towards high EM and low naive CD8 T cell frequencies in the ATG-G group. This shift was observed up to $d+180$ after alloHSCT, suggesting indirect long-term effects resulting from ATG-G treatment. Low shares of naive CD8 T cells could be the consequence of a rapid transition into the memory pool or thymic impairment due to ATG-G as previously suggested for CD4 T cell regeneration [1]. The rapid increase of EM CD8 T cells appears to be driven mainly by homeostatic mechanisms in the ATG-G depleted host, since concomitant high rates of CD8 T cell proliferation were found around day 15 in the ATG-G group only. This hypothesis is supported by consistently higher proliferation rates of CD45RA$^-$ CD8 T cells as compared to CD45RA$^+$ CD8 T cells within the ATG-G group. However, it has previously been shown that changes towards an EM phenotype from other subsets such as naive CD8 T cells are also induced by T cell receptor stimulation and that CM CD8 T cells undergo various phenotypic changes upon cytokine stimulation, amongst others towards an EM phenotype [6]. It cannot be excluded that both these mechanisms contribute to the expansion of the EM CD8 T cell subset. Yet, the same reports also support the notion that EM CD8 T cells are especially receptive to cytokine stimulation [6] thus favoring the hypothesis of homeostatic proliferation.

In line with larger clinical studies demonstrating ATG-G efficacy especially for prevention of severe acute GvHD (reviewed in [7]), acute GvHD-rates in both patient groups in our study were similar even though ATG-G treated patients were transplanted with MUD grafts as opposed to MRD grafts in the noATG-G group and thus were at higher risk of GvHD (acute GvHD ATG-G group versus noATG-G group: Grade I and II 58% versus 63%, Grade III and IV 17% versus 13%). Consequently, the initial T cell depletion by ATG-G limited GvHD in MUD transplantations as intended. Yet, this also suggests that the significant expansion of EM CD8 T cells in the ATG-G group is not driving GvHD. Results from mouse studies equally suggest that memory CD8 T cells are less potent in mediating acute GvHD than naive CD8 T cells [8]. EM CD8 T cells appear to be favorable over CM CD8 T cells in this respect in mice [9,10]. Likewise, *in vitro* studies with human material from healthy

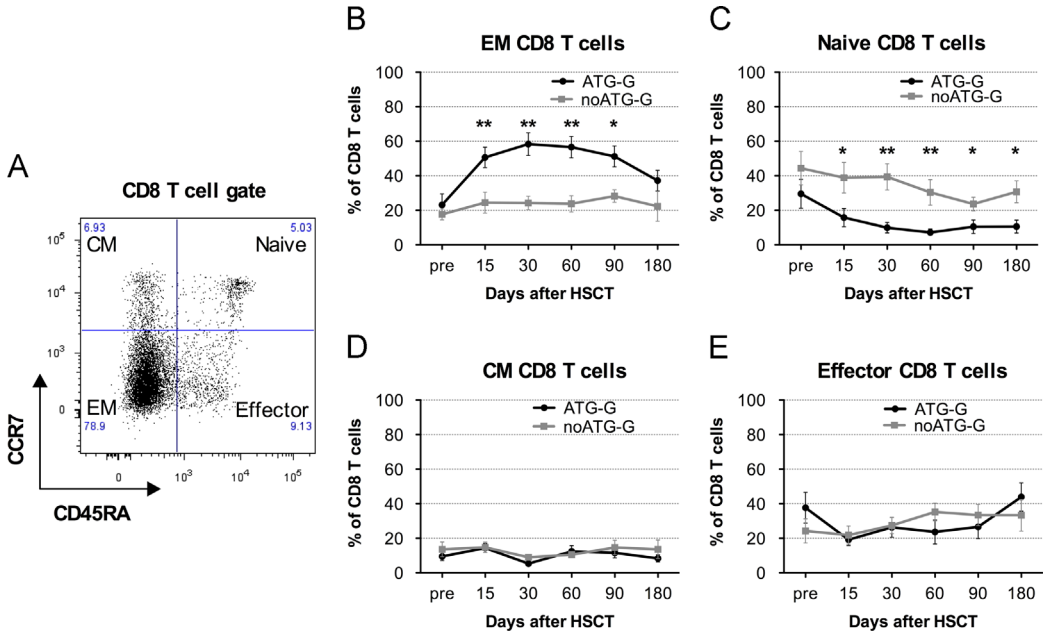

Fig. 2. Reconstitution of CD8 T cell subsets after alloHSCT. (A) Gating strategy according to Sallusto et al. (B–E) Frequencies of CD8 T cell subsets in ATG and noATG groups. Mean values of frequencies (% ± SEM) are shown. For statistical evaluation the Mann Whitney U test was used. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$.

donors indicate an increased alloreactivity of naive CD8 T cells as compared to memory CD8 T cells under certain conditions [11].

In summary, our data suggest that rapid recovery of CD8 T cells after ATG-G induced T cell depletion in alloHSCT patients is due to peripheral homeostatic proliferation of EM CD8 T cells and not due to accelerated regeneration of naive CD8 T cells. This leads to a characteristic shift to EM CD8 T cells that is not associated with higher rates of GvHD.

Authorship and Disclosures

FW designed and performed experiments, analyzed and interpreted results and clinical data and wrote the paper, KR designed experiments, provided clinical data and interpreted results, AH performed experiments and analyzed results, MD designed and performed experiments, AM analyzed and interpreted results, IWB, LU and BD provided and interpreted clinical data and provided important conceptual insight, AT provided important conceptual insight, contributed to analyzing results and helped in writing the paper, IKN designed experiments, analyzed and interpreted results and wrote the paper. All authors approved the manuscript.

References

[1] Na IK, Wittenbecher F, Dziubianau M, Herholz A, Mensen A, Kunkel D, et al. Rabbit antithymocyte globulin (Thymoglobulin(R)) impairs the thymic output of both conventional and regulatory CD4+ T cells after allogeneic hematopoietic stem cell transplantation in adult patients. *Haematologica* 2013;98(1):23–30.

[2] Mueller T. Mechanisms of action of thymoglobulin. Transplantation 2007;84:S5–S10.

[3] Krenger W, Blazar BR, Hollander GA. Thymic T-cell development in allogeneic stem cell transplantation. Blood 2011;117:6768–76.

[4] Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 1999;401:708–12.

[5] Waller EK, Langston AA, Lonial S, Cherry J, Somani J, Allen AJ, et al. Pharmacokinetics and pharmacodynamics of anti-thymocyte globulin in recipients of partially HLA-matched blood hematopoietic progenitor cell transplantation. Biology of Blood and Marrow Transplantation 2003;9:460–71.

[6] Geginat J, Lanzavecchia A, Sallusto F. Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines. Blood 2003;101:4260–6.

[7] Theurich S, Fischmann H, Shimabukuro-Vornhagen A, Chemnitz JM, Holtick U, Scheid C, et al. Polyclonal anti-thymocyte globulins for the prophylaxis of graft-versus-host disease after allogeneic stem cell or bone marrow transplantation in adults. Cochrane Database System Review 2012;9:CD009159.

[8] Dutt S, Baker J, Kohrt HE, Kambham N, Sanyal M, Negrin RS, et al. CD8+CD44(hi) but not CD4+CD44(hi) memory T cells mediate potent graft antilymphoma activity without GVHD. Blood 2011;117:3230–9.

[9] Zheng H, Matte-Martone C, Jain D, McNiff J, Shlomchik WD. Central memory CD8+ T cells induce graft-versus-host disease and mediate graft-versus-leukemia. Journal of Immunology 2009;182:5938–48.

[10] Chen BJ, Cui X, Sempowski GD, Liu C, Chao NJ. Transfer of allogeneic CD62L-memory T cells without graft-versus-host disease. Blood 2004;103:1534–41.

[11] Distler E, Bloetz A, Albrecht J, Asdufan S, Hohberger A, Frey M, et al. Alloreactive and leukemia-reactive T cells are preferentially derived from naive precursors in healthy donors: implications for immunotherapy with memory T cells. Haematologica 2011;96:1024–32.