T-cell immune response controls the high incidence of adenovirus infection in adult allogenic hematopoietic transplantation recipients

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Supplementary Material

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METHODS

Study design

A prospective cohort study of consecutive cases was conducted at the University Hospital Virgen del Rocío, Spain, from February 2016 to May 2018. All patients over 14 years old who received an allogeneic HSCT and signed the informed consent were included. Patients were excluded if they did not give written informed consent or if the severity of the initial clinical status precluded drawing blood samples. The study was approved by the Ethics Committee for Clinical Research of the University Hospital Virgen del Rocío, Seville, Spain (C.I. 1058-N-15).

Patient inclusion and follow-up

The study was carried out during the first 100 days after transplantation. At the time of admission for allo-HSCT, informed consent was requested. During the follow-up, blood samples were collected to quantify the HAdV viral load, weekly from day -7 to day +50

and biweekly from then until day +100 after transplantation (13 samples). In addition, three blood samples, at days +21, +56 and +100 after transplantation, were collected to evaluate the HAdV-specific T-cell immune response development. Those patients with an alternative donor (cord blood or haploidentical) and/or developed subsequent GVHD grade III-IV were considered high-risk patients for HAdV infection.

Determination of HAdV viral load

HAdV viral load was determined in plasma using the ADENOVIRUS R-GENE® Kit (bioMérieux, Madrid, Spain). Plasma samples were processed for viral genome extraction using the MagNA Pure Compact Nucleic Acid Isolation kit (Roche, Madrid, Spain). The isolated DNA was used as a template for quantitative real-time PCR, and a negative control without DNA was used as a control for PCR contamination. HAdV viremia was analysed at any level of HAdV DNAemia, in peripheral blood, and in the subgroup of patients with HAdV DNAemia $\geq 5x10^2$ DNA copies/mL (1). The episodes of HAdV viremia were classified as transient (blips) when HAdV was detected in one isolated sample or persistent when HAdV was detected in at least two consecutive samples.

Sequence-based typing of HAdV positive samples

Samples positive for HAdV were amplified and sequenced following the reported primers and protocol by M. Okada *et al.* (2). Nucleotide sequences of PCR products were compared to reported sequences using the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST/).

Determination of HAdV-specific cellular immune response

HAdV-specific T-cell immune response was determined using intracellular cytokine staining and flow cytometry, following a previously reported protocol with some modifications (3). Briefly, 500 µL of whole blood were stimulated with 1 µg/mL of PepMix HAd-3 hexon protein and 1 µg/mL of PepMix HAd-5 penton protein (JPT Peptides Technologies GmbH, Berlin, Germany). Unstimulated samples were used as a negative control and as positive control whole blood samples were stimulated with 1.5 µg/mL *Streptomyces conglobatus*, ionomycin, calcium salt, and 25 ng/mL 4-alpha-phorbol12-myristate13-acetate (Sigma Aldrich, Madrid, Spain). All samples were co-stimulated with 1 µg/mL CD28/CD49d (Beckton Dickinson, Madrid, Spain) and treated with 10 µg/mL of brefeldin A (Beckton Dickinson) to prevent cytokine secretion. After incubating the sample for 4 hours at 37 °C with 5% CO_2, 5 ml of FACS Lysis (Beckton Dickinson) was added and incubated for 10 minutes at room temperature (RT). Cells were centrifuged at 500 g for 5 minutes at 4 °C and washed 1x with phosphate buffered saline (PBS). For staining, cells were incubated in the dark for 20 minutes at RT with 0.04 µg/µL PE anti-human CD69, 0.1 µg/µL PerCP/Cy5.5 anti-human CD4, 0.1 µg/µL PE/Cy7 anti-human CD8 and 0.5 µg/µL BV510 anti-human CD3 (Biolegend, San Diego, USA). Cells were fixed adding 50 µL of IntraPrep reagent 1 (BeckmanCoulter, Madrid, Spain) and incubating for 15 minutes. After washing 1x with PBS, cells were permeabilized adding 50 µL of IntraPrep reagent 2 and incubated for 15 minutes with the following monoclonal antibodies: 0.025 µg/µL APC anti-human IL-2, 0.05 µg/µL FITC anti-human IFN-γ and 0.05 µg/µL BV421 anti-human. Cells were washed 1x with PBS and resuspended in 300 µL of PBS. Samples were then analyzed on a LSR Fortessa cytometer by quantifying 40,000 events of CD3+ cells from the total population of lymphocytes. The percentages of activated CD4+ and CD8+ cells that express IFN-γ, TNF-α and IL-2 were normalized to the negative control. The development of a HAdV-specific T-cell immune response

was defined as the expression of any of the evaluated cytokines (IFN-γ, TNF-α, and IL-2) by activated CD4+ or CD8+ cells.

Statistical analysis

A descriptive analysis of clinical characteristics of patients and HAdV infection episodes was performed. Categorical variables were expressed as percentages. Continuous variables were expressed as median and interquartile range (IQR) or mean and standard deviation (SD). Categorical variables were compared by the Chi-square test or Fisher exact test and continuous variables by the Student's t test or Mann-Whitney test. Differences were considered significant for p values < 0.05. Statistical analyses were performed using SPSS version 15.0 software (SPSS, Chicago, IL, USA) and graphics were obtained using GraphPad Prism 5 (GraphPad, La Jolla, CA, USA).

Supplemental Table 1. Demographics and clinical characteristics of the 95 HSCT recipients.

	N (%)
Female	39 (41.1)
Age, years mean (SD)	47.8 (13.4)
Hematologic Disease	
– Acute Myeloid Leukemia	28 (29.5)
– Myelodysplastic Syndrome	20 (21.1)
– Non-Hodgkin Lymphoma	12 (12.6)
– Hodgkin Lymphoma	10 (10.5)
– Acute Lymphoblastic Leukemia	11 (11.6)
– Others	14 (14.7)
Donor	
– Non-related donor	49 (51.6)
– HLA-identical sibling	38 (40.0)
– Haploidentical donor	8 (8.4)
Conditioning	
– Reduced intensity	72 (75.8)
– Myeloablative	23 (24.2)
ATG[#] administered	10 (10.5)
CD34+ (x10^6/kg), mean (SD)	5.9 (1.6)
Cytomegalovirus positive serology	
– Donor / Receptor	54 (56.8) / 81 (85.3)
Female Donor to male Receptor	17 (17.9)
GVHD[†] prophylaxis	
– Tacrolimus + Sirolimus	57 (60.0)
– Tacrolimus + Methotrexate	26 (27.4)
– Others[§]	12 (12.6)
Febrile neutropenia	52 (54.7)
Acute GVHD	42 (44.2)
– GVHD III-IV	22 (52.4)
– Systemic corticosteroids	25 (59.5)
Cytomegalovirus infection	29 (30.5)
– Cytomegalovirus disease	4 (13.7)
Mortality	10 (9.5)

[#]ATG: anti-timocytic globulin; [†]GVHD, graft versus host disease; [§]Cyclosporin (CsA), methotrexate, cyclophosphamide plus CsA and mophethil mycophenolate (MMF), or tacrolimus plus antitimocytic globulin (ATG) and metotrexate.

Supplemental Table 2. Demographics, transplantation features and clinical characteristics of patients with and wihout HAdV viremia post-transplantation.

Variable	Patients with viremia, n = 58	Patients without viremia, n = 37	*p*
Female	24 (41.4%)	15 (40.5%)	0.94
Conditioning (reduced intensity)	41 (70.7%)	31 (83.8%)	0.15
Antitimocytic globulin	5 (8.6%)	5 (13.5%)	0.44
Female donor / Male receptor	10 (17.2%)	8 (21.6%)	0.59
No related donor	28 (48.3%)	21 (56.8%)	0.42
High risk HSCT (Haploidentical & GVHD† III-IV)	21 (36.2%)	9 (24.3%)	0.22
GVHD	29 (50.0%)	13 (35.1%)	0.16
GVHD (III-IV)	16 (27.6%)	6 (16.2%)	0.20
GVHD Prophylaxis (Tacrolimus and Sirolimus)	33 (56.9%)	24 (64.9%)	0.44
Cytomegalovirus infection	17 (31.5%)	12 (32%)	0.46
Cytomegalocirus treatment (Ganciclovir)	12 (20.7%)	10 (27.0%)	0.36
Mortality	5 (8.6%)	5 (13.5%)	0.45

†GVHD, graft versus host disease.

Supplemental Table 3. HAdV-specific T-cells immune response. Patients (%) with/without HAdV viremia expressing IL-2, TNF-α and IFN-γ at days +21, +56 and +100 after transplantation by HAdV-specific CD4+ and CD8+ T-cells ($^{\#}$ $p < 0.05$).

	CD4+			CD8+		
	IL-2	**TNF-α**	**INF-γ**	**IL-2**	**TNF-α**	**INF-γ**
+21	5.3/6.0	23.6/12.1	12.7/6.0	12.7/0.0#	20.0/3.0#	12.7/6.0
+56	26.8/5.0#	58.5/45.0	36.6/35	21.6/20.0	31.7/35.0	34.1/20.0
+100	41.5/20.8	75.6/62.5	36.6/41.6	24.4/25.0	26.8/50.5	43.9/41.7

Supplemental Table 4. HAdV-specific T-cell immune response in patients with viremia (A) *vs.* those without viremia (B). HAdV-specific CD4+ and CD8+ T-cells (%) expressing IL-2, TNF-α and IFN-γ on days +21, +56 and +100 after transplantation. Results are shown as mean ± SD ($^{\#}p < 0.05$).

A

	CD4+			CD8+		
	IL-2	**TNF-α**	**INF-γ**	**IL-2**	**TNF-α**	**INF-γ**
+21	0.007±0.03	0.03±0.06	0.02±0.06	0.01±0.04#	0.02±0.04#	0.02±0.05
+56	0.02±0.05#	0.07±0.07	0.06±0.09	0.03±0.06	0.05±0.09	0.04±0.06
+100	0.05±0.07	0.13±0.11	0.05±0.07	0.03±0.05	0.05±0.08	0.06±0.09

B

	CD4+			CD8+		
	IL-2	**TNF-α**	**INF-γ**	**IL-2**	**TNF-α**	**INF-γ**
+21	0.006±0.02	0.01±0.05	0.009±0.03	0.00±0.00#	0.006±0.03#	0.01±0.04
+56	0.005±0.02#	0.05±0.06	0.03±0.04	0.02±0.04	0.03±0.04	0.02±0.04
+100	0.02±0.05	0.1±0.1	0.04±0.05	0.02±0.05	0.06±0.07	0.05±0.07

Supplemental Table 5. HAdV-specific T-cell immune response in patients with viremia ≥ $5x10^2$ copies/mL (A) or < $5x10^2$ copies/mL (B). HAdV-specific CD4+ and CD8+ T-cells (%) expressing IL-2, TNF-α and IFN-γ on days +21, +56 and +100 after transplantation. Results are shown as mean ± SD (patients with viremia ≥ $5x10^2$ copies/mL *vs.* < $5x10^2$ copies/mL, $^{\#}p < 0.05$).

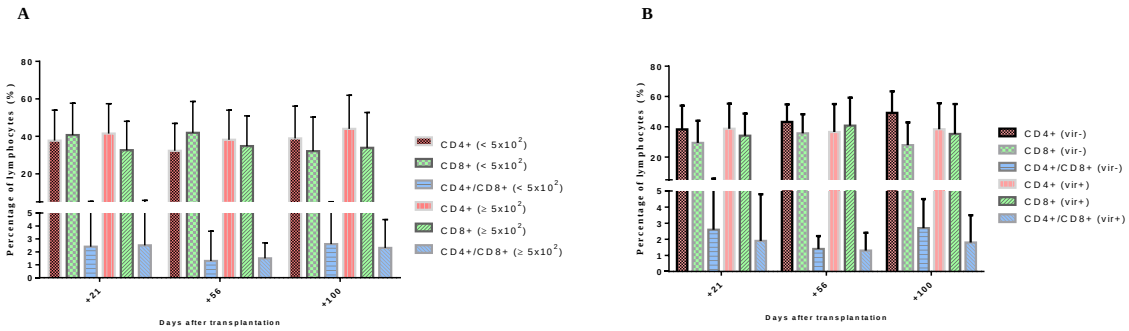
A

	CD4+			CD8+		
	IL-2	**TNF-α**	**INF-γ**	**IL-2**	**TNF-α**	**INF-γ**
+21	0.005±0.02	0.03±0.05	0.005±0.02	0.02±0.07	0.02±0.04	0.03±0.07
+56	0.03±0.6	0.07±0.07	0.04±0.09	0.03±0.06	0.04±0.08	0.04±0.06
+100	0.1±0.09$^{\#}$	0.2±0.1$^{\#}$	0.06±0.08	0.03±0.05	0.05±0.09	0.1±0.1$^{\#}$

B

	CD4+			CD8+		
	IL-2	**TNF-α**	**INF-γ**	**IL-2**	**TNF-α**	**INF-γ**
+21	0.008±0.03	0.03±0.06	0.03±0.09	0.02±0.05	0.02±0.05	0.01±0.04
+56	0.03±0.06	0.09±0.08	0.08±0.1	0.04±0.08	0.08±0.1	0.05±0.07
+100	0.03±0.06$^{\#}$	0.13±0.1$^{\#}$	0.05±0.08	0.02±0.04	0.04±0.08	0.05±0.08$^{\#}$

Supplementary Figure 1. Lymphocytes CD4+ and CD8+ (%) and CD4+/CD8+ ratio in patients with HAdV viremia < $5x10^2$ and ≥ $5x10^2$ copies/mL (A) and with (vir+) and without (vir-) HAdV viremia (B).



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