Organ siderosis and hemophagocytosis during acute graft-*versus*-host disease

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Supplementary Data

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Materials and Methods

Patients

In this retrospective analysis, all patients with the diagnosis of ALL, AML, severe aplastic anemia, CML, multiple myeloma and NHL receiving allo-HSCT at Charité Berlin, Campus Benjamin Franklin, between 2003 and 2008 and surviving longer than 60 days were included. The study has been approved by the Ethics Committee and all patients gave their informed consent to scientific analysis of their data in accordance with the declaration of Helsinki. In total, 104 patients were assessed. Control of ferritin levels was performed in all patients as part of the routine work up and was available in all patients. To be able to analyze ferritin data we decided to create three cohorts: 1) Ferritin < 5000µg/l = low to medium, 2) Ferritin 5000µg/l – 10.000µg/l = high, Ferritin > 10000µg/l = very high. Of patients with a ferritin of greater than 10000µg/l, bone marrow aspirates at the time of diagnosis were reassessed for the occurrence of hemophagocytosis.

Measurement of liver iron in preclinical models

C57BL/6 mice were conditioned with treosulfan and cyclophosphamide and transplanted syngenically (control) or with Balb/c splenocytes as previously described.[1] On day 9, mice were sacrificed and liver iron was determined by graphite furnace atomic absorption spectrometry. Organs were embedded in formalin, fixed and stained (Berliner Blue) according to standard protocol.

Gene expression profiling in liver during GVHD in preclinical models

To investigate the gene expression profiling during GVHD, we compared gene expression of aprox. 22.000 genes in allo-BMT recipients to gene expression in syn-BMT recipients at day 14 after BMT. Lethally irradiated recipients received 5 x 10^6 cells from TCD-BM and 1 x 10^6 enriched donor splenic T cells. RNA were isolated and cRNA were then hybridized to the mouse-ref8 array. Data are average fold change in livers of lethally irradiated allo-BMT recipients as compared with lethally irradiated syn-BMT recipients.

Protein expression profiling in liver during GVHD in preclinical models

C57BL/6 recipients received busulfan (20 mg/kg/day) for 5 days and cyclophosphamide (100 mg/kg/day) in the last 3 days additionally. Followed by a two-day resting phase, at day 0, C57BL/6 recipients were injected via the lateral tail vein with 1.5 x 10^7 bone marrow cell (BMC) and 2 x 10^6 splenocyte from LP/J (allogeneic) or C57BL/6 (syngeneic) as donor, respectively. Animals were evaluated for clinical symptoms of GVHD as described previously.[2] At day +14 after BMT, liver of four mice per group (allogeneic and syngeneic) was snap frozen. Proteins were isolated with denaturation buffer (6 M Urea, 2 M Thiourea

and 20 mM HEPES, pH 7.5) on the dry ice. Protein and peptide quantitation information were extracted from MaxQuant 1.2.2.5. All the samples were searched against the IPI mouse database version 3.84 with 60.012 entries (ftp://ftp.ebi.ac.uk/pub/databases/IPI). Cleavage specificity for main search was set for trypsin/P. Search parameters were two missed cleavage sites, cysteine carbamidomethylation as fixed modification and methionine oxidation as variable modification. Quantification data of labeled peptides were measure considering N-termini and lysine dimethylation on light (+28Da), medium (+32Da) or on heavy (+36Da) modification per free primary amine. The results were filtered to 1% false discovery rate at peptide level by MaxQuant.

1. Heimesaat MM, Nogai A, Bereswill S, et al. MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. Gut. 2010;59(8):1079-1087.
2. Riesner K, Kalupa M, Shi Y, Elezkurtaj S, Penack O. A preclinical acute GVHD mouse model based on chemotherapy conditioning and MHC-matched transplantation. Bone Marrow Transplant. 2015;

Results

		Maximal Ferritin Value [µg/l]		
		<5000	5000-10000	>10000
	n (% of total)	71 (68,3%)	22 (21,2%)	11 (10,6%)
Age		40 (18-81)	38 (19-66)	35 (19-56)
Diagnosis [n]	ALL	12	2	1
	AML	42	16	8
	sAA	3	2	0
	CLL	5	2	1
	MM	6	0	0
	NHL	2	0	0
Karnofsy-Index prior Tx	70-80	21,7%	47,8%	36,4%
	90-100	78,3%	52,2%	63,6%
Ferritin prior Tx	Mean [µg/l]	1129 (10-3681)	1594 (81-6781)	2042 (610-3867)
Donor	Haploidentical	0,0%	4,3%	9,1%
	Related	38,9%	13,0%	27,3%
	Syngeneic	0,0%	4,3%	0,0%
	Unrelated	61,1%	78,3%	63,6%
ATG	Yes	57,7%	68,2%	60,0%
	No	42,3%	31,8%	40,0%
Conditioning	Myeloablative	20,8%	26,1%	36,4%
	RIC	79,2%	73,9%	63,6%
CMV	High risk	24,3%	52,2%	18,2%
	Other	75,7%	47,8%	81,8%
aGVHD (Grade)	0	41,7%	21,7%	9,1%
	1	13,9%	26,1%	18,2%
	2	29,2%	26,1%	9,1%
	3	9,7%	21,7%	63,6%
	4	5,6%	4,3%	0,0%
cGVHD	n.a.	27,78%	21,74%	27,27%
	No	41,67%	34,78%	36,36%
	limited	18,06%	17,39%	9,09%
	extended	12,50%	26,09%	27,27%
Any GVHD		69,44%	86,96%	100,00%

Suppl table 1: Patient characteristics and distribution of maximal serum ferritin values.

Pt.	Age	Days after transplant	Ferritin [µg/l]	Splenomegaly	Fever	Cytopenia *	sIL2-R **	Triglyceridemia	NK-Cell Dysfunction **	Hemophagocytosis in BM	HLH-Score ***	Transaminitis #	Hyperbilirubinemia #	Elevated LDH #	Hypoalbuminemia #	Hyponatriemia #	EBV-Positivity ##	Elevated Creatinin	Elevated CRP	Other morbidities
#1	59	22	10367	y	y	y	n.a.	y	n.a.	n.a.	5 of 5	y	y	y	y	n	n	n	y	Severe aGvHD, Mucositis, Fever
#2	30	380	11153	y	y	y	n.a.	n.a.	n.a.	n	4 of 4	y	y	y	n	n	n.a.	y	y	Aspergillus infection (proven), severe aGvHD, MAHA
#3	59	530	13493	n	y	n	n.a.	n	n.a.	y	3 of 6	y	y	n	y	n	n	y	y	2nd transplant after relapse, aspergillus pneumonia (probable)
#4	54	158	14329	n	y	n	n.a.	n.a.	n.a.	y	3 of 5	y	y	n	y	n	n.a.	n	y	Relapse, severe aGvHD
#5	47	274	17410	n	n	n	n.a.	n.a.	n.a.	y	2 of 5	y	y	y	y	n	n.a.	n	y	severe cGvHD, unclear infection
#6	43	44	17500	n	y	n	n.a.	y	n.a.	n.a.	3 of 5	y	y	y	y	n	n	n	y	Unclear cerebral confusion, muscle weakness, aGvHD (2°); CMV infection
#7	40	700	20266	n	y	n	n.a.	n.a.	n.a.	y	3 of 5	y	y	n	n	n	n	n	y	Unclear confusion, gait problems, fever, aGvHD
#8	65	91	21898	n	y	n	n.a.	n.a.	n.a.	y	3 of 5	y	n	n	n	n	n.a.	y	y	Relapse, sepsis
#9	51	510	24649	n	y	n	n.a.	y	n.a.	y	4 of 6	y	y	y	n	n	y	n	y	chronic GvHD (extended), pneumocystis jirucreni infection
#10	46	617	26105	n	y	n	n.a.	y	n.a.	y	4 of 6	y	y	n	y	n	y	y	y	cGvHD (extended) with ulcerating and superinfected skin lesions, relapsing pneumocystis infections
#11	21	76	31608	n	y	n	n.a.	y	n.a.	y	4 of 6	y	y	y	y	n	y	n	y	Aspergillus penumonia (probable), intracerebral hemorrhage, severe aGvHD

* At least 2 of the following: Hemoglobin<9,0g/dl; Platelets<100/nL; Neutrophils<1,0/nl)

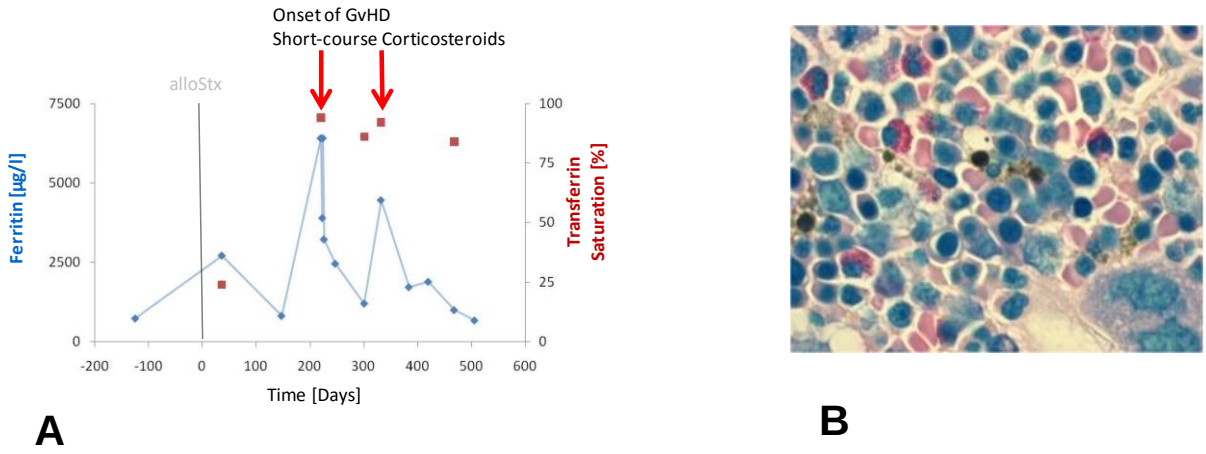
** IL-2r and NK-cell function were not assessed during the observation period

*** According to HLH-2004 diagnostic criteria; positive parameters of parameters assessed.

Supportive criteria for hemophagocytosis

Pt.#10 positive EBV-PCR; Pt. #11 positive EBV EA

Suppl. Table 2: Clinical parameters of allo-HSCT recipients with ferritin level>10000µg/l during GVHD. Abbreviations: n.a. = not applicable, y = yes, n = no.



Suppl. Fig 1: Rapid occurrence of hyperferritinemia and organsiderosis. Female patient undergoing allo-HSCT with rapid increase of ferritin and transferring saturation associated with GVHD. Treatment of GVHD resulted in prompt decrease of ferritin (A). In histological sections, organsiderosis was present (B)