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Disturbed B cell and DC-Homeostasis in Pediatric cGVHD Patients-Cocultivation Experiments and Review of the Literature

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Abstract

B cells and DCs are suspected to play an important role in the pathogenesis of cGvHD, which is a serious complication of HSCT with high morbidity. It is characterized by immune responses of donor immune cells against recipient-derived antigens. Pathogenesis is not yet fully understood, however reconstitution of B cells after HSCT has similarities to physiologic ontogeny. Immunophenotyping and co-cultivation-experiments of B cells and DCs from pediatric patients with cGvHD as well as healthy donors were conducted. Significant differences between patients and healthy donors were observed with increased memory, transitional, CD69+ and CD86+ phenotype and lower levels of naïve B cells due to apoptosis. Co-cultivation revealed this to be primarily B cell-dependent without major effects of and with DCs. There was a decreased CD11c- phenotype in patients and less apoptosis of DCs. Our data suggest a disturbed homeostasis in B cells with increased memory phenotype in patients, whereas DCs could not influence these differences, therefore DCs are not imposing as promising targets. B cell-dependent approaches should be further investigated.

Introduction

Allogeneic HSCT is an established therapy for high-risk malignancies such as relapsed leukemia and lymphomas, but also for various severe non-malignant diseases. GvHD is a complication of hematopoietic stem cell transplantation, with donor immune cells forming responses against host antigens, resulting in an immunological rejection of the recipient's tissues. The reaction of donor-derived immune cells against the recipient's malignant cells describes the graft-versus-tumor or -leukaemia (GvL) effect [1] and composes the curative potential of HSCT. Responses against host antigens and immunological rejection of the recipient's tissues describe Graft-versus-host disease (GvHD). Two distinct forms of GvHD can be differentiated. Initially, acute GvHD had been defined as occurring within the first 100 days, chronic GvHD (cGvHD) after 100 days post-HSCT. With a deeper knowledge of the pathogenesis of both acute and chronic GvHD though, and with a better understanding of the differences on cellular and clinical levels, the definition has been extended, as pathogenesis seems to be different. Incidence of cGvHD may continuously follow aGvHD, occur after resolved aGvHD or develop de novo. Various complications can arise from the disease and its management still constitutes a big challenge.

Pediatric patients with cGvHD show significantly lower physical functioning than HSCT recipients without cGvHD [2], impairment of growth and loss of quality of life. Even if cGvHD is known to increase GVL effects, this benefit is outweighed by the increased mortality associated with the disease [3,4]. Consequently, the primary goal of post-transplantation therapy is to prevent GvHD while enhancing desired GvL effects, thus increasing survival and decreasing disease-relapse. Still today, in spite of more than 50 years of experience with HSCT and about 25,000 allogeneic HSCTs each year, [5] cGvHD is, after disease-relapse, the most important reason for post-transplant mortality [6]. Up to 70% of adult patients develop cGvHD [1], while the incidence in children is lower (20-50%), whereas rates have increased since the use of peripheral blood stem cells [7] and donors unrelated to the patients [6]. Known risk factors for the development of cGvHD include previous aGvHD, stem cell source (PBSC), increasing age of host, CMV-status, TBI and sex-mismatch [6]. However, the concrete mechanisms of the development of cGvHD are still not entirely known. While pathogenesis of aGvHD has been studied extensively and seems to be predominantly T cell-mediated, in recent studies the significance of B cells in cGvHD pathogenesis has been in focus [8]. Hypotheses exist, that cGvHD is a mainly Th2-mediated disease with autoimmune features

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Table 1:

	#1	#2	#3	#4	#5	#6	#7
Sex	m	m	m	m	m	m	m
Age (Years)	12	4	7	20	7	15	8
Diagnosis	MDS	AML	ALL	ALL	AIE	AML	LCH
Days Since HSCT	332	1074	755	302	2068	484	2784
Status before HSCT	SD	PD	PR	CR	SD	SD	PD
HLA Status	Ident	Ident	Ident	Ident	Haploident	Ident	Ident
Donor	Sibling	Unrelated	Fremd	Sibling	Parental	Sibling	Fremd
Type of HSCT	PBSCT	PBSCT	PBSCT	PBSCT	PBSCT	PBSCT	PBSCT
Sex Mismatch	No	No	Yes	No	Yes	Yes	Yes
Relapse	No	Yes	No	No	No	No	No
aGvHD	Yes	Yes	Yes	Yes	No	No	Yes
Localisation	>2	>2	Skin	Skin, Liver	>2	Skin	Skin

[9]. The possible essential steps of its pathogenesis could be triggered by damage to the thymus following conditioning prior to HSCT or, more significantly, aGvHD. Thus, a decrease in negative selection of $CD4^+$ T cells may occur, leading to a deviated immune response with increased production of IL-4, IL-5 and IL-11, being part of the Th2 cytokine pattern. Tissue fibroblasts can be stimulated both directly by Th2-released IL-2, IL-10 and TGFβ1, and indirectly via activation of macrophages, which produce PDGF and TGFβ1. This process can be enhanced by low Treg levels resulting in further deviation of the immune system towards Th2 and Th17 response [10]. Th17 cells, a $CD4^+$ T cell subset expressing the IL-23-receptor [11], can act as B cell helpers and have been shown to increase the formation of germinal centres as well as promoting proliferation and activation of B cells with their signature cytokines IL-17, IL-21 and IL-22 [12]. This dysregulation of B cell homeostasis can, as well as high levels of BAFF, result in B cell autoreactivity [10]. In summation, all these steps contribute to systemic effects similar to autoimmune diseases affecting multiple organs as skin, nails, mouth, eyes, muscles, gastrointestinal tract, liver, lungs, kidneys, heart and bone marrow [13-16]. However, an improvement of therapy opportunities is desperately needed, as standard therapy consisting of glucocorticoids with or without tacrolimus or ciclosporin [1], has many side effects-especially in pediatric patients [6] and we are still lacking specific therapies. Several biomarkers have been introduced and proposed for the identification of patients at risk of cGvHD, including Tregs, B cells, BAFF, IL-10 [17], as well as development of HY antibodies [18].

While various ways of interaction between DCs and T cells not only via antigen presentation are known [19], their influence on B cells has just lately become the focus of investigation. Indeed, many of the aforementioned mechanisms directly involved in the pathogenesis of autoimmunity are complemented or redundantly synergize with effects of DCs and B cells on each other. In response to TLR stimulation or BCR ligation, pDCs can enhance auto reactive B cell proliferation and production of anti-snRNP auto antibodies as well as B cell survival [20]. Furthermore, dendritic cells have been shown to be the most important APCs. In mouse models anti-CD40 activated B cells were unable to fully prime $CD4^+$ T cells *in vivo* without DCs expressing the correct restriction element MHC II I-E [21]. This would rather implicate an auxiliary role of B cells in optimizing $CD4^+$ T cell priming. Therefore only concerted presentation of antigens by both dendritic and B cells leads to an optimal and effective $CD4^+$ T cell-immunity *in-vivo* [21]. Additionally, pDCs can induce differentiation of CD40-activated B cells into antibody-producing plasma cells via sequential secretion of IL-6 and type I IFN [22,23]. This explains, how these cytokines, together with BAFF may partially contribute to the effects of pDCs on B cell activation in addition to cell-to-cell contact [20,24]. Besides these means of communication, a direct involvement of the ICAM-1-LFA-1 pathway in the signaling between pDCs and auto reactive B cells has been described [20]. As for type I IFN, it is able to upregulated CD38 on B cells and enhance secretion of BAFF and APRIL by B cells, monocytes and mDCs, confirming its importance in the context of cGvHD [25,26]. Follicular DCs are involved in the development of somatically mutated and switched memory cells [27]. Following activation via TLR7/8-L and, less pronounced, via TLR9, pDCs have been shown to be able to induce upregulation of BLIMP1 and X-box binding protein 1, to increase B cellproliferation as well as the differentiation into $CD27^{high}$ plasmablasts [28] which indicates their involvement in B cellhomeostasis.

The aims of our study were to compare patterns of B cells and DCs from patients with cGvHD and healthy donors. Furthermore, apoptosis was measured and co-cultivation was used to observe possible interactions and evaluate the effects of B cell activation on immune cells.

Materials and Methods

We performed immunophenotyping of B cells and DCs from 7 pediatric patients (Table 1) with cGvHD and 13 healthy donors. Mononuclear cells were isolated from peripheral blood via Ficoll, followed by positive selection of $CD19^+$ and $CD14^+$ cells via MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). B cells were cultivated with IL-10 (Sigma, Taufkirchen, Germany), $CD14^+$ cells matured and differentiated to fast DCs within 48 hours with GM-CSF, IL-4, IL-1b, IL-6, TNFα (CellGenix, Freiburg, Germany) and PGE2 (Pharmacia Limited, Kent, UK) [29].

Cocultivation

Co-cultivation of B cells and DCs was performed in order to determine effects of cell-type and -origin. At day 3 co-cultivation of B cells and DCs was initiated based on characterization of the cells, namely auto-auto (B cells patient + DCs patient), auto-allo (B cells patient + DCs donor), allo-auto (B cells donor + DCs patient) and allo-allo (B cells donor + DCs donor).

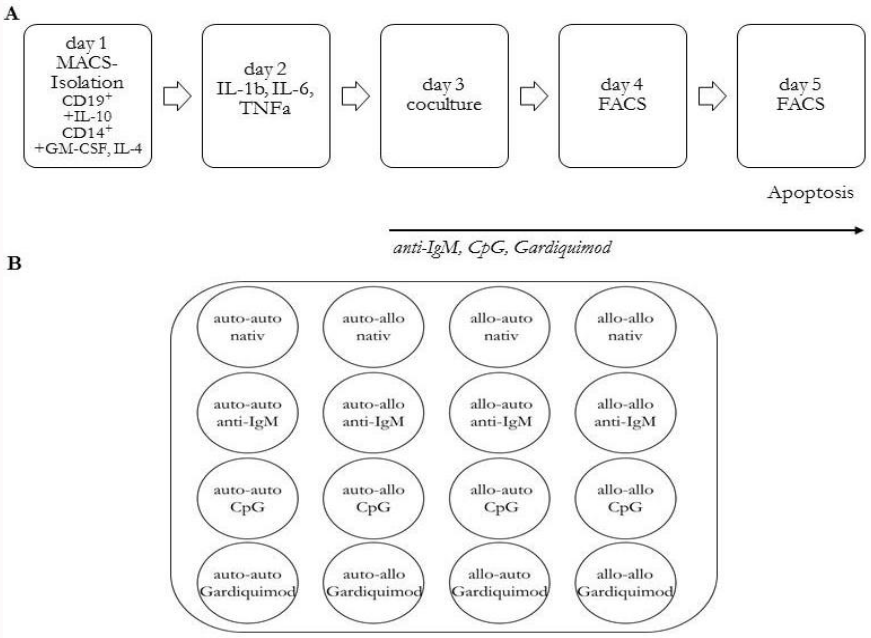

Figure 1: Experiment setting (A) day 1: Isolation of CD19+ and CD14+ mononuclear cells via MACS, adding respective agents for B cell culture und DC-maturation, FACS day 2: adding agents for DC-differentiation, day 3: cocultivation, adding B cell activation agents and FACS with Apoptosis measurement, day 4: repeating B cell activation and FACS, day 5: FACS with Apoptosis measurement and Co-cultivation schematic (B) with 16 well design for co-cultivation.

B cell activation

Additionally, we investigated B cell activation by further dividing our samples into 4 equal parts and adding anti-IgM (Jackson Laboratories, Pennsylvania, USA), CpG and Gardiquimod (InvivoGen, San Diego, USA) or leaving blank at days 3 and 4 (Figure 1). Immunophenotypingwas performed at days 1, 3, 4 and 5.

Statistical analysis

All p-values were two-sided, considered significant below .05 and determined via independent T-tests. We compared the means of relative proportions of cells determined via immunophenotyping. All calculations were performed with SPSS (version 20.0.0; IBM, Ehningen).

Results

B cells

No considerable differences between patients and healthy donors were observed at days 1 and 3. Not until 96 hours after cell culture were we able to measure significantly diverse cell counts. An increased transitional, CD69$^+$ and CD86$^+$ (Figure 2) as well as decreased mature naïve phenotype was observed at days 4 and 5 in patients with cGvHD compared to healthy controls. Higher levels of CD34$^+$ memory cells and lower levels of BAFF-R$^+$ naïve cells were measured at day 5, coincident with increased apoptosis of naïve and non-switched memory B cells (Table 2).

When taking the co-cultivation into consideration we can see these observations confirmed. In none of the subsets do we see a significant difference in cell counts between patients and auto-allo, indicating no effect of DC-origin on B cells. Similarly, we did observe significant differences between healthy donors and auto-allo in every B cell subset except for BAFF-R$^+$naïve B cells (Table 3).

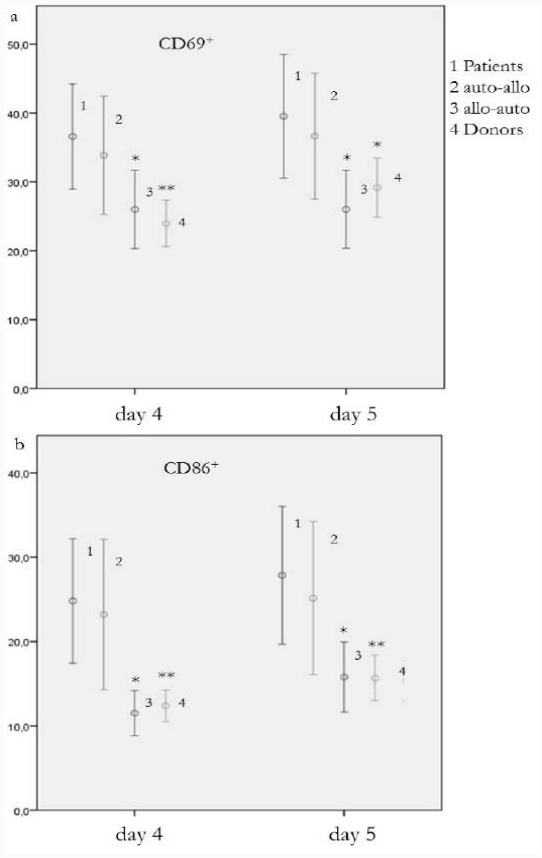

Figure 2: Comparison of percentages of CD69$^+$ and CD86$^+$ B cells in co-cultures with 95% confidence interval: a CD69$^+$ B cells b CD86$^+$ B cells, * p-value < .05, ** p-value ≤ .001

DCs

We observed a decreased CD11c$^-$ phenotype in patients at day 5 and less apoptosis in DCs. Other DC subsets did not differ significantly (Table 2).

Table 2:

Cell Subset		Day 4				Day 5			
		Mean	Median	SD	p-Value	Mean	Median	SD	p-Value
$CD34^+$ Memory ($CD24^+$ $CD38$) B Cells	Patients Donors	39.30 29.24	26.80 18.95	35.32 31.55	.196	48.32 27.85	40.85 16.05	40.25 29.86	.012
Transitional ($CD24^{++}$ $CD38^{++}$) Cells	Patients Donors	3.44 1.46	1.35 1.05	6.02 1.53	.028	3.54 1.67	2.15 1.20	6.16 1.94	.046
$CD69^+$B Cells	Patients Donors	36.58 23.96	31.20 24.35	19.71 12.15	.001	39.52 29.17	34.85 25.95	23.21 15.45	.019
$CD86^+$B Cells	Patients Donors	24.82 12.38	17.20 11.00	19.03 6.70	<.001	27.85 15.68	19.00 14.30	21.04 9.70	.001
Mature Naive ($CD24^{int}CD38^{inl}$) B Cells	Patients Donors	15.71 30.48	14.35 24.10	11.81 24.62	.004	18.57 29.39	16.00 22.70	12.14 21.60	.017
BAFF-R^+ Naive (IgD^+ CD27) B Cells	Patients Donors	65.16 77.55	92.65 94.55	41.79 32.44	.146	59.23 81.49	79.40 97.30	42.69 27.10	.006
CD11c-DCs	Patients Donors	44.69 46.98	48.55 45.60	80.86 84.74	.530	46.92 54.38	39.70 57.00	73.68 81.93	.028
Annexin V^+ Naïve (IgD^+ CD27) B cells	Patients Donors	- -	- -	- -		36.92 25.57	12.05 9.20	39.57 31.67	.015
Annexin V^+ Non Switched Memory (IgD^+ $CD27^+$) B cells	Patients Donors	- -	- -	- -		52.76 48.10	66.70 37.25	40.90 32.06	.018
Annexin V^+ DCs	Patients Donors	- -	- -	- -		42.10 57.63	37.85 57.50	21.67 15.12	.001

Co-cultivation reveals significant differences only between healthy donors and auto-allo (Table 3).

Discussion

Interestingly, reconstitution of B cells after HSCT has similarities to physiologic ontogeny [30] yet, the B cell compartment in patients after HSCT differs from that of healthy adults [31]. Compared to other immune cells, reconstitution of B cells is the slowest, with reduced levels in up to 31% of pediatric patients even 12 months after transplant [32]. Firstly, levels of memory followed by transitional and naïve B cells rise [33,34]. Recovery of B lymphocytes in children has been observed to be significantly faster after unrelated transplant of cord blood than BMT, although without impact on overall survival [35]. PBSCT results in higher counts of B lymphocytes in the peripheral blood early after engraftment compared to BMT, whereas there is no difference later [36]. Peripheral blood B cells don't reach normal counts until up to one year after HSCT and consist of only donor-derived lymphocytes [30,37]. GvHD is an important factor in delayed reconstitution of lymphocyte pools, and especially B cells after HSCT [27,32,38]. Furthermore, development of chronic GvHD correlates with delayed recovery of B lymphocytes [32]. Concomitantly, high counts of B cell precursors correlate with a significantly lower incidence of cGvHD, indicating unaltered B cell lymphopoiesis [39]. Increased numbers of transitional [37] and naïve B cells [40] are found after HSCT. Early after transplant, the B cell CDR3 repertoire is restricted, but after 12 months post-HSCT the variability of naïve B lymphocyte pattern isn't significantly different from healthy controls, whereas a restricted repertoire of memory B cells still exists [41]. Up to 6 months after HSCT, IgH CD3 repertoire disparities between memory and naïve B cells have been detected, indicating compromised reconstitution of $CD27^+$ memory B lymphocytes [37,40,42]. In pediatric patients, $CD27^-$ IgM^{high} cells have been observed to constitute the largest population of B lymphocytes in the first 12 months after HSCT, with levels decreasing over time. This subset has been further divided into transitional and non-transitional cells, which in turn were differentiated via $CD45RB^{MEM55}$ [31]. In the first two years, levels of memory B cell are very low whereas naïve B cells are predominant and B lymphocyte reconstitution resembles the physiologic development in children [27]. Furthermore, the level of $CD27^+$ IgM^- memory cells has been found to proportionally increase, whereas $CD27^+$ IgM^{high} memory B cells would not increase and stay lower than compared to a control group [31]. It has been proposed, that the occurrence of new B cells after HSCT may serve as a predictive determinant of later B cell levels [43].

Bcells and cGvHD

In models for several autoimmune diseases, including rheumatic arthritis and JIA,autoreactive B cells, emerging from failure of tolerance checkpoints, play an important role [1,44]. Elevated levels of TNFα and IL-6, commonly associated with classic autoimmune diseases, can also be found in cGvHD patients [11]. Delayed or disturbed B cell-reconstitution and elevated plasma B cell–activating factor levels are associated with increased numbers of circulating $CD27^+$ B cell subsets [45]. Furthermore, this observation is associated with significantly higher B cell-protein contents [46], which comprise antigen-experienced B cells with a correlating commitment to plasma cell differentiation [47]. Indeed did we find higher levels of memory B cells in the patient population as compared to healthy donors. In addition, cGvHD patients who demonstrate clinical improvement and positive response to treatment have robust recovery of the peripheral naïve B cell pool [45,48]. This suggests, concomitant with an observed increase of IgD^+ $CD38^{high}$ $CD27^-$ in healthy HSCT patients [45], that the return to B cell homeostasis might be critical in the prevention of autoimmune cGvHD [49] and indeed, in our patients we observed less naïve IgD^+ $CD27^-$ B cells, as well as increased levels of transitional cells. This might be due to delayed and impaired B cell reconstitution in cGvHD patients. $CD34^-$ $CD20^+$ B cells, known to positively correlate, where as $CD34^+$ $CD19^+$ progenitor B cells in the

Table 3:

Cell Subset				p-Value Day 4	p-Value Day 5
CD34+ Memory B Cells	Patients	-	Auto-Allo	0.54	0.844
		-	Allo-Auto	0.474	0.23
	Donors	<	Auto-Allo	0.045	0.004
		<	Allo-Auto	0.034	0.248
Teansitional Cells	Patients	-	Auto-Allo	0.334	0.509
		-	Allo-Auto	0.077	0.296
	Donors	>	Auto-Allo	0.046	0.019
		>	Allo-Auto	0.689	0.432
CD69+ B Cells	Patients	-	Auto-Allo	0.629	0.646
		>	Allo-Auto	0.027	0.012
	Donors	<	Auto-Allo	0.011	0.092
		-	Allo-Auto	0.508	0.376
CD86+ B Cells	Patients	-	Auto-Allo	0.777	0.652
		>	Allo-Auto	0.001	0.009
	Donors	<	Auto-Allo	0.002	0.013
		-	Auto-Allo	0.587	0.96
Mature naïve B Cells	Patients	-	Auto-Allo	0.611	0.757
		<	Allo-Auto	0.007	0.042
	Donors	>	Auto-Allo	0.008	0.025
		-	Allo-Auto	0.264	0.446
BAFF-R+ Naive B Cells	Patients	-	Auto-Allo	0.98	0.443
		-	Allo-Auto	0.654	0.227
		>	Auto-Allo	0.136	0.062
	Donors	-	Allo-Auto	0.342	0.194
CD11c DCs	Patients	-	Auto-Allo	0.179	0.92
		-	Allo-Auto	0.639	0.564
	Donors	>	Auto-Allo	0.256	0.005
		-	Allo-Auto	0.98	0.137
Annexin V+ Naïve B cells	Patients	-	Auto-Allo	-	0.856
		-	Allo-Auto	-	0.818
	Donors	-	Auto-Allo	-	0.304
		-	Allo-Auto	-	0.3
Annexin V+ Non Switched Memory B cells	Patients	-	Auto-Allo	-	0.882
		-	Allo-Auto	-	0.592
	Donors	-	Auto-Allo	-	0.738
		-	Allo-Auto	-	0.863
Annexin V+ DCs	Patients	-	Auto-Allo	-	0.195
		-	Allo-Auto	-	0.817
	Donors	-	Auto-Allo	-	0.144
		>	Allo-Auto	-	0

transplant would correlate inversely with GvHD incidence [50]. In patients with cGVHD, less $CD24^{hi}$ CD^{27+} BcellsandIL-10–producing $CD24^{hi}$ CD^{27+} Bcells have been detected [42]. Nevertheless, after HSCT no significant correlation between B cell subsets in the bone marrow and cGvHD or survival could be detected [51]. With the knowledge, that induction of cGvHD depends on interaction of $CD4^+$ T cells with B cells, it surprises, that the absence of B cells in secondary recipients didn't prevent cGvHD after $CD4^+$ T cells already had contact with B cells [13]. Auto antibodies found in patients with cGvHD are of various types, including ANA, anti-dsDNA, anti-smooth muscle, anti mitochondrial and anti cardiolipin antibodies [52,53]. In patients with extensive GvHD, auto antibodies specifically targeting PDGFR and mediating excessive production of collagen have been identified, their levels matching the severity of skin involvement or lung fibrosis [16]. The occurrence of auto antibodies could be associated with a higher incidence of cGvHD and a lower relapse rate [53]. However, even if studies find correlations of antibodies with disease severity [54], their occurrence is inconsistent [55]. In addition to the described interactions of B cell subsets and cGvHD, we detected significantly higher levels of $CD69^+$ and $CD80^+$ B cells in patients as compared to healthy donors, indicating higher levels of activation. Activated alloreactive and autoreactive B cells are associated with cGvHD [56]. With no significant differences in B cell subsets concerning apoptosis, our co-cultivation experiments do not support the observations made in naïve and non-switched memory B cells.

DCs and cGvHD

A decrease in GvHD severity after depletion of $CD11c^{high}$ donor DCs has been observed [57]. In addition, different manifestations seem to be dependent on distinctive APC profiles as, for example, expression of CD80/86 in T cells for induction of cutaneous cGvHD, which could be mediated by both donor and host APCs, whereas gut cGvHD was mainly dependent on donor APCs and required both CD80/86 and CD40 signaling [58]. CD83, highly expressed on DCs, is suggested to contribute to pathways including activation of $CD4^+$ and $CD8^+$ T cells as well as B cell homeostasis [59]. PDCs are characterized by a $CD11c^{int}$ B220+ phenotype and are capable of producing high amounts of type I IFN as well as exerting potent T cell stimulation when matured via CD40 or TLR [25,60,61]. This stresses the importance of donor DCs, as 80% of peripheral blood DCs are replaced by donor cells within 14 days after transplantation in human recipients, reaching more than 95% by day 56 [62]. Reconstitution of DCs starts at about 2 to 3 weeks after HSCT, with numbers of myeloid DCs normalizing, while pDCs don't reach normal levels even in 12 months [27]. No significant association between DC chimerism and cGvHD has yet been found [63], but there is evidence, that host DCs can present host-derived antigen to donor T cells and donor DCs have activating effects on alloreactive $CD8^+$ T cells [64]. Nevertheless, a profound depletion of host DCs and B cells could not prevent GvHD induction [65]. Donor $CD11b^-$ $CD11c^+$ pDCs have been observed to enhance Th1, $CD4^+$ and type I $CD8^+$ CTL immune polarization of donor T cells as well as promoting GVL effects without enhancing GvHD, while $CD11b^+$ $CD11c^+$ cDCs would induce Th2 and type II $CD8^+$ CTL immune polarization [66]. This complements the observation that adding donor myeloid or $CD11c^{high}$ pDCs to the hematopoietic graft results in increased severity of GvHD [67]. Our co-cultivation experiments reveal significant differences in $CD11c^-$ DCs only between healthy donors and auto-allo, indicating a mitigating effect of patient B cells on donor DC homeostasis. Experimental depletion of host $CD11^+$ DCs wasn't able to prevent the disease [65]. Instead, transplantation of CD11b depleted bone marrow hematopoietic grafts leads to increased levels of donor spleen-derived $CD4^+$ memory T cells mediating augmented GVL effects and IFNγ in the recipient [68]. However, higher counts of $CD123^+$ $CD4^+$ DCs in the bone marrow graft have been found to be associated with decreased incidence of cGvHD but increased relapse in recipients [69]. Lack of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs can induce severe autoimmunity [70]. In the presence of TGFβ and all-trans retinoic acid, mouse DCs can indeed induce antigen-specific and immunosuppressive $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs capable of persisting for months and attenuating GvHD [71]. Interestingly, in patients with cGvHD, Th17 and $CD4^+$ $CD25^+$ $Foxp3^+$ T cells exhibit an inverse proportionality [11]. Levels of Th17 cells in patients with active cGvHD are significantly elevated in comparison to healthy donors; furthermore, patients with inactive GvHD have very low counts of Th17 cells in the peripheral blood, making these cells good indicators for disease status [11]. In mouse models, treatment with regulatory DCs after allogeneic HSCT can mediate an increase in levels of antigen-specific $CD4^+$ $CD25^+$ $Foxp3^+$ T cells and thus prevent cutaneous cGvHD in mice by generating alloreactive Tregs from donor derived T cells [72]. Furthermore, experimental therapeutic extracorporeal photopheresis increased levels of $CD4^+$ $CD25^+$ $Foxp3^+$ and modulate cGvHD activity [73]. Yet, the genotype of the donor for the (GT)n Polymorphism in the Promoter/Enhancer of Focp3 has no correlation with the development of cGvHD [74]. Type I IFN acts in autoimmune pathogenesis by negatively affecting differentiation of Th2 and Th17 cells [64,75] and inducing antibody responses [26]. The immuno modulatory effects of type I IFN include increased maturation of DCs and upregulation of expression of MHC I and II, as well as enhanced levels of CD80/86, BAFF and APRIL [25]. In patients after allogeneic HSCT, $CD4^+$ $CD123^+$ pDC precursors were able to produce type I IFN [76] and in mice, pDCs expressing MHCII are able to sufficiently prime donor $CD4^+$ T cells to induce GvHD [61]. On the other hand, secretion of IFNγ by murine donor T cells resulted in increased expression of Indoleamine-2,3-dioxygenase, involved in the regulation of gastrointestinal GvHD, [77]in donor pDCs, thus altering T cell balance and limiting GvHD without inhibition of GVL effects [78]. Furthermore, high levels of IL-15 on day +7 post transplant have been observed to correlate significantly with a lower risk of cGvHD [79], being involved in autoimmune diseases as a modulator of inflammation [80] and enhancing GVL effects [81]. We observed no effects of DCs on B-cell-dependent pathways, possibly due to less $CD11c^-$ regulatory DCs and high levels of apoptosis.

Biomarkers

Besides a disturbed B cell-homeostasis [49,51], some previously described biomarkers have been associated with an involvement of specific organs, such as soluble CD13 and BAFF for hepatic, and anti-dsDNA antibodies for joint, sclerodermatous and ocular cGvHD [52]. In pediatric patients, high levels of IL-2Rα and HGF could be significantly associated with GvHD, whereas elevated IL-8 would decrease the risk of GvHD [82]. Concentrations of CXCL9 above the median have been found to be associated with cGvHD within the first three months of diagnosis [83]. CXCL10 and CXCL11 have lately been identified as promising diagnostic markers for both aGvHD and cGvHD [84]. BAFF also has been suggested as a biomarker for cGvHD [49,85], it acts as a survival factor for B cells, as high levels of BAFF have been shown to result in increased size and metabolism of B cells [46]. Elevated levels of BAFF in patients with cGvHD positively

affect numbers of both, pre-germinal center and post-germinal center plasmablast- and plasmacell-like B cell subsets [45]. In transgenic mice models, over expression of BAFF prevents apoptosis of self-reactive B cells, leads to an enhancement of anti-dsDNA-specific B cell-maturation as well as secretion of antibodies [86] and results in autoimmune phenomena [87]. Interestingly, BAFF-receptor deficient mice would not develop autoimmunity, indicating a prominent role of this factor in disease pathogenesis [88]. Levels of BAFF are significantly higher in patients with clinical manifestation of cGvHD than in patients without cGvHD [89]. An elevated BAFF/B cell-ratio in patients with cGvHD has been observed due to both persistent elevation of BAFF concentration and B-lymphopenia, characterized by delayed recovery of B cell homeostasis and a selective defect in numbers of naive $CD27^-$ cells [45]. Despite that, a decrease of soluble BAFF levels could predict the clinical response to cGvHD [52]. The possibility of BAFF-mediated activation of AKT and ERK pathways in patients with cGvHD has been proposed, resulting in decreased B cell-apoptosis by lower levels of anti apoptotic Bim via NF-κB pathway [46,90]. IL-10 is an important factor in autoimmune pathogenesis as observed in experimental autoimmune encephalomyelitis, inflammatory bowel disease, collagen-induced arthritis and systemic lupus erythematosus [91]. Maturation of DCs under the influence of IL-10 induces tolerogenic DCs capable of inducing anergic $CD4^+$ and $CD8^+$ cells, inhibiting Th1 and Th2 responses [60]. B cells expressing B7 are necessary to mediate expression of IL-10 and Foxp3 by $CD4^+$ $CD25^+$ Treg cells in experimental autoimmune encephalitis, thus supporting recovery from the disease [92]. In mouse models, transferred IL-10-producing B cells were able to reduce cGvHD severity due to reconstitution of regulatory B cell subsets and have been shown to have a suppressive role in the development of sclerodermatous cGvHD [93], as well as mediating the decrease of allogeneic donor T cell proliferation and expansion [94]. In patients with cGvHD, an impaired production of IL-10 has been observed [42]. The detection of allogeneic HY-antibodies 3 months after female to male HSCT may predict incidence and severity of cGvHD as well as non relapse mortality [95].

Approaches to targeted therapy

Present B cell targeted therapies include anti-CD20, -CD22, -IL-12/23, -BAFF and -BAFF-receptor-3 antibodies [1,96-98]. A series of studies have analyzed the use of rituximab in human cGvHD with response rates ranging from 50 to 83% [54]. After treatment with rituximab, levels of precursor B cells were significantly higher in patients with stable or improved disease showing the correlation between clinical response and B cell-homeostasis [99]. On the other hand, it has been suggested that rituximab, failing to reduce BAFF levels, would induce an even more abnormal BAFF/B cell-ratio and support activated B cells [45] and its application as a prophylactic agent is still controversially discussed [18,99,100]. Dendritic vaccination is a promising approach to induce direct tumor-targeted immune responses after HSCT in order to enhance GVL effects. Ideally, immune responses against the malignant cells mediated by antigen-specific DCs would not have effects on cGvHD [101]. However, GvHD negatively affects the desired reactions to vaccines [102]. Nonetheless, the setting of HSCT is ideal for application of DCs, because, as mentioned earlier, donor DCs tend to fully replace host DCs after transplantation [62]. There are several approaches to dendritic vaccination against neoplasms, including generation of DCs ex vivo by culturing progenitor cells with specific cytokines and infusion of these DCs in patients, tumor-associated antigens aimed at DCs, and DC-derived exosomes [19]. Disturbed B cell-homeostasis and increased autoreactive B cells–critical in the development of cGvHD [103] are mediated, amongst others, by type I IFN-producing DCs and increased BAFF levels. Both could be influenced by *in-vitro* stimulated pDCs. Further innovative approaches in GvHD therapy include tyrosine kinase inhibitors like Imatinibvia Inhibition of c-Abl activity possibly blocking autoreactive B cell responses [104] and Ibrutinib, an inhibitor of both Bruton's Tyrosine Kinase and IL-2 Inducible T-cell Kinase, enzymes upstream of BCR and TCR signaling pathways, having shown promising results in acting as a prophylactic agent in murine cGvHD models [105].

Conclusions and Perspectives

Summarizing these observations, an impaired reconstitution of the immune cell homeostasis, in particular B cells, seems to be the basis of cGvHD pathogenesis, characterized by an increased memory, transitional, $CD69^+$ and $CD86^+$ phenotype and lower levels of naïve B cells due to Apoptosis.However, despite extensive investigations, there is little evidence that findings have facilitated a cohesive understanding, have translated into clinically useful tools, or have induced new therapeutic strategies. Validated markers for the risk of cGvHD development may permit avoidance of donors prone to cause cGvHD, alteration of transplant or therapeutic approaches to mitigate these risks. Useful and validated biologic markers associated with treatment-response and prognosis might positively influence current clinical practice and augment the range of therapeutic intervention. An advanced understanding of genetic polymorphisms in several cytokines, donor-recipient HLA-matching, MHC haplotypes and minor histocompatibility mismatch [106] may help to understand pathophysiological development of cGvHD [107,108]. As various interactions between B cells and DCs exist, DC-mediated priming of B cells or dendritic vaccinations seem to imply new therapeutic possibilities, even if our results dispute a role of DCs in altered B cell homeostasis in cGvHD patients. A priority area for future research lies in well-characterized clinical populations in order to obtain adequate power for studies in this field. Translation of experimental data into a clinical setting might provide useful tools for assessing risk for cGvHD and response, and may identify novel therapeutic strategies to reduce morbidity and improve prognosis.

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