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IL12 and IFNγ secretion by donor mononuclear cells in response to host antigens may predict acute GVHD after HSCT

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ABSTRACT

One persistent problem of allogeneic hematopoietic stem cell transplantation (HSCT) is acute graft versus host disease (GVHD). The role of cytokines in the pathogenesis of GVHD has been acknowledged.

We aimed, in the current study, to investigate the possibility of prediction of acute GVHD through investigating the pattern of interleukin 12 (IL12) and interferon gamma (IFNγ) production of both patients' origin and donors' origin. A total of 45 patients, receiving allogeneic peripheral blood (PB) stem cells from an identical sibling, were included in the study. Patients' plasma was collected after conditioning, during aplastic phase (representing patients' origin) and after engraftment (representing donors' origin). In addition an aliquot from the graft was used as responders in mixed lymphocyte culture (MLC) for 3 days with patients' mitomycin-treated mononuclear cells as stimulators. Culture supernatant was used for detection of IL12 and IFNγ of donors' origin. Fourteen patients developed acute GVHD. In culture supernatant, IL12 was detectable in 7/14 cases with and in none of 31 cases without acute GVHD ($p = < 0.001$). The corresponding figures for IFNγ were 10/14 and 3/31 with significantly higher IFNγ level in cases with than in cases without acute GVHD ($p = 0.001$). At engraftment the corresponding figures were 7/14 and 5/31 for IL12 and 11/14 and 7/31 for IFNγ with significantly higher cytokine levels in cases with acute GVHD ($p = 0.008$ and $p = 0.001$ respectively). At a cutoff of 0.89 pg/ml, IL12 in culture supernatant may predict acute GVHD with absolute specificity of 100% and a sensitivity of 50%. In conclusion, IL12 and IFNγ of donors' origin not of patients' origin may predict the occurrence of acute GVHD. The MLC model may allow prediction of acute GVHD upfront before conditioning of the patient or mobilization of the donor.

1. Introduction

Allogeneic hematopoietic stem cell (HSC) transplantation is a widely used therapeutic modality. Graft versus host disease (GVHD) continues to be the main concern of transplanters and the hope is to be able to segregate GVHD from the closely associated graft versus leukemia (GVL) or more broadly versus malignancy effect (Ferrara et al., 2009; Flowers et al., 2011; Fanning et al., 2013). A major role of cytokines in the pathogenesis of GVHD has been acknowledged with a lot of controversial reports. The controversy involves (i) the types of cytokines claimed to have detrimental or protective effect, (ii) the variability in the effect of the same cytokine depending if it is of donor or host origin and (iii) the variability according to the time of its administration in experimental animal models (Ferrara, 2002).

There is substantial evidence to implicate that cytokines play a major role in GVHD induction and grade (Ferrara, 2002; Carayol et al., 1997) and its use as a therapeutic target has been recently addressed (Kumar et al., 2017). GVHD was reported to be associated with increased production of IFN γ (Wang and Yang, 2014), IL2 (Wang et al., 1995) and IL10 (Takatsuka et al., 1999). Both IL 10 and IL5 have been used as predictors of GVHD (Imoto et al., 2000; Tanaka et al., 1997).

In general, Th1 response- induced cytokines are claimed to be contributing to the occurrence and severity of acute GVHD while those associated with Th2 response are claimed to be protective. The balance between Th1 and Th2 was claimed to be the decisive factor (Tanaka et al., 1997; Kamel et al., 2013).

IL12 is effective at inducing IFNγ by activated T cells; secretion of the latter by the T cells further enhances IL12 production and secretion

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by dendritic cells (DCs) creating a classical positive feedback loop for enhancement of IL12 production and further skews the response towards Th1 (Watford et al., 2003). While IL12 and IFNγ promote a Th1 response, these cytokines also inhibit Th2 responses (Watford et al., 2003).

If still not yet preventable, prediction of its occurrence will help to control or ameliorate the severity of GVHD especially if prediction can be done before the actual transplant.

Accordingly, we studied (i) in vitro production of IL12 and IFNγ by graft cells in response to recipient antigens in a mixed lymphocyte culture (MLC) setup mimicking the in vivo situation, (ii) IL12 and IFNγ pattern in the host prior to transplant representing the cytokine storm in response to conditioning regimen as well as at selected time points post-transplant to reflect the interaction between donor's immunocytes and host tissues in the true in vivo environment. Comparison and correlation between in vitro and in vivo pattern of IL12 and IFNγ in patients with and without GVHD may throw light on their role in the pathogenesis of this serious problem and their potential value in prediction of GVHD.

2. Material and methods

2.1. Patients

The study included 45 patients who received fully matched allogeneic peripheral blood stem cell transplantation (PBSCT) from a sibling donor at Nasser Institute. They included 26 males and 19 females with an age range from 6 to 41 with a median of 22 years. Seventeen patients had AML, 15 had CML, 4 had ALL, 5 had aplastic anemia, 3 had MDS and one patient had Fanconi's anemia.

Conditioning regimens and GVHD prophylaxis were administered as previously reported (Mahmoud et al., 2008).

2.2. Methods

The work was performed according to Helsinki declaration. The protocol was approved by the IRB of the NCI, Cairo University and a written informed consent was obtained from all patients or their guardians.

A blood sample was obtained from the patient before conditioning, mononuclear cells separated by Ficoll-Hypaque density gradient centrifugation and cryopreserved. On the day of transplant, the cryopreserved cells were thawed and mitomycin treated to serve as stimulators while the mononuclear cells of the graft served as responders in a MLC setup as previously described (Kamel et al., 2013). Viability test was performed for both thawed patient's cells and graft cells. It varied between 82 and 94% with a median of 87% for the thawed patient's cells and was > 95% in the graft cells. Culture plates were incubated for 3 days at 37^{O}C and 5% CO2; the culture supernatant was collected and stored at -80 ^{O}C till tested. Plasma samples were obtained from patients at day 0, during aplastic phase and at engraftment.

IL12 and IFNγ were measured by microbead array technology using Luminex 200 and Flourokine MAP kit provided by R&D Company (Human multianalyte profiling base kit A and Human multianalyte profiling base kit B; R&D Systems, Inc. Minneapolis, USA).

Follow up of patients was carried out for one year at least; development of acute and chronic GVHD was recorded.

2.3. Statistical analysis

SPSS statistical package version 16 was used for data analysis. Numerical data were expressed as mean ± SD, median and range. Qualitative data were expressed as frequency. Chi square test (Fisher's Exact test) was used for the relation between qualitative variables; for quantitative data, Mann Whitney test was used. ROC curve was performed to determine the cut off values for the different markers; sensitivity and specificity were calculated. Spearman's correlation was used for relation between numeric variables

3. Results

Fourteen/45 (31.11%) patients developed acute GVHD at 28–72 with a median of 43 days; four grade I, six grade II and four grade III. Seven (15.56%) patients developed chronic GVHD; 3 of them on top of acute GVHD. Patients with chronic GVHD showed no significant differences in any of the tested parameters.

3.1. Cytokines of donors' origin in relation to acute GVHD

Both IL12 and IFNγ showed marked variability between individual cases. Positive correlation between both cytokines was encountered in culture supernatant (r = 0.75, P = < 0.001) as well as in patients' plasma at engraftment (r = 0.57, P = < 0.001).

In culture supernatant IL12 was detectable in 7/14 (50%) cases who developed acute GVHD; the missed 7 cases included the 4 cases with grade I and two grade II GVHD cases. It could not be detected in any of the 31 cases who did not develop acute GVHD (P = < 0.001).

IFN γ was detected in 10/14 (71.43%) cases who developed acute GVHD as compared to 3/31 cases without acute GVHD. The three cases showed a level of 1.1, 8.1 and 80.01 pg/ml. To be noted, even these 3 positive cases that did not develop acute GVHD had lower levels of IFNγ when compared to the high figures encountered in patients who developed acute GVHD (P = < 0.001).

In patients' plasma at engraftment, IL12 was detectable in 7/14 cases with acute GVHD. It was undetectable in 26/31 cases without GVHD. IFNγ was detectable in 11/14 (78.57) cases with acute GVHD. It was undetectable in 24/31 (77.42) cases without GVHD. The levels of IL12 and IFNγ, of donor origin, in the 14 cases that developed GVHD are presented in Table (1) and the levels in positive cases are presented in Table (2). The difference was found to be significantly different between patients who did and those who did not develop acute GVHD (P = < 0.001).

At a cut off value of 15.9 pg/ml in either culture supernatant or patients' plasma at engraftment, IFNγ showed a sensitivity of 64.3%, a specificity of 96.8% and a total accuracy of 80.4% (Fig. 1). Nine/10 cases that had a level ≥ the cutoff in the culture supernatant developed acute GVHD as compared to 5/35 with levels below the cutoff (p = 0.001). While 9/12 cases that had a level ≥ the cutoff in patients' plasma at engraftment developed acute GVHD as compared to 5/33 with levels below the cutoff (p = 0.001).

At a cutoff value of 0.89 pg/ml, the level of IL12 in culture supernatant showed a sensitivity of 50.0%, absolute specificity of 100.0%

Table 1
IL12 and IFNγ Levels (pg/ml) of donor origin in 14 cases that developed GVHD.

Case No	Age Years	Diagnosis	GVHD Grade	Culture Supernatant		At Engraftment	
				IL12	IFNγ	IL12	IFNγ
1	25	CML	G2	< LDL	< LDL	< LDL	< LDL
4	22	CML	G2	< LDL	< LDL	< LDL	< LDL
5	29	AML	G2	< LDL	6.2	< LDL	12.2
6	18	CML	G2	< LDL	< LDL	< LDL	< LDL
8	31	AML	G2	2.00	41.66	77.92	10.52
15	29	CML	G3	11.6	651.2	8.9	224.9
21	34	CML	G1	1.78	23.7	6.3	15.9
24	45	CML	G3	463.5	19000	608.5	360.9
25	23	AML	G3	12.81	71.2	< LDL	96.2
36	29	MDS	G2	14.6	118.6	51.9	88.3
37	41	CML	G3	72.3	159.3	88.3	427
39	35	AML	G1	< LDL	< LDL	< LDL	15.99
42	10	AML	G1	< LDL	29.14	< LDL	56.99
45	33	ALL	G1	< LDL	15.97	3.89	11

< LDL: less than lower detection limit.

Table 2
IL12 and IFNγ levels (pg/ml) of Donor Origin in Relation to Acute GVHD post Allogeneic Peripheral Blood Stem Cell Transplantation.

GVHD Status	Culture Supernatant		Post Engraftment	
	IL12	IFNγ	IL12	IFNγ
Acute GVHD: 14 cases				
+ve cases	7 (50%)	10 (71.4 %)	7 (50%)	11 (78.57%)
Range	2.0–463.5	6.2–19.000	3.89–608.5	11–427
Median	14.6	133.5	51.8	77.9
No GVHD: 31 cases				
+ve cases	0 (0%)	3 (9.6%)	5 (16.13%)	7 (22.58%)
Range	0.00–0.00	1.1–80.01	2.0–6.88	0.27–26.67
Median	0.00	8.1	2.93	15.8
P.	< 0.001	< 0.001	< 0.008	< 0.001

Table 3
IL12 and IFNγ (pg/ml) levels of Patient's Origin in Relation to Acute GVHD post Allogeneic peripheral Blood Stem Cell Transplantation.

GVHD Status	Post Conditioning		Aplastic Phase	
	IL12	IFN γ	IL12	IFN γ
Acute GVHD: 14 cases				
+ve cases	3 (21.43%)	2(14.29%)	3 (21.43%)	6 (42.86%)
Range	2.46–9.32	2.03 & 23.7	3.41–61.33	9.43–470.31
Median	7.9		5.87	38.75
No GVHD: 31 cases				
+ve cases	2 (6.45%)	5 (16.13%)	4 (12.90%)	7 (22.58%)
Range	2.06 & 23.7	0.27–266.0	1.1-14.83	1.01– 202.4
Median	–	26.69	3.6	29.63
P	0.366	0.88	0.3	0.14

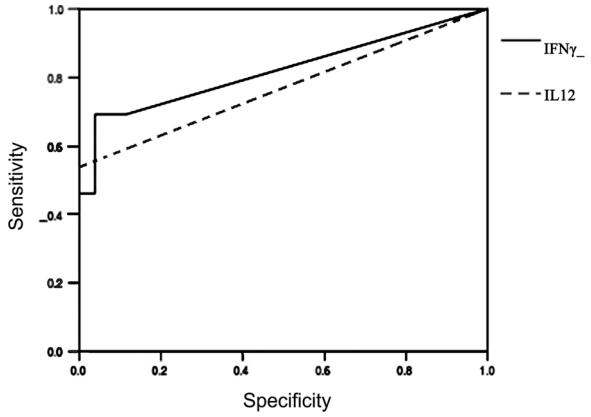

Fig. 1. ROC curves for level of IL12 and IFNγ in mixed lymphocyte culture supernatant in patients with and without acute GVHD after allogeneic PBSCT from a sibling.

and a total accuracy of 83.3% (Fig. 2). All 7 cases that had a level ≥ the cutoff developed acute GVHD as compared to 7/38 with levels below the cutoff (p = 0.000). At a cutoff value of 1.0 pg/ml, the level of IL12 at engraftment showed a sensitivity of 50.0%, a specificity of 83.9% and a total accuracy of 72.3%. Seven/12 cases that had a level ≥ the cutoff developed acute GVHD as compared to 7/33 with levels below the cutoff (p = 0.023).

3.2. Cytokines of patients' origin in relation to acute GVHD

Both IL12 and IFNγ showed marked variability between individual cases.

Positive correlation between both cytokines was encountered in patients' plasma at both post-conditioning (r = 0.86, P = < 0.001) and during aplastic phase (r = 0.54, P = < 0.001).

No significant difference was encountered in the levels of either cytokine between cases who did and those who did not develop acute GVHD (Table 3).

Post-conditioning, IL12 was undetectable in 11/14 (78.6%) cases who developed as compared to 27/31(87.1%) cases who did not develop acute GVHD.

On the other hand, IFNγ was undetectable in 12/14 (85.7%) cases who did as compared to 26/31 (83.9%) cases who did not develop acute GVHD.

In patients' plasma during the aplastic phase, IL12 was undetectable in 11/14 (78.6%) cases with as compared to 27/31 (87%) cases without acute GVHD. IFN γ was undetectable in 9/14 (64.3%) cases with as compared to 24/31 (77.4%) cases without acute GVHD. Table (3) presents the cytokine levels in positive cases and Table (4) presents the findings in the 14 cases that developed acute GVHD.

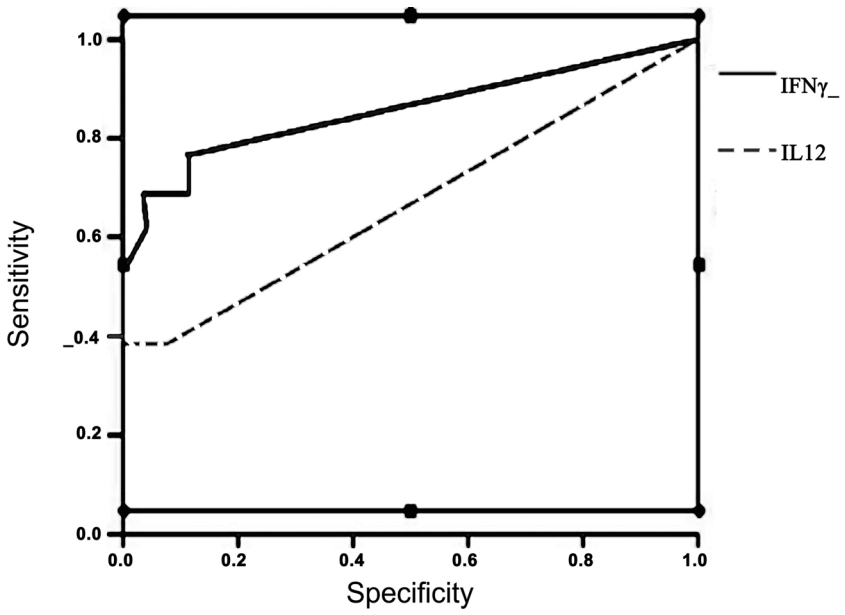

Fig. 2. ROC curves for level of IL12 and IFNγ in patient's plasma post-engraftment in patients with and without acute GVHD after allogeneic PBSCT from a sibling.

Table 4
IL12 and IFNγ Levels (pg/ml) of patient's origin in 14 cases that developed GVHD.

Case No	Age Years	Diagnosis	GVHD Grade	After Conditioning		Aplastic phase	
				IL12	IFNγ	IL12	IFNγ
1	25	CML	G2	< LDL	< LDL	< LDL	< LDL
4	22	CML	G2	< LDL	< LDL	< LDL	< LDL
5	29	AML	G2	< LDL	< LDL	< LDL	< LDL
6	18	CML	G2	< LDL	< LDL	< LDL	< LDL
8	31	AML	G2	< LDL	< LDL	< LDL	< LDL
15	29	CML	G3	< LDL	< LDL	< LDL	< LDL
21	34	CML	G1	< LDL	< LDL	< LDL	< LDL
24	45	CML	G3	9.32	< LDL	5.87	< LDL
25	23	AML	G3	7.9	23.7	< LDL	470.31
36	29	MDS	G2	< LDL	< LDL	< LDL	46
37	41	CML	G3	< LDL	< LDL	61.33	56.3
39	35	AML	G1	< LDL	< LDL	< LDL	31.5
42	10	AML	G1	< LDL	< LDL	< LDL	5.22
45	33	ALL	G1	2.46	2.03	3.41	9.43

< LDL: less than lower detection limit.

3.3. Other factors contributing to acute GVHD

We analyzed the known risk factors for development of acute GVHD including age, diagnosis, conditioning regimen, female donor to male recipient and CD34 + ve dose. None was significant in the current cohort (Table 5).

4. Discussion

GVHD continues to be a major complication after allogeneic HSCT(Nomura et al., 2009; Zeiser and Blazar, 2017). A lot of articles have addressed the potential factors contributing to risk and severity of acute GVHD (Ferrara et al., 2009; Flowers et al., 2011; Fanning et al., 2013; Kamel et al., 2018a, b).

The pathophysiology of GVHD can be described as a three phase process. The first phase is the afferent phase associated with release of proinflammatory cytokines in response to conditioning regimen-induced tissue damage (Zeiser et al., 2004). These act to up regulate the expression of MHC antigens and cell surface adhesion molecules on host antigen presenting cells (APCs), which mediate an alloimmune response by mature donor T cells. This cytokine storm is an important mediator of the occurrence and severity of acute GVHD, and cytokines interactions directly Influence this scenario. However, the balance between pro and anti Inflammatory cytokine releases in determining GVHD is complex and most probably Influenced by many transplant variables including the type of conditioning regimen, stem cell source and number of T cells within the graft as well as the type of GVHD prophylaxis (Bader et al., 2004). It is worth mentioning that though proinflammatory cytokines play a crucial role in acute GVHD induction, yet they may sometime protect from relapse which further complicates the impact of cytokines on the ultimate outcome of HSCT. In an elegant study by Mathew et al (Mathew et al., 2018) it was shown that sorafenib induced a graft vs. leukemia effect via increased IL-15 production by FLT3-ITD + leukemia cells and was associated with better survival. The second phase of acute GVHD is the induction and expansion phase. It is the triggering and activation of donor derived T cells by recipient and donor APC as well as the inflammatory cytokines (Bader et al., 2004). Activated T cells result in the production of IL 2 and IFNγ (or Th1 response). Finally, the effector phase is characterized by activated donor T cell mediated cytotoxic damage against host cells through Fas–Fas ligand interaction, perforin granzyme and TNFα. The latter has a central role in the pathophysiology, stimulating cytokine production leading to the clinical manifestations of acute GVHD (Zeiser et al., 2004; Bader et al., 2004).

Table 5
Risk factors of acute GVHD in 46 allogeneic Hematopoietic stem cell transplant recipients from an identical donor.

Parameter	GVHD		p
	Yes (No: 14)	No (No: 31)	
Donor/Recipient gender:	6	16	0.33
Male–Male	1	6	
Male–Female	1	4	
Female–Female	6	5	
Female–Male			
Age groups: Years	1	2	0.96
< 18	4	8	
18–25	9	21	
> 25			
Conditioning regimen	7	15	0.85
Bu/CY	1	5	
TBI/CY	4	9	
FLU/CY	2	2	
Others			
CD34 count x10^6/kg	6.7 ± 2.92	6.71 ± 2.08	0.59
%CD3 count in graft MNC	63.19 ± 14.87	63.61 ± 9.23	0.86
CMV activation: No (%)	2/14 (14.29%)	6/32 (18.75%)	0.71

* Not included in statistics.
Bu: Busulfan, Cy: Cyclophosphamide, TBI: Total Body irradiation, Flu: Fludarabine, CMV: Cytomegalo-virus, AML: Acute myeloid leukemia, CML: Chronic myeloid leukemia, MDS: Myelodysplastic syndrome, ALL: Acute lymphatic leukemia.

Both Inflammatory and anti Inflammatory cytokines have been reported to be associated with acute GVHD. Though, their role and possible mutual interactions during acute GVHD are not well understood, yet, cytokines are acknowledged as a main player at the core of donors' immune response to host antigens (Henden and Hill, 2015). In general Th1 cytokines are associated with development of acute GVHD while Th2 are claimed to be protective. The hypothesis that Th1 cytokines may be potent in stimulating cell mediated immune mechanisms involved in acute GVHD was supported, several decades ago, by reports stating that localized production of IFNγ within acute GVHD target organs is mediated by IL12 production from macrophages and that anti IL12 monoclonal antibody blocks acute GVHD development in vivo in mice (Williamson et al., 1996). Accordingly, we hypothesized that, if the Th1 cytokine production could be evaluated upfront, in an in vitro model, it might be possible to predict the possibility of development of acute GVHD before transplantation. We also wanted to evaluate the contribution of the cytokines from donor or recipient origin to development of acute GVHD. Thus we performed a MLC experiment in which, host mononuclear cells and those of the donors' were co-cultured for three days in a trial to mimic the interaction of host and donor cells after engraftment; Mitomycin treated patient's cells acted as stimulators while graft cells acted as responders in MLC set up. MLC between identical siblings is expected to be negative with regards to lymphocyte proliferation; in the classical MLC there is no H^3 thymidine uptake. The release of cytokines in a number of cases denotes that even without proliferation, donor lymphocytes reacted to the host antigens with the production of an array of cytokines. Explanation of this immune response is difficult; however one possibility may be that after thawing the used cryopreserved patients' cells might contain some dead cells exposing different antigens. This might not be the case when using fresh living cells as the ones used in classical MLC. Yet this may be more similar to the situation in vivo with the post conditioning tissue damage. Even without post-thawing death, some cells will be disintegrated during the 3 day incubation period of the MLC. The finding that some of the cases that developed acute GVHD showed no cytokine production further highlights the complexity and the variability of mechanisms underlying the development of acute GVHD.

We also measured plasma concentration of both cytokines at day 0 and during aplastic phase (host origin) as well as at engraftment (donor origin).

Significant positive correlation was encountered between IL12 and IFNγ levels in culture supernatant and in patients' plasma at all time points. This is in harmony with the fact that IFNγ is a Th1 cytokine mainly produced by T cells, NK cells and macrophages in response to IL12 (Ethuin et al., 2004). Both IL12 and IFNγ of donor origin in culture supernatant and at engraftment were associated with the development of acute GVHD. While the level of those of patients' origin at day 0 and during aplastic phase were comparable in patients who developed or did not develop acute GVHD. The pathogenesis of GVHD has been attributed, at least partly, to the cytokine storm occurring post-conditioning in response to tissue damage. Apparently IL12 and IFNγ are not among the players in this scenario which is probably executed by inflammatory cytokines namely IL1, IL6 and TNFα (Henden and Hill, 2015). Lack of effect of cytokines of patients' origin has been reported as well for IL10 (Tawara et al., 2012)

Our assumption that acute GVHD may be predicted before transplantation is highly supported by the in vitro production of IL12 and IFNγ in the MLC setup. In culture supernatant, IL12 was detected in 7/14 (50.0%) cases that developed acute GVHD and in none of the 31 cases who did not develop acute GVHD. Thus IL12 production in culture supernatant predicts the occurrence of acute GVHD while its absence, though mostly favors that acute GVHD will not develop, does not absolutely exclude this possibility. In other words it is a good positive but not negative predictor of acute GVHD. However it is worth mentioning that among the negative cases were the four grade I and two grade II acute GVHD cases.

The absence of IL12 in the other seven cases may be explained by the fact that IL12 is mainly produced by myeloid DCs and that peripheral blood monocytes are considered the main pool of DC precursors (Rissoan et al., 1999) and the fact that, at the effector level, naive CD4 positive lymphocytes are the principal target for IL12 which induces their differentiation into Th1 effectors (Trinchieri, 2003). A study made by Mohty et al, (Mohty et al., 2005) investigated the recovery of monocytes in relation to clinically significant acute GVHD; they found more rapid recovery of monocytes before clinical onset in patients with grade II to IV acute GVHD than in patients with grade 0 to I acute GVHD (P = 0 0.005). Accordingly the number of monocytes in the graft in our MLC may be a contributing factor; it would be interesting to study the percentage of monocytes in the graft and investigate their relation to IL12 production in culture. In the same study that was done by Mohty et al. (Mohty et al., 2005), high IL12 level measured one month after allogeneic SCT was significantly associated with grades II to IV acute GVHD (P = 0.001), which corresponds to our findings regarding IL12 of donors' origin in post engraftment phase. However IL12 at engraftment does not have the same predictive power as that of culture supernatant, as it was encountered in 7/14 (50%) cases who developed acute GVHD as compared to 5/31 (16.13%) cases who did not develop acute GVHD.

On evaluating the level of IL12 of patients' origin; in the post conditioning phase, it was detected in 3/14 (21.43%) in cases who developed acute GVHD as compared to 4/31 (13.0%) in cases who did not develop acute GVHD (P = 0.36).

These findings are in concordance with the findings of a study made by Reddy et al. (Reddy et al. (2005)), as there was no association between IL12 levels and the risk of GVHD (P = 0.51) when plasma IL12 levels were measured before transplantation (patients' origin).

In this study, the level of IFNγ was found to be significantly higher in culture supernatant of patients who developed acute GVHD it was encountered in 10/14(71.4%) compared to only 3/31 (9.6%) cases who did not develop acute GVHD (P < 0.001). To be noted, even these 3 cases who did not develop acute GVHD had lower levels of IFNγ when compared to the high figures encountered in patients who developed acute GVHD (P < 0.001). It has been shown that IFNγ is an important cytokine in the efferent phase of acute GVHD and that serum IFNγ is increased in patients with acute GVHD (Niderwieser et al., 1990).

In concordance with our findings, Rembereger et al., 2003) had reported a significantly higher levels of IFNγ among patients who had moderate to severe acute GVHD compared to patients with little, if any, GVHD when IFNγ was measured after engraftment i.e. donor's origin (P < 0.03), which corresponds to the post engraftment phase in our study.

IFNγ alone can efficiently up regulate the class II antigen presenting pathway and thus promotes peptide specific activation of CD4 + T cells. IFNγ treatment further up regulates class II MHC molecules in cells constitutively expressing class II MHC, such as B cells, DCs, and cells of the monocyte macrophage lineage (professional APCs) (Mach et al., 1996).

IFNγ is also able to induce class II MHC expression in cells that do not constitutively express these genes (non professional APC's) (Billiau et al., 1988).

However, in our study, the level of IFNγ in phases corresponding to patients' origin was comparable in both groups with or without acute GVHD. In post conditioning phase it was detected in 2/14(14.3%) of cases who developed acute GVHD compared to 5/31(16.13%) cases who did not develop acute GVHD (P = 0.88). While, in aplastic phase it was detected in 6/14 (42.86%) cases who developed acute GVHD compared to 7/31 (22.58%) cases who did not develop acute GVHD (P = 0.14).

It has to be taken in consideration that the in vivo effect of IFNγ is multifaceted. Among other cytokines, IFN-γ is known to induce up-regulation of chemokines and receptors that play an important role in determining the severity of GVHD (He et al., 2007). On the other side, IFN- γ is essential for the development of T-regs; also expression of PD1 on lymphocytes and its receptor PD-L1 expression on non-lymphoid tissue were reported to be IFN-γ dependent and contribute to the constraint of lymphocyte-mediated tissue damage late in the acute GVHD setting (Blazar et al., 2003). Therefore, in the setting of HSCT, cytokines (including IFN-γ) participate in positive and negative-feedback loops in both lymphocyte and nonlymphocyte populations (Zeiser and Blazar, 2017; Zeiser et al., 2004).

IFN γ plays an important role in the maintenance of T cell homeostasis (Seder and Ahmed, 2003). and regulation of T regulatory cells (Yang et al., 2005). The mechanism of suppressive effect of T regulatory cells involves secretion of the immunosuppressive cytokine IL10 upon activation in vitro (Nakamura et al., 2001), as well as in vivo, where IL10 production by $CD4^+CD25^+$ T cells seemed to be essential in preventing intestinal inflammation (Asseman et al., 1999). This may emphasize the importance of the balance between different cytokines in the in vivo situation.

Cytokines, in general, are locally produced and act mainly on the cells in the immediate vicinity. This highlights the general limitation of in vivo plasma cytokine measurements. Accordingly the in vitro MLC setup might be a better indicator for cytokine production.

Trials to predict occurrence and severity of GVHD are ever going. Paczesny et al. (Paczesny et al. (2009)), screened plasma of transplanted patients at different time points after transplantation and within 24 h of GVHD diagnosis. Using logistic regression analysis, they reported that a 4 biomarkers (HGF, TNFR1, IL-8, and IL-2Rα,) panel was effectively associated with acute GVHD.

Using 4 biomarkers, Hartwell et al. (Hartwell et al. (2017)) suggested an early algorithm to predict lethal GVHD and survival. This model was also capable of predicting the risk of severe GVHD after SCT before the onset of GVHD symptoms.

Budde et al. (Budde et al. (2017)) used a panel including lymphocytes and cytokines for prediction of graft-versus-host disease. Five-parameter biomarker score including $CD4^+$ and $CD8^+$ T cells, $CD19^-$ $CD21^+$ B cells, CD4/CD8 ratio, as well as soluble IL-2 receptor was used to predict GVHD.

Min et al (Min et al., 2017) used a composite biomarker panel (including: 1- elafin, 2- regenerating islet-derived 3-α, 3- soluble tumor necrosis factor receptor-1, 4- soluble interleukin-2 receptor-α and 5-hepatocyte growth factor) for prediction of severity and diagnosis of

acute GVHD with T-cell-depleted allogeneic stem cell transplants. The panel significantly differentiated between patients with and without acute GVHD. Martinez-Laperche et al, (Martínez-Laperche et al., 2018) suggested a predictive approach including clinical variables (gender and gender mismatch, age, CD34+ cell source, conditioning regimen) as well as cytokine gene polymorphisms. Clinical variables included donor and recipient gender, recipient age, female donor/male recipient, stem-cell source, conditioning regimen; in addition to 25 SNPs in 12 cytokine genes. They concluded that combining clinical and genetic data gave better prediction of GVHD

Kim et al. (Kim et al. (2012)) proposed SNP-based risk models, also including clinical and genetic variables, associated with acute, but not chronic GVHD.

Plasma micro RNA (miRNA) has also been suggested as a non-invasive biomarker for prediction of occurrence and severity of acute GVHD (Xiao et al., 2013). A 4 miRNA model (miR -199a-3p, miR-377, miR-93, and miR-423,) was claimed to predict the probability of acute GVHD occurrence and severity. their high expression was associated with poor overall survival (45). Also, donor or recipient miR-146a polymorphism (rs2910164) was connected to higher frequency of grade III- IV acute GVHD (Stickel et al., 2014, 2017).

All previous studies addressed prediction of occurrence and severity of GVHD after transplantation. In the current study, we are reporting, for the first time, the possibility of prediction of acute GVHD upfront if in the model we used, the graft cells, as a stimulator, are substituted with donors' mononuclear cells in a MLC before conditioning of the patient or stem cell mobilization of the donor. With 100% specificity IL-12 level in the culture supernatant is a good positive predictor. Though, with 50% sensitivity, it is not a perfect negative predictor, yet we have to take in consideration that 6/7 of the missed cases included the four grade I and two grade II GVHD. Also in those cases with expected acute GVHD, an alternate donor may be considered. Unlike IL12, IFNγ in culture supernatant was not an absolute positive predictor; however using the calculated cutoff it showed 96.8% sensitivity missing only one case, which makes it still a valuable marker. The small number of cases did not allow analysis of combination of both IL12 and IFNγ in the same case.

In conclusion, we have demonstrated that IL12 and IFNγ of donor origin were associated with acute GVHD. In the MLC setup, production of IL12 was an absolute positive but not negative predictor of the occurrence of GVHD. The in vitro MLC setup might allow prediction of acute GVHD before setting a date for the transplant. Promising as they are, the results here are obtained from a small number; a validation cohort is essential to consolidate these data.

Declaration of interest

None.

Authorship

A.M.K. contributed to the design of the experiments, interpretation of results and writing of the manuscript, N.M.E. contributed to the analysis on luminex, E.K.A. performed the experiment, R.A.E. contributed to patients care and collection of clinical data, M.A.S. contributed to patients care and collection of clinical data, PW contributed to study design and supervised luminex data analysis, H.K.M. contributed to patients care and collection of clinical data.

CRediT authorship contribution statement

Azza M Kamel: Conceptualization, Data curation, Funding acquisition, Project administration, Writing - review & editing. **Nahla M Elsharkawy:** Investigation, Data curation. **Eman K Abdelfattah:** Investigation, Methodology. **Raafat Abdelfattah:** Project administration, Data curation. **Mohammed A Samra:** Project administration, Data curation. **Paul Wallace:** Conceptualization, Supervision, Validation. **Hossam K Mahmoud:** Project administration, Data curation.

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