Cytopenia and leukocyte recovery shape cytokine fluctuations after myeloablative allogeneic hematopoietic stem cell transplantation

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ABSTRACT

Background

Allogeneic hematopoietic stem cell transplantation is associated with profound changes in levels of various cytokines. Emphasis has been placed on conditioning-associated mucosal damage and neutropenia and associated bacterial translocation as the initiating conditions predisposing to acute graft-*versus*-host disease. The post-transplant period is, however, also associated with increases in certain homeostatic cytokines. It is unclear how much the homeostatic drive to lymphocyte recovery and the production of cytokines from the engrafting donor immune system determine cytokine fluctuations in the peri- and immediate post-transplant period. The aim of this study was to examine the contributions of the conditioning regimen, donor engraftment, infections, and graft-*versus*-host disease to fluctuations in cytokines involved in homeostasis and inflammation.

Design and Methods

We examined the levels of 33 cytokines in relation to peri- and post-transplant events such as conditioning regimen, chimerism, and acute graft-*versus*-host disease in myeloablative, non-T cell-replete HLA-identical sibling donor stem cell transplantation for hematologic malignancies.

Results

We identified two cytokine storms. The first occurred following conditioning and reached peak levels when all the leukocytes were at their lowest concentrations. The second cytokine storm occurred concurrently with hematopoietic reconstitution and subsided with the achievement of full donor lymphocyte chimerism.

Conclusions

Our results indicate that both recipient-related and donor-related factors contribute to the changes in cytokine levels in the recipient following allogeneic hematopoietic stem cell transplantation. *The study reported here was performed using plasma samples drawn from patients enrolled in the ClinicalTrials.gov-registered trials NCT00467961 and NCT00378534*

Key words: cytokines, post-transplantation, conditioning, allogeneic stem cell transplantation.

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The online version of this article has a Supplementary Appendix.

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is associated with events which perturb the normal steady state of circulating cytokines. Animal studies suggest that immunosuppressive and myeloablative conditioning regimens can increase inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor-α (TNFα) and establish a cycle of lymphocyte activation which contributes to the subsequent development of graft-*versus*-host disease (GvHD).[1] In man the profound neutropenia and mucosal damage associated with myeloablative conditioning has been assumed to cause similar cytokine fluctuations triggered by lipopolysaccharides derived from gut bacteria. Studies of the post-transplant humoral cytokine milieu also identified homeostatic increases in some cytokines such as granulocyte colony-stimulating factor (G-CSF),[2] IL-15,[3,4] and IL-7[4,5] during the period of cytopenia as well as cytokine patterns associated with the GvHD alloresponse.[4,6] While emphasis has been placed on the role of mucosal damage from the conditioning regimen in determining early cytokine fluctuations it is unclear how much contribution to the cytokine changes that favor GvHD comes from the homeostatic drive to lymphocyte recovery and the production of cytokines from the engrafting donor immune system. Here we studied the peri- and post-transplant cytokine dynamics in patients undergoing myeloablative conditioning and T-cell-depleted or T-cell-manipulated SCT to explore the contribution of the conditioning regimen, donor engraftment, infections, and GvHD to fluctuations in 33 cytokines (homeostatic, inflammatory and anti-inflammatory cytokines, and chemokines).

Design and Methods

Study population

All patients and donors studied participated in Institutional Review Board-approved protocols 07-H-0136 and 06-H-0248 (ClinicalTrials.gov identifiers NCT00467961 and NCT00378534, respectively). Patients received a 7-day conditioning regimen consisting of fludarabine, 25 mg/m^2 daily on days -8 to -4, fractionated total body irradiation at a dose of 150 cGy x 8 fractions on days -7 to -4, and cyclophosphamide 60 mg/kg on days -3 and -2. On day 0 the patients received a CD34 cell-selected HSCT from an HLA identical sibling donor; the graft was depleted of T lymphocytes (residual CD3$^+$ cell dose, 5×10^4/kg) (Miltenyi, Inc.). GvHD prophylaxis consisted of low dose cyclosporine A (plasma levels 100-200 μg/mL), and any GvHD that did develop was treated with steroids, and, in some patients, with methotrexate, budesonide, and/or rituximab. Standard prophylaxis with sulfamethoxazole/trimethoprim and valacyclovir was given and pre-emptive treatment with valganciclovir was given immediately if cytomegalovirus antigenemia was detected. Plasma samples were collected twice weekly from day -8 until 100 days post-transplantation and stored at -80°C until analysis. Establishment of donor hematopoiesis was routinely assessed at days 14, 30, 45, 60, 90 and later by short tandem repeat polymerase chain reaction analysis of chimerism in CD3$^+$ and CD14/15$^+$ cells.[7]

Sample preparation and analysis

Plasma samples from 20 patients and eight transplant donors were collected into heparin. Samples were stored as 300 μL aliquots in cryovials at -80°C until analysis. Levels of 33 hematopoietic and non-hematopoietic cell-derived cytokines (*Online Supplementary Table S1*) with known involvement in inflammatory, anti-inflammatory, homeostatic, chemotactic, and tissue repair processes were determined in duplicate using the Luminex multiplexing platform (Austin, TX, USA) with detection reagents from Bio-Rad (Hercules, CA, USA). Samples with cytokine levels that were below the assay's lower limit of detection were assigned the values of the midpoint between the lower limit of detection and zero.

Statistical methods

Log-transformed leukocyte counts, C-reactive protein and cytokine levels were used to reduce the variability of individual measurements and improve normality of the data. For the analysis of the conditioning effect pre-transplant, a linear mixed-effects model was used to estimate the mean fold change of each variable from 1 week prior to HSCT (day -7) to the time of transplantation (day 0).[8] An estimate of a positive mean slope indicates an increase of the marker level pre-transplant following the conditioning, whereas a negative mean slope indicates a decrease of the marker level. To estimate the mean curve and subject-specific trajectory of each variable over time, a non-parametric mixed-effects regression spline model was used.[8,9] Cytokines without significant fluctuations during the study were identified by examining the 95% confidence bands of their estimated mean curves. To describe the correlation between leukocytes and cytokines or between cytokines, the non-parametric Spearman's rank correlation was used. The resampling-subject bootstrap was used to compute the *P* value accounting for correlation among repeated measurements from each patient.[9] Cox proportional hazards regression models were used to examine the association between the maximum cytokine levels within 2 weeks post-transplant and development of each of the three outcomes (full donor chimerism, acute GvHD and cytomegalovirus reactivation). Statistical significance was set at a two-sided *P* value less than 0.05. Analyses were performed using the R statistical programming software (*www.r-project.org*).

Results

Patients studied and transplant outcome

The characteristics and major outcomes of the 20 patients studied are summarized in Table 1. Seven patients over the age of 55 years received reduced dose conditioning of 400 cGy. Seven patients received a T-cell-depleted graft (protocol 06-H-0248) and 13 received a selectively depleted graft (protocol 07-H-0136).[10] During conditioning, lymphocyte and monocyte counts fell rapidly whereas granulocytes showed a median 3-fold (range, 1.5-6.3) increase over 2 days (related to the dexamethasone administered during the radiation treatment) followed by a rapid decline to zero over the subsequent 5 days (Figure 1A). All patients established donor myeloid chimerism by day 14, and all 20 patients achieved greater than 95% donor T-cell chimerism at a median of 30 days (range, 14-270 days). The median time to neutrophil recovery was 12 days (range, 9-17 days). The median lymphocyte count on day 30 post-HSCT was 781/μL (range, 384-1805/μL). Low-grade (I-II) acute GvHD developed in 17 patients at a median of 35 days (range, 21-108 days), predominantly with skin involvement (Table 1). Cytomegalovirus reactivation occurred in 11 patients on one or two occasions between days 20-55 (median, day 34) after transplantation. Most patients developed a fever with concomitant rise of C-reactive protein following the transplant (Figure 1B), which became undetectable again within 2-4 weeks.

Plasma collection

The cytokine profile of a total of 329 plasma samples obtained every 3-4 days from the start of conditioning was studied (*Online Supplementary Table S1*).

Categorization of cytokine changes

Five cytokine patterns were observed (Figure 1): (i) Cytokines detected in less than 15% of samples or detected below the median level of 15 pg/mL: IL-1β, IL-2, IL-4, IL-5, IL-7, IL-8, IL-10, IL-13, IL-12p70 and macrophage inflammatory protein (MIP)-1α; (ii) cytokines detectable in more than 90% of samples at normal levels without significant fluctuations: stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), IL-17, and MIP-1β; (iii) cytokines positively correlated with leukocyte counts and showing the lowest levels at the time of transplant: IL-1ra and RANTES (regulated upon activation normal T-cell expressed and secreted); (iv) cytokines inversely correlated with leukocyte count reaching a peak within 1 week after transplantation: monocyte chemotactic protein-1 (MCP-1), G-CSF, IL-6 and IL-15; (v) cytokines showing a gradual increase from transplant until day 35: hepatocyte growth factor (HGF), stem cell growth factor beta (SCGFβ), interferon-inducible γ-induced protein-10 (IP10), IL-18, platelet-derived growth factor-BB (PDGF-BB), IL-9, eotaxin, IL-1ra, leukemia inhibitory factor (LIF), and RANTES.

Cytokine fluctuations during the conditioning regimen

We tested the hypothesis that the conditioning regimen induces a surge in inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and IL-1β.[11] We took a global approach to determine whether these and other cytokines with homeostatic, inflammatory, and anti-inflammatory functions showed any change from the start of conditioning to the day of transplantation. The results are shown in Table 2 and Figure 1. Three cytokine patterns were observed in this pre-transplant period: (i) no change during conditioning: TNFα, IL-1β, IL-12p70, IFN-α2, IL-2, and IL-7; (ii) decreased during conditioning: IFNγ, IL-18, IL-1ra, and C-reactive protein levels; (iii) increased more than 10-fold during conditioning: MCP-1 and IL-15. IL-10 was undetectable during this period. G-CSF and IL-6 levels did not change significantly between day -7 and day 0; however, further analysis revealed a non-linear trend during this period (Figure 1A). Thus, the conditioning regimen was associated with a steady-state or a decrease in inflammatory cytokines.

Peri-transplant leukopenia is associated with a cytokine storm

The leukopenic phase from conditioning until day 14 after HSCT was characterized by significant transient increases in the neutrophil homeostatic cytokines IL-6 and G-CSF, peaking at week 1 post-transplant and declining as

Table 1. Patients' characteristics.

Patient	Sex	Age	Diagnosis	Day after SCT			Acute GvHD			Chronic GvHD			Day CI stopped
				Donor % Chimerism ≥ 95	DLI	CMV	Grade	Day of diagnosis	Treatment	Grade	Day of diagnosis	Treatment	
1	M	47	MDS	45			II	57	CI, Steroids	Extensive	109	CI, Steroids	90
2	M	28	CML	60		37							92
3	M	68	MDS	270	126[¥]	34				Limited	210	CI, Steroids	482
4	M	36	APL	30			II	21	CI, Steroids	Extensive	100	CI, Steroids, RTX	588*
5	F	67	MDS/AML	120	54[¥]		II	22	CI, Steroids				161
6	F	41	ALL Ph+	30			II	24	Steroids	Extensive	100	Steroids, RTX	26
7	M	66	AML	270	147**		I	28	CI, Steroids	Limited	100	CI, Steroids	> 911
8	M	46	AML	30	556[δ]		II	35	CI, Steroids	Extensive	113	Steroids, RTX	> 665
9	F	46	AML	14			I	40	CI, Steroids				120
10	M	65	MDS	120	85[¥]		II	108	Steroids	Extensive	180	Steroids, Etanercet, RTX	295[±]
11	F	58	AML	180	66[¥]					Extensive	143	Steroids, Etanercet, RTX	196[±]
12	M	44	CML	30		21	I	21	Steroids				98
13	F	31	AML	30	85**	55	I	23	Steroids				36
14	F	42	AML	30	212**	37	II	27	CI, Steroids	Limited	100	CI	187
15	F	38	ALL Ph+	30		20	II	30	CI, Steroids, RTX	Limited	102	CI, Steroids, Daclizumab, Infliximab	449[±]
16	F	30	MDS	30		27	II	44	CI, Steroids	Limited	107	CI, Steroids, RTX	421[±]
17	M	68	CLL	45		37	I	59	CI, Steroids	Extensive	150	CI, Steroids, RTX	139[±]
18	M	53	AML	30		34	I	300	CI, Steroids	Limited	300	CI, Steroids	810*
19	F	59	AML	14	196**	23	I	100	CI, Steroids, RTX	Limited	100	Steroids, CI, RTX	181
20	M	41	MDS	30	404[δ]	27	II	84	Steroids	Limited	210	CI, Steroids, RTX	967

*M: male; F: female; MDS: myelodysplastic syndrome; CML: chronic myelogenous leukemia; APL: acute promyelocytic leukemia; AML: acute myelogenous leukemia; ALL: acute lymphoblastic leukemia; CLL: chronic lymphocytic leukemia; DLI: donor lymphocyte infusion; CMV: first day of cytomegalovirus reactivation; Steroids, includes prednisone, methylprednisolone, budesonide; CI: calcineurin inhibitor; RTX: rituximab; *CI continued from acute GvHD treatment; [±]patient died while receiving CI; DLI given for: [¥]falling donor chimerism, ** relapse of malignancy, [δ]recurrent viral reactivation. All patients achieved full myeloid donor chimerism on or before day 14*

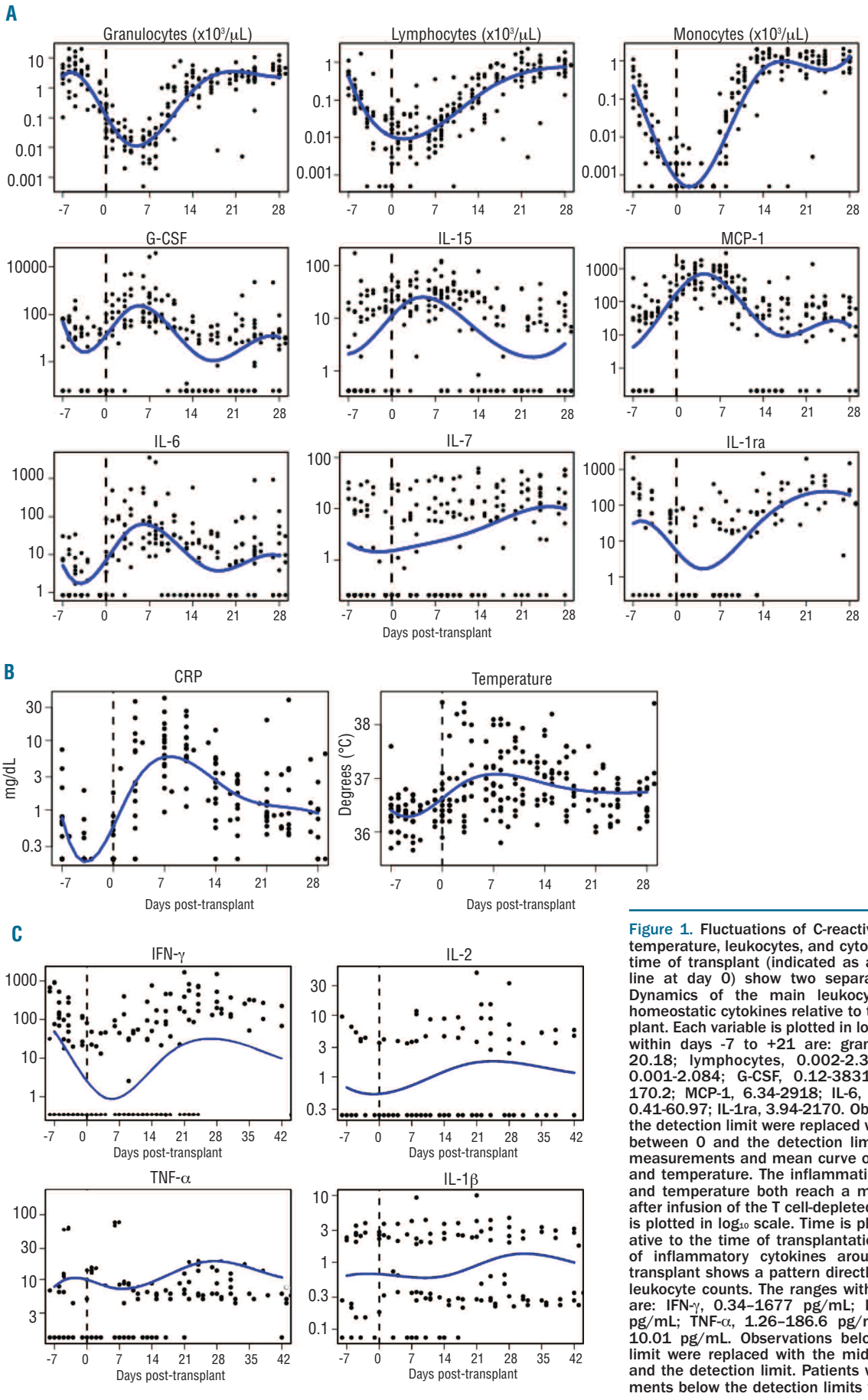

Figure 1. Fluctuations of C-reactive protein (CRP), temperature, leukocytes, and cytokines around the time of transplant (indicated as a broken vertical line at day 0) show two separate patterns. (A) Dynamics of the main leukocyte subsets and homeostatic cytokines relative to the time of transplant. Each variable is plotted in $\log_{10}$ scale. Ranges within days -7 to +21 are: granulocytes, 0.002-20.18; lymphocytes, 0.002-2.307; monocytes, 0.001-2.084; G-CSF, 0.12-38310; IL-15, 0.84-170.2; MCP-1, 6.34-2918; IL-6, 1.72-3524; IL-7, 0.41-60.97; IL-1ra, 3.94-2170. Observations below the detection limit were replaced with the midpoint between 0 and the detection limit. (B) Individual measurements and mean curve over time for CRP and temperature. The inflammation indicator CRP and temperature both reach a maximum a week after infusion of the T cell-depleted transplant. CRP is plotted in $\log_{10}$ scale. Time is plotted in days relative to the time of transplantation. (C) Dynamics of inflammatory cytokines around the time of transplant shows a pattern directly correlated with leukocyte counts. The ranges within days -7 to 42 are: IFN-γ, 0.34–1677 pg/mL; IL-2, 0.24–48.77 pg/mL; TNF-α, 1.26–186.6 pg/mL; IL-1β, 0.08–10.01 pg/mL. Observations below the detection limit were replaced with the midpoint between 0 and the detection limit. Patients with all measurements below the detection limits were excluded.

granulocytes returned (Figure 1A). There was an inverse correlation between neutrophil numbers and G-CSF and IL-6 (neutrophils and G-CSF, Spearman's correlation r = -0.57, $P<0.001$; neutrophils and IL-6 r = -0.58; $P<0.001$). A similar inverse correlation was observed between lymphocyte count and IL-15 (r = -0.32; $P=0.01$), a cytokine which is required for NK-cell and T-cell recovery (Figure 1A).[3,12-15] Peak levels of these homeostatic cytokines occurred within 7 days of transplantation, suggesting a close homeostatic relationship between the cytokine and its target (Figure 2).

In contrast, levels of the lymphopoietic cytokine IL-7 correlated directly with leukocyte numbers (r = 0.39; $P=0.005$), suggesting that IL-7-producing cells were contained within the graft. C-reactive protein levels increased after transplantation and peaked at a median of 7 days after HSCT (range, 3-82 days), correlating significantly with IL-6 levels (r = 0.74; $P<0.001$), and confirming a regulatory role of IL-6 in determining C-reactive protein levels. Furthermore, peak body temperature appeared at a median of 8.5 days (range, 0-62 days), following closely after the IL-6 peak.

Cytokine changes associated with donor lymphocyte engraftment and later post-transplant events

In a univariate Cox regression analysis of cytokine peak levels occurring within 2 weeks post-transplantation and the occurrence of chimerism, cytomegalovirus reactivation, and acute GvHD, we found that peak IP-10 levels were significantly associated with early full donor chimerism. A trend was noted for eotaxin, PDGF-BB, IL-1β, LIF and IL-17 (all $0.05< P <0.1$). None of the cytokines was significantly correlated with the onset of acute GvHD or cytomegalovirus reactivation (all $P>0.1$). In contrast to the uniform fluctuations in homeostatic cytokine peak levels, inflammatory cytokines displayed wide patient-dependent variation, suggesting that other frequent post-transplant events not monitored here (including changes in hepatic and renal function, transfusions, polypharmacy, infections, and their treatment) contributed to individual day-to-day cytokine changes.

Table 2. Estimated cytokine levels and fold changes during conditioning.

Cytokine (pg/mL)	Median level at day -7	Median level at day 0	Mean fold change from day -7	P*
Increasing during conditioning				
IL-15	2.1	23.3	11.2	0.0003
MCP-1	9.6	167.6	17.5	<0.0001
Decreasing during conditioning				
IFN-γ	186.5	0.9	0.013	<0.0001
IL-1ra	263.4	31.0	0.12	0.002
RANTES	2013	233.4	0.12	0.063
SCGFb	32229	3816	0.11	0.01
IP-10	673.0	228.6	0.34	0.006
HGF	332.1	110.8	0.33	<0.0001
IL-18	278.7	69.9	0.25	0.028

*Note: *P value for testing whether the fold change from day -7 to day 0 was significantly greater than one (increasing trend) or less than one (decreasing trend). Patients who had all cytokine measurements below the detection limit during conditioning have been excluded.*

Discussion

Allogeneic HSCT profoundly disrupts various homeostatic mechanisms and inflicts damage upon organs, simultaneously eliciting homeostatic, repair, and immune responses. Some of the "cytokine fingerprints" of these processes have been studied here, including cytokines driving cell-mediated immunity (IL-1β; IL-12p70; IL-18; LIF; TNFα; IFNγ; IL-17; IL-9) and humoral immunity (IL-2; IL-4; IL-5; IL-10; IL-13); chemokines (RANTES; eotaxin; MIP-1α and -β; MCP-1; IL-8) and cytokines with inflammatory and chemotactic functions (IL-17); and homeostatic and tissue repair cytokines (SCF; SCGF; HGF; FGF-basic; IL-7; IL-15; G-CSF; IL-6; GM-CSF; VEGF; HGF). We correlated their levels and dynamics with clinical (GvHD; fever) and clinical laboratory (C-reactive protein; leukocyte counts) data. Our main findings are: (i) the conditioning regimen caused a decrease rather than increase in inflammatory cytokine levels; (ii) two cytokine surges were identified (Figure 2), the first coinciding with pancytopenia and the second with leukocyte reconstitution. The first cytokine storm involved predominantly hematopoietic and lymphopoietic homeostatic cytokines, whereas the second involved inflammatory and anti-inflammatory cytokines as well as chemokines. Predictably, therefore, we found an inverse correlation not only between granulocyte numbers and G-CSF levels, but also between granulocyte numbers and IL-6 levels, suggesting the latter's involvement in granulocyte reconstitution following myeloablation;[2] (iii) importantly, we found that the switch to donor chimerism and not the occurrence of GvHD correlated most closely with a surge in IP-10.

The transplant conditioning regimen causes widespread damage to healthy tissues, including the gastrointestinal tract with its diverse commensal microbiome.[16,17] In a preclinical T-cell-replete HSCT model Ferrara and co-workers gave fractionated total body irradiation to mice, followed within a few hours by a bone marrow and spleen T-cell-replete MHC-mismatched transplant.[18] Infused donor T cells caused GvHD, but only at the higher dose of total body irradiation which correlated with higher levels of lipopolysaccharides in the circulation. Transplantation of conditioned mice with a graft from lipopolysaccharide-resistant or -sensitive donors showed that mice receiving lipopolysaccharide-resistant donor cells developed significantly less GvHD,[19] pointing toward a role for lipopolysaccharides in the induction of GvHD. Further studies showed that blocking lipopolysaccharides could prevent GvHD,[20] leading to the conclusion that conditioning regimen-induced damage to the gastrointestinal tract was primarily responsible for experimental GvHD.

Unlike the murine transplant model, our patients received a conditioning regimen consisting of both total body irradiation and chemotherapeutics, spread out over 7 days, which causes a slow decline of leukocyte numbers (Figure 1) until the day of transplantation when the T-cell-depleted or allo-depleted transplant is infused from an MHC matched donor. Under these conditions, immune cells responsive to bacteria-derived danger molecules were at a nadir at the time of transplantation (Figure 1). Furthermore, patients received cyclosporine for GvHD prophylaxis, which could blunt the T-cell response.[21] These differences may explain the lack of increase in inflammatory cytokines during conditioning and contrast with findings in murine studies in which total body irradiation followed immediately by infusion of MHC-mismatched immunocompetent

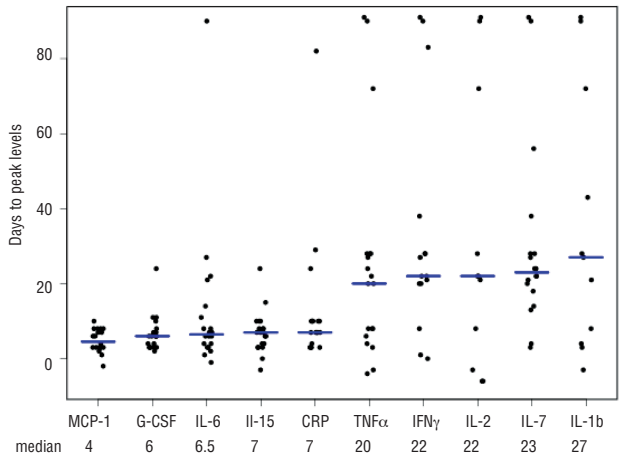

Figure 2. Days to peak levels of C-reactive protein (CRP) and cytokines. Patients with all measurements below detection limits have been excluded.

cells correlated with rises in IL-1 and IL-6. It, therefore, appears that the radiation-induced myeloablation we used did not cause as extensive tissue damage as is generally assumed, although bacterial invasion across a neutropenic mucosa remains a potential mechanism for bacterial lipopolysaccharide-driven cytokine surges during conditioning. It was not possible in this small study to relate peri-transplant changes of cytokines with the subsequent development of GvHD. Furthermore, our findings do not exclude the possibility that irradiation activates tissue resident antigen-presenting cells,[22] subsequently triggering GvHD. It should be borne in mind that cytokine changes are likely to vary considerably according to the regimen and the type of transplant (for example, a reduced intensity regimen was shown to induce increased TNFα levels compared to myeloablative therapy[23]). Our findings concern T-cell-depleted or manipulated myeloablative transplants without heavy immunosuppression. Furthermore, T-cell depletion was achieved by selecting $CD34^+$ cells, thus removing NK cells, B cells, monocytes, and other peripheral blood cells in addition to the T cells, all of which may have affected the quality of the cytokine dynamics post-HSCT. It will be necessary to study other transplant regimens including reduced intensity stem cell transplantation, autologous stem cell transplantation and T-cell-replete transplants to better characterize the contribution of alloreactive T cells, and regimen intensity to peri-transplant cytokine fluctuations.

Many studies have sought to relate levels of cytokines and soluble receptors with GvHD in the T-replete HSCT setting but currently there is no consensus.[24-35] The wide discrepancies may be attributable to the diversity of the diseases transplanted, the transplant conditioning (notably inclusion of antithymocyte globulin);[23] genetic diversity, including cytokine (receptor) gene polymorphisms,[36-40] infection, veno-occlusive disease and lymphocytopenia in the case of transient IL-7 and IL-15 increases.[3-6,29,41,42] However, the achievement of full lymphoid chimerism has so far not been identified as a significant moment for cytokine changes. Nevertheless, the strong correlation of the acquisition of full donor T-cell chimerism with subsequent graft-*versus*-host and graft-*versus*-leukemia reactions would make it likely that cytokine fluctuations might be detected at this time.

It was surprising that in this study IL-7 levels did not follow the same pattern as the other homeostatic cytokines IL-15, G-CSF, and IL-6. IL-7 is produced by non-hematopoietic stromal and epithelial cells in the thymus, bone marrow, intestine, and lymphoid tissue,[43] but also by dendritic cells[44,45] and B cells.[46] Others have found an inverse correlation between IL-7 and lymphocyte numbers post-HSCT.[4,5] Our data suggest that the cells that produced this important lymphopoietic cytokine were contained within the graft, and that the non-hematopoietic tissues were not a significant source of IL-7 in our patients.

To conclude, the absence of a cytokine storm during the conditioning regimen in these T-cell-depleted myeloablative HSCT recipients suggests a revision of the concept that total body irradiation-based regimens cause an important release of inflammatory cytokines. Rather, HSCT is characterized by two cytokine storms, the first occurring during pancytopenia, and the second with immune reconstitution, possibly coinciding with the replacement of the patient's immune system by that of the donor. Our findings support the idea that immune reconstitution might benefit from cytokine therapy with IL-15 which could improve both early NK-cell and T-cell recovery – factors which we have found to be beneficial to the outcome of HSCT.[47,48]

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

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