Plasma levels of IL-7 and IL-15 in the first month after myeloablative BMT are predictive biomarkers of both acute GVHD and relapse

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Introduction

Long-term clinical remissions of hematologic malignancies after allo-SCT largely rely on the GVL effect, but a major limitation is GVHD. In a fully HLA-matched setting, both the GVL effect and GVHD are mediated primarily by mature T cells that are able to differentiate into antileukemia/antilymphoma effectors or to inflict immune damage in host tissues, respectively, whereas natural killer (NK) cells can also exert antitumor effects.1,2 After the transplant conditioning regimen, patients present with a iatrogenic lymphopenia. Although innate immunity, including NK cells, recovers rapidly,3,4 the slow process of T-cell recovery is dominated for many months by homeostatic peripheral expansion (HPE) of mature T cells brought by unmanipulated allografts.4–6

Homeostatic peripheral expansion is dependent on homeostatic cytokines, predominantly IL-76–8 and IL-15.9 Severe lymphopenia results in reduced consumption of these cytokines, whereas the inflammatory environment induced by the preparative regimen could enhance IL-15 release.10 Resultant increased IL-7 and IL-15 levels expose infused donor T cells to concentrations favorable to their expansion and persistence in the recipient. Allo-SCT failure might then result either from insufficient facilitation of tumor immunity because of insufficient HPE or, conversely, from substantial expansion with too strong an immune reaction against host alloantigens, causing GVHD.

A few reports have associated acute GVHD with systemic levels of either IL-711 or IL-15,12–15 whereas others have found no association,16,17 but no study has analyzed the two homeostatic cytokines together, and we are not aware of any report on the possible association of their levels with malignancy relapse. This study prospectively investigated plasma levels of IL-7 and IL-15 in a homogeneous group of 40 patients in CR of their hematologic malignancy who underwent fully HLA-matched BMT after myeloablative conditioning. Peak IL-7 level was found to be a significant predictor of acute GVHD, and peak IL-15 level a significant predictor of malignancy relapse.

Subjects and methods

This study focused only on patients who received BM grafts after myeloablative conditioning, as post transplant immune reconstitution depends on the degree of conditioning-induced lymphopenia and the source of stem cells. A total of 40 consecutive patients were enrolled between October 2006 and January 2009 after receiving the written informed consent and with institutional ethics committee approval.
All patients were in CR of their hematologic malignancy. All cases were as homogeneous as possible in terms of patient/donor characteristics and transplantation modalities; all patients received an unmanipulated A-, B-, Cw-, DR- and DQ-HLA-matched BM graft after myeloablative conditioning. Three patients received antithymoglobulin.

Myeloablative conditioning included either radiation and chemotherapy or chemotherapy alone, and then patients were administered standard GVHD prophylaxis with CY and short-term MTX (on days +1, +2 and +6). Prophylaxes against *Pneumocystis jirovecii*, *Toxoplasma gondii*, fungal and HSV infection were also administered during the neutropenic phase. Patients were monitored prospectively in terms of clinical complications, including graft rejection, GVHD, infection and relapse, with a median follow-up of more than 1 year. Acute GVHD was graded according to standard criteria. Patient and graft characteristics are presented in Table 1.

### Measurement of IL-7 and IL-15 levels

EDTA-anticoagulated blood samples were obtained before conditioning, on the day of transplantation before infusion of the allograft, then 7, 14, 18, 25, 30, 60 and 90 days post transplantation. To ensure optimal recovery, we aliquoted plasma shortly after collection and stored it at −80 °C until measurement of cytokine levels. Samples were analyzed using high-sensitivity ELISAs as per the manufacturer’s protocol (IL-7 highly-sensitive and IL-15 Quantikine ELISA kits; R&D systems, Minneapolis, MN, USA). A total of 38 healthy subjects who gave their consent served as controls.

### Flow cytometry

To determine absolute numbers of CD3⁺, CD3⁺ CD4⁺, CD3⁺ CD8⁺ T cells and CD3⁻ CD56⁺ NK cells (CD3 CD56⁺dim and CD3⁻ CD56⁺bright), we stained peripheral blood samples from day 30, 60 and 90 post-graft with direct conjugates of fluorochrome-labeled monoclonal antibodies, using whole-blood lysis and fluorobeads in a flow-cytometry-based technique. Naive and memory T-cell subsets were enumerated according to their expression pattern of CD45RA and CCR7 markers.

### Statistical analysis

Comparisons between groups were based on the Mann–Whitney U-test and correlations were based on Spearman’s R. The median levels of IL-7 and IL-15 on day 14 post-graft were used to stratify patients for Kaplan–Meier analyses. Differences between strata were evaluated by log-rank tests. Multivariate analyses using Cox proportional-hazards models were used to consider day +14 IL-7 and IL-15 levels and those clinical characteristics (as presented in Table 1) that were significant according to univariate analyses. The final multivariate models were determined using a P-value of 0.05 for the inclusion and exclusion of variables. All analyses were performed with SPSS software (version 11.5, SPSS Inc., Chicago, IL, USA).

### Results

#### Dynamics of plasma IL-7 and IL-15 levels in BM recipients

Before conditioning (day −15), all patients had plasma levels of IL-7 and IL-15 within or slightly above the normal range (Table 1). After initiation of the myeloablative conditioning regimen, both cytokines proceeded along similar kinetic courses and evolved inversely to absolute T-cell counts (Figure 1). IL-7 and IL-15 levels peaked both on day +14 at medians of 11.9 and 33.3 pg/ml, respectively, and gradually returned close to normal values thereafter. Day +14 IL-7 and IL-15 levels were positively correlated with the occurrence of acute GVHD (Table 1).
and strongly correlated ($R = 0.731; P = 0.00005$), and these peak values were closely correlated to preceding (day 0, day $+7$) and following (day $+18$, day $+25$) levels of the respective cytokine, but not to its preconditioning level. Homeostatic cytokine levels were not correlated with recipients’ ages, and comparisons of patients administered myeloablative conditioning with or without TBI or with or without antilymphocyte serum fell short of significance. Neither the CD34$^+$ cell dose nor the T-cell dose infused correlated with post-graft IL-7 and IL-15 levels.

By day $+14$, interpatient variations of IL-7 and IL-15 levels were wide (range $3.8-30.2$ and $14.3-66$ pg/ml, respectively), which is 1.1- to 15.4-fold their respective preconditioning level. These broad ranges were explained by individual differences in circulating T-cell numbers, as all 40 patients had extremely small or null lymphocyte counts before (day 0, day $+7$) and at this time point (33 patients with null counts, 7 with 100–300 lymphocytes per μl; Figure 1). Presence or absence of a few circulating lymphocytes by day $+14$ was not associated with any of the characteristics presented in Table 1. In particular, there was no relation to whether the myeloablative regimen included TBI or antilymphocyte serum. We therefore examined the relationship of plasma IL-7 and IL-15 levels to transplant outcomes.

**IL-7 and IL-15 levels according to transplant-related events**

A total of 19 BM recipients developed acute GVHD. Their highest levels of IL-7 and IL-15 were observed on day $+14$ at medians of 15.8 and 38.7 pg/ml, respectively, which are 7.3- and 7.8-fold their respective preconditioning level. Although IL-7 and IL-15 levels peaked at the same time point, day $+14$ levels of both cytokines were significantly elevated as compared with values from patients without acute GVHD ($P = 0.008$ and 0.04, respectively; Figure 2a). IL-7 levels correlated negatively with the time to onset of acute GVHD ($R = 0.43; P = 0.030$) and positively correlated to the grade ($R = 0.61; P = 0.0015$). Grades 2–4 occurred in 13 patients (Table 1), with IL-7 peaking by day $+14$ at a median of 16.4 pg/ml ($P = 0.004$ as compared to patients with grades 0–1). IL-15 levels peaked also in patients with acute GVHD (grades 1–4: median, 40.2 pg/ml), but their correlation with the grade bordered on significance ($R = 0.401; P = 0.052$). Because of extremely small or null T-cell numbers by day $+14$, the distribution of subsets among T cells could not be accurately determined. By day $+30$, which is the median time to acute GVHD (Table 1), patients who already had or who subsequently developed acute GVHD had greater absolute counts of T cells and their CD4$^+$ subset (medians of 286 vs 210 CD3$^+$ cells per μl; $P = 0.01$ and 82 vs 53 CD3$^+$ CD4$^+$ cells per μl; $P = 0.0495$). Most T cells showed a memory-type phenotype (medians of 89% of all CD4$^+$ and 91% of all CD8$^+$ T cells, respectively), irrespective of recipients’ age. Absolute CD8$^+$ T-cell counts exhibited broad ranges throughout follow-up and did not achieve formal statistical significance.

Using the median value determined on day $+14$ in all 40 recipients for stratification, we found the cumulative incidence of grades 2–4 acute GVHD proved to be...
significantly higher if by day +14 patients had heightened IL-7 levels (Figure 2b). A high level of IL-7 on day +14 was confirmed as the strongest predictive factor of subsequent risk of grades 2-4 acute GVHD by multivariate Cox regression analysis: hazard ratio (HR) = 5.38; 95% confidence interval (CI) = 1.28–22.67; P = 0.022 (overall model fit: P = 0.001). Other characteristics that had univariate significance for this outcome either did not remain in the multivariate model (female to male grafts vs any other combination, definite CMV reactivation rate at any time post-graft) or had lower significance (sibling vs any other combination, definite CMV reactivation rate at any time post-graft) or had lower significance (sibling vs any other combination, definite CMV reactivation rate at any time post-graft) or had lower significance (sibling vs any other combination, definite CMV reactivation rate at any time post-graft). Peak IL-15 levels did not appear in the model, presumably because of their strong correlation to IL-7 levels and the lower univariate significance of IL-15 in association with acute GVHD. Likewise, day +30 CD4+ T-cell counts did not remain in the model. No significant associations of cytokine levels were found with the occurrence of CMV reactivation (N = 11) or chronic GVHD (N = 12).

IL-7 and IL-15 levels in patients who relapsed
Malignant relapse occurred in 10 recipients (Table 1). As shown in Figure 3a, patients who went on to relapse had significantly reduced levels of IL-15 by day +14 (median of 21.3 pg/ml, which is only 3.4-fold the preconditioning level; P = 0.024). Their IL-7 levels also tended to be lower (median of 8.9 vs 13.9 pg/ml, at 5.2-fold the preconditioning level), without achieving formal statistical significance. By day +30 these patients tended to have lower CD8+ T-cell counts (median of 100 vs 138 cells per µl) and less CD3+CD56+ NK cells (median of 77 vs 83 cells per µl), without achieving formal statistical significance at this or later time points. The cumulative incidence of relapse proved to be higher if by day +14 patients had plasma IL-15 levels below the median from all 40 recipients (Figure 3b). Neither occurrence of relapse nor peak levels of IL-7 and IL-15 were significantly associated with malignancy type (myeloid vs lymphoid), disease status at conditioning (first CR vs second remission of acute leukemia), donor type and other characteristics shown in Table 1. Accordingly, when entering malignancy type and disease status at conditioning as covariates in Cox regression analysis, only IL-15 level on day +14 was predictive of subsequent risk of relapse (HR = 0.93; 95% CI = 0.86–1.00; P = 0.035). A competitive risk model was not used, because there was only one death from nonrelapse mortality, and the Cox model remained significant when recalculated entering all but this particular patient (HR = 0.93; P = 0.043).

Discussion
Successful T-cell recovery can lead to GVL effects contributing to potential cure of hematologic malignancies, but GVHD remains a considerable obstacle to improved clinical outcomes. Given the key role of homeostatic cytokines in initial T-cell recovery, this study prospectively analyzed plasma levels of IL-7 and IL-15 over sequential time points before and after allo-SCT. All patients were as homogeneous as possible in terms of patient/donor characteristics and transplantation modalities, being uniformly given myeloablative conditioning, unmanipulated BM grafts with 10 of 10 HLA-matched, and the same GHVD and infection prophylaxis regimens.

Overall, IL-7 and IL-15 levels evolved inversely to absolute T-cell counts, confirming previous reports that analyzed separately IL-7,11,16,17 or IL-15,3,12–15 Both cytokine levels rose after initiation of the myeloablative regimen, both peaked by day 14 after transplantation and were positively and strongly correlated, irrespective of any demographic and clinical characteristics. At this early time point all patients were profoundly lymphopenic, most (33/40) with null lymphocyte counts, consistent with the current model that T-cell depletion increases the bioavailability of homeostatic cytokines.6,8 Constitutive IL-7 production by stromal cells is not thought to be substantially affected by external stimuli, but IL-15 production by recipient cells is subject to modulation by an inflammatory milieu that is conceivably induced by an intensive conditioning regimen and the ensuing cytokine storm. Time course and peak levels of IL-7 and IL-15 were similar,
regardless of whether the myeloablative conditioning had included or not TBI or antilymphocyte serum. Peak IL-7 and IL-15 levels preceded the progressive augmentation of circulating T-cell counts, confirming previous studies that investigated IL-7, IL-15, and IL-18. These observations agree well with the important function of these cytokines as major regulators of HPE of naive and memory T cells and with the role of IL-15 in NK cell development. This series included some patients less than 18 years of age, but peak cytokine levels did not correlate with patients' age. Kinetics of T-cell recovery were similar over the first 3 months post transplant, irrespective of patients' age, confirming previous results. HPE is known to provide the larger contribution to immune reconstitution during the first months post transplant, whereas naive T-cell recovery, which depends on active thymopoiesis, is a very slow process, even in children. Between days +30 and +90, the majority of T cells were characterized as having a memory-like phenotype, consistent with the phenotypic changes known to be associated with the HPE process. Furthermore, a recent trial of IL-7 administration in nonhematologic cancer patients showed that the degree of lymphocyte expansion was dose dependent, but with no relationship to age.

Higher peak levels of IL-7 and IL-15 were associated by univariate analyses with the development and grading of acute GVHD. By applying a multivariate model, we found a high IL-7 level by day 14 after transplantation was the factor most strongly associated with the probability of developing grades 2-4 acute GVHD, whereas the IL-15 factor was lost. Increased IL-7 levels preceding the development of GVHD have recently been reported after reduced-intensity conditioning and PBSC transplantation. In experimental models, the effect of IL-7 on the severity of acute GVHD is modulated by the intensity of the conditioning regimen. Our data provide evidence that measurement of IL-7 levels could assist in predicting risk of acute GVHD after fully myeloablative conditioning.

The positive correlation between peak levels of IL-7 and IL-15 and subsequent occurrence and severity of acute GVHD is likely explained by the substantial HPE that infused T cells in patients with heightened levels of these homeostatic cytokines. Consistent with that, these patients had recovered more T cells, and particularly CD4 + T cells, by 30 days post-graft, which is the median time to onset of acute GVHD. Some studies have reported on less transplant-related mortality in patients who recover T cells, and particularly CD4 + T cells, more rapidly. CD4 + T-cell absolute numbers are usually higher in the first months after PBSC infusion, which provides higher T-cell doses than BM grafts, but the incidence of acute GVHD does not appear to be higher. Thus, it may be not the size of the lymphocyte inoculum but the ratio among their subsets that determines this outcome. Marrow and PBSC grafts provide overall similar relative frequencies of naive and memory T-cell subsets, although with broad interdonor variations. Some T cells accumulated after infections may cross-react with alloantigens, but naive or central memory T cells, or both, contain precursors alloreactive to HLA. In a fully molecular HLA-matched setting, receiving a high percentage of CD4 + T cells expressing CCR7 (composed of naive and central memory T cells) correlates with the incidence of acute GVHD, suggesting that at least one of these two subsets contains precursors alloreactive to minor histocompatibility antigens. IL-7 is critical for promoting naive and central memory T-cell expansion when coupled with cognate antigen-mediated signals, which could promote host reactivity of donor T cells, prevent their activation-induced cell death and exaggerate their differentiation to Th1 effectors. The impact of elevated IL-7 levels on alloresponses might be amplified by IL-7 receptor-α gene polymorphisms, some of which have been associated with transplant-related mortality. Furthermore, alloresponses initiated during HPE could not be efficiently constrained by regulatory T cells, as expansion of human regulatory T cells is less amplified by IL-7 administration than that of conventional T cells.

The strong association of elevated IL-7 peak levels with increased development and severity of acute GVHD also suggests that administration of recombinant human IL-7 to improve immune reconstitution might not always be beneficial.

No association was evidenced between plasma IL-7 and IL-15 levels and development of infection or chronic GVHD. Chronic GVHD is a late complication, and then the protracted renewal of thymopoiesis cannot be neglected. Experimental studies indicate that pathogenic T cells generated from donor stem cells are responsible for the evolution from acute to chronic GVHD. IL-7 may enhance thymopoiesis and it does expand recent thymic emigrants, but thymic productivity appears to be reduced in patients with chronic GVHD.

Lower peak levels of IL-15 were associated by univariate and multivariate analyses with subsequent occurrence of malignancy relapse, and peak levels of IL-7 also tended to be low in these patients. All patients had standard-risk hematologic malignancies, and in this 10/10 HLA-matched setting, relapse rates did not differ whether receiving sibling or unrelated donor grafts, as expected. Only the peak IL-15 level appeared to be predictive for relapse, although this outcome was not significantly affected by entering the covariates malignancy type and first vs beyond first CR. This suggests the possibility of protracted HPE, particularly that of the CD8 + T-cell pool as IL-15 can preferentially promote expansion of this cell population. Poor T-cell recovery, particularly diminished numbers of CD8 + T cells and of their most differentiated phenotypes, has been associated with higher relapse rates, presumably by reducing the development of GVL effectors. All patients were given fully HLA-matched allografts, but NK cells could reportedly exert protective antitumor effects, at least against myeloid malignancies.

We cannot exclude that reduced levels of IL-15 might also affect the development and function of NK cell populations, although we found no relationship between relapse rate and NK cells counts (whether CD56 + dim or CD56 + bright).

In conclusion, this prospective study indicates that peak IL-7 and IL-15 levels during the lymphopenic phase after myeloablative conditioning differ among allograft patients. Further investigations are warranted to explore why the production of these homeostatic cytokines varies among
patients at the peritransplantation period, irrespective of the modalities of the myeloablative regimen. This study included a relatively small number of patients, but who were as homogeneous as possible in terms of transplantation modalities. This study must be extended to a larger patient population, comparing different stem cell sources and conditioning regimen intensities. These data lend support to the predictive value of systemic IL-7 levels for the risk of acute GVHD after both BM (this report) and peripheral blood allo-SCT, and our study is the first to associate a reduced peak level of IL-15 with a greater risk of malignant relapse. A precocious determination of plasma level of IL-7 and IL-15 by day 14 post-graft could therefore help predict subsequent occurrence of major events that complicate allo-SCT: acute GVHD and malignancy relapse, and help build an algorithm for their individualized prophylaxis.

Conflict of interest
The authors declare no conflict of interest.

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