A pilot study of single nucleotide polymorphisms in the interleukin-6 receptor and their effects on pre- and postransplant serum mediator level and outcome after allogeneic stem cell transplantation

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Running head: SNPs in the IL-6R and allogeneic stem cell transplantation

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Summary:

Background: Interleukin 6 (IL-6) is an important regulator of immunity and inflammation in many diseases. Single nucleotide polymorphisms (SNPs) in the IL-6 gene influence outcome after allogeneic stem cell transplantation (ASCT), but the possible importance of SNPs in the IL-6 receptor has not been examined. We therefore investigated whether SNPs in the IL-6R gene influenced biochemical characteristics and clinical outcomes after ASCT.

Patients and methods: We investigated the IL-6 promoter variant rs1800975 and the IL-6R SNPs rs4453032, rs2228145, rs4129267, rs4845374, rs4329505, rs4845617, rs12083537, rs4845618, rs6698040 and rs4379670 in a 101 population-based cohort of allotransplant recipients and their family donors.

Results: Patients being homozygous for the major alleles of the IL-6R SNPs rs2228145 and rs4845618 showed high pre-transplant CRP serum levels together with decreased sIL-6R levels; the decreased IL-6R levels persisted 6 months post-transplant. In contrast, patients being homozygous for the minor allele of the IL6-R SNP rs4379670 showed decreased pretransplant CRP levels. Furthermore, the IL-6R rs4845618 donor genotype showed an association with severe acute graft versus host disease (GVHD), whereas the donor genotype of the IL-6 SNP rs1800795 was associated with decreased survival 100 days post-transplant. Finally, the recipient genotype of the IL-6R SNP rs4329505 showed a strong association with 2-years non-relapse mortality and this effect was highly significant also in multivariate analysis.

Conclusion: IL-6 and IL-6R SNPs influence the clinical outcome after allogeneic stem cell transplantation.

INTRODUCTION

The balance between pro- and anti-inflammatory cytokines during the pre- as well as the post-transplant period influences the risk of acute or chronic graft-versus-host disease (GVHD) after allogeneic stem cell transplantation (ASCT) [1, 2]. Several studies have also demonstrated that the gene expression and activity of single cytokines differ between healthy individuals due to single nucleotide polymorphisms (SNPs) and this is an independent risk factors for GVHD [3]. SNPs in the Interleukin-6 receptor (IL-6R) gene influence the balance between pro and anti-inflammatory IL-6 activities [4, 5]. The SNP rs1800795 in the promoter region of the IL-6 gene results in increased IL-6 levels and is associated with an adverse outcome after allotransplantation [6-10]. In addition, it has recently also been shown that polymorphism in Janus kinas 2, that is directly downstream of the IL-6R, are associated with increased risk of GVHD [11]. Experimental studies support the hypothesis that there are close links between increased IL-6 activity, T-cell development and end organ damage after ASCT [12-15]. Finally, IL-6 is a pleiotropic cytokine that is involved in regenerative processes especially in the liver and the gastrointestinal tract, two organs that are commonly affected in GVHD [16-19] .

IL-6R exists both in a membrane-bound (mIL-6R) and a soluble form (sIL-6R). Only specific cell types express mIL-6R, and initiation of downstream signaling through binding of IL-6 to the surface expressed mIL6-R/gp130 complex is termed cis or classical IL-6 signaling [20]. On the other hand, sIL-6R can bind to surface-expressed gp130 that is expressed by most cells and a complete IL-6/IL-6R/gp130 complex can thereby be formed and initiate downstream signaling even in cells that do not express the IL-6R themselves [20]. This is termed trans-signaling; such signaling seems important for initiation of many proinflammatory IL-6 effects [20] and can be specifically inhibited by a soluble form of gp130 (sgp130). sIL-6 receptor is formed through proteolytic cleavage of the membrane-bound receptor by ADAM (A Disintegrin and Metalloprotease) proteases [5, 21]. The sIL-6R levels increase during inflammation due to upregulation of ADAMTS-17; the inhibitory capacity of sgp130 is thereby overwhelmed and IL-6 trans signaling is initiated [21].

The SNP Ala358Asp/rs2228145 alters the amino acid sequence of the IL-6R chain at the site of cleavage by ADAMTS17, and this SNP will thereby alter the rate of proteolytic sIL-6R release [5],

Individuals that are hetero- or homozygous for the rs2228145 allele will therefore have significantly higher levels of sIL-6R compared to individual that are homozygous for the ancestral allele [4]. Both experimental and epidemiological data suggest that rs2228145 modulates IL-6 trans-signaling, and this seems to be associated with altered CRP levels, severity of autoimmune diseases and risk of cardiovascular disease [22, 23]. Other IL-6R SNPs have also shown independent effects on the levels of sIL-6R as well as other soluble inflammatory mediators [4, 24], but the molecular mechanisms behind these effects have not been elucidated.

To the best of our knowledge the effects of IL-6R SNPs on the cytokine network and the outcome after allotransplantation have not been examined previously. Inhibition of the IL-6/JAK2/STAT pathway is now considered as a possible strategy for prophylaxis and treatment for graft versus host disease (GVHD) [25]. A more detailed characterization of the possible biological importance of IL-6R SNPs will then be needed as a part of the scientific basis for further clinical studies of IL-6 targeting therapy in allotransplant recipients. In the present study, we therefore investigated how specific IL-6R SNPs influence pre- and post-transplant serum levels of C reactive protein (CRP), IL-6 sIL-6R, sgp130 as well as the risk of acute and chronic GVHD after ASCT.

PATIENTS AND METHODS

Patients

The study was approved by the local Ethics Committee (REK VEST 2013/ 634 and REK VEST 2015/1410; Regional Ethics Committee III, University of Bergen, Norway). Only patients with an available family donor were transplanted at our center during the study period (from January 2006 until June 2016), and this represents all allotransplanted patients with family donors from a defined geographical area of Norway (Norwegian Health Regions III, IV and V). No transplantations with other donor types were included. The decision to proceed to transplantation was taken by the Norwegian Advisory Board for Stem Cell Transplantation and based on national guidelines.

Design of the study

Primary goal. The primary goal in our present study was to investigate the effects of different SNPs on (i) pre- and post-transplant serum levels of CRP, sIL-6R and sgp130, and (ii) clinical outcomes.

Selection of SNPs. More than 1000 SNP have been identified in the IL-6 receptor gene [26]. The selection of SNP to be analyzed was based on review of the available literature, and in addition we required that all selected SNPs should have a minor allele frequency (MAF) of at least 10%. The IL-6R SNP rs2228145 was included because it is important for the release rate of sIL-6R, it has well-defined biological effects [5] and influences outcome in several inflammatory disorders e.g. rheumatoid arthritis, asthma and cardiovascular disease [27-29]. To evaluate if other independent SNPs in the IL-6R gene are important for serum levels or posttransplant outcomes we used the same tagging SNPs as described previously by Lopez-Lasanta et al [29]. In summary, each of the SNPs rs4379670, rs6698040, rs4845374, rs4453032 and rs4845618 independently tags approximately 100 different SNPs (r^2 above 0,7) within the IL-6R gene.

We identified one cohort of allotransplant recipients where the effects of 8 IL-6R SNPs had been examined [30, 31]. Two of these SNPs were excluded from our study due to an expected minor allele frequency below 0.1 %, and 5 of the other SNPs were excluded because they show strong linkage disequilibrium (r^2 >0,7) with the tagging SNPs rs4379670 or rs4845618 according to the SNP Annotation and Proxy Search database [32]. The last of these 8 SNPs was rs4845617 that had weak linkage disequilibrium both with the selected tagging SNPs, and therefore was included in our study. Finally, based on our review of the literature we also included the two SNPs rs4329505 and rs12083537 because they are independent of rs2228145 with respect to influence on CRP levels, responses to anti-IL6 treatment and risk of autoimmune disease [33-35].

The IL-6 promoter region SNP rs1800975 influences IL-6 expression and outcome after allotransplantation and was therefore included in our study [8-10, 31].

Clinical outcomes. The clinical outcomes were defined as (i) risk of acute GVHD requiring high-dose steroid treatment; (ii) transplant-related mortality at day +100 post-transplant (acute GVHD is defined as a complication mainly occurring before day +100); (iii) transplant-related mortality 2 years after transplantation (relapse usually occurs during the first two years after allotransplantation); and (iv) the

risk of chronic GVHD requiring systemic immunosuppression. Acute and chronic GVHD were diagnosed according to generally accepted criteria [36]. All patients with acute GVHD were evaluated using the Glucksberg score, and patients who required intravenous treatment with at least 1 mg/kg/day of methylprednisolone (or equivalent steroid dose) for acute GVHD were considered to have serious (i.e. grade 2-4) acute GVHD. For evaluation of chronic GVHD we performed a landmark analysis where we only included patients that were alive at day +100 posttransplant without relapse. Patients that were previously treated with high-dose steroids for aGVHD were also excluded from the analysis to reduce the heterogeneity of this group and ensure that only patients with classic chronic GVHD were included and not patients that could have chronic overlap [37]. Furthermore, this selection of patients ensured that all patients included in the landmark analysis were only treated with one systemic immunosuppressive drug (cyclosporine A) and started taper off cyclosporine A at day +100; we thereby we had a very simple and reliable readout of chronic cGVHD that could be defined as development of a clinical picture consistent with chronic GVHD requiring systemic immunosuppression either by an additional immunosuppressive agent/treatment to cyclosporine A or by prolongation of the ongoing cyclosporine A prophylaxis. Standard comorbidity index scores (HCT-CI and EBMT-score) were not systematically implemented or register until after 2012 and were therefore available only for a minority of patients.

Preparation of plasma samples and analysis of cytokine levels

Pretransplant samples were collected either on the day of the pre-transplantation evaluation or on the day of admission for stem cell transplantation (median 23 days pretransplant, interquartile range (IQR) 14 days). Blood samples and clinical information (i.e. data on relapse, GVHD, and other transplant-related complications) were collected every third month during the first year posttransplant and thereafter once a year.

Serum was prepared from venous blood within 2 hours after sampling. The serum was transferred to cryotubes and stored at -80°C until analyzed. Bio-Plex kits were used for analysis of IL-6, sgp130 and sIL-6R (sCD126) levels (Bio-Rad, Hercules, CA, USA), and all samples were analyzed using the Luminex®200™ Bio-Rad platform. All other blood tests included in this study were performed

immediately after sampling at Laboratory for Clinical Biochemistry, Haukeland University Hospital, and the same methods and technology were used throughout the study period for all these analyses.

DNA sample preparation and genotyping

For all donor's and patient's DNA was extracted from peripheral blood or heparinized bone marrow and thereafter purified using QiaSymphony DSP DNA kit (Qiagen, Venlo, The Netherlands). DNA quantity and quality was measured using a Nanodrop ND-1000 spectrophotometer and aliquoted into 96 wells plates at a target concentration of 2-20 ng per well. The rs6698040 was analyzed using a TaqMan assay whereas all other SNPs were analyzed using KASP assays with ViiA7 instrument (Life Technologies).

Statistical analyses

Allele frequencies, r^2 and possible deviation from the Hardy-Weinberg equilibrium were calculated using Haploview version 4.2 (Broad Institute, Cambridge, MA, USA; downloaded from http://www.broad.mit.edu/mpg/haploview). All other statistical analyses were performed using the Stata Version 14 (StataCorp. 2009; Stata Statistical Software, College Station TX) and GraphPad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA). Spearman's correlation for bivariate samples was used for correlation analyses, continuous variables were compared using non-parametric tests (Kruskal–Wallis one-way analysis of variance/Man-Whitney-U test). The Chi-Square tests and Fisher's exact tests were used to compare categorized variables. Differences were regarded as statistically significant when p-values <0.05.

Overall survival was calculated using the Kaplan-Meier product limit method. For competing risk analysis cause of death was either classified as relapse-related or treatment-related. Crude and adjusted subdistribution hazard ratios (SHR) were calculated using cumulative incidence regression methods as described by Fine and Gray [38], for therapy related mortality TRM at two different time points (day +100 post-transplant and 2 years after transplantation). Risk of developing acute GVHD was calculated in a similar manner with death from relapse or other causes as competing risk factors. In advance it had

been defined that age and covariates with *p*-value <0.1 in univariate analyses should be included in the adjusted model.

The effect of each SNP on GVHD or transplant-related mortality was primarily evaluated as homozygous major allele versus heterozygous/homozygous minor allele. For some SNPs with previous proven significance of major allele or for SNPs with low minor allele frequency (less than 15 persons with homozygous minor allele) homozygous minor allele versus heterozygous/homozygous major allele was compared.

RESULTS

Patient and donor characteristics

During the observation period 105 allotransplantations were performed at our institution. Two patients were retransplanted due to leukemia relapse (1 acute myeloid leukemia (AML) and 1 acute lymphoblastic leukemia (ALL) patient); both patients were retransplanted with their original donor but with a different myeloablative conditioning regime. Only data from the first transplantation were included in our analyses. Thus, our study is based on the data from 103 allogeneic stem cell transplantations.

The characteristic of the 103 patients and their donors are summarized in Table 1 together with the expected SNP frequencies [26]. DNA was available for 101 of the 103 recipients and for 101 of the 103 donors. Granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood stem cell grafts were used except for four patients with aplastic anemia and a patient transplanted with stem cells from a donor below 15 years of age. These five patients received bone marrow grafts. Except for the patients with aplastic anemia only 2 additional patients received anti-thymocyte globulin (ATG) in addition to standard GVHD prophylaxis with methotrexate and Cyclosporine A. A majority of 92 patients received conditioning treatment with fludarabine/busulfan or cyclophosphamide/busulfan. Busulfan was given intravenously in all these patients.

Two subsets of SNPs show strong linkage disequilibrium

The results from the genotyping of 10 SNPs are summarized in Table 2. The overall genotype recall and the recall for each of the SNPs was ≥98%; those patients that were not successfully genotyped were usually the same patients for each SNP and this observation suggests that the DNA quality for these patients was inferior. No deviations from Hardy Weinberg equilibrium were observed. Calculated r^2 and relative chromosome positions are shown in Figure 1. As expected, high linkage disequilibrium (LD) was observed between (i) rs4453032, rs2228145 and rs4129267; and between (ii) rs4845374 and rs4329505. For these two SNP groups we therefore report only the results for the SNPs rs2228145 and rs4845374, respectively.

The IL-6/IL-6R SNPs have no major influence on serum CRP levels in healthy peripheral blood stem cell donors

The donors were regarded as healthy controls, and prior to the stem cell mobilization with G-CSF they all had an expected low serum CRP level (median 1 mg/L, range 1-21 mg/L, IQR 1), i.e. an undetectable level below < 1 mg/L was observed for half of the donors and the level was within the normal range for 90% of them. Furthermore, after G-CSF treatment at the time of peripheral blood stem cell harvesting the serum CRP level of the donors showed a significant increase (n=96, median 8 mg/L, range 1-49 mg/L, IQR 10, p-value <0.01). There was a significant correlation between premobilization CRP level and the levels after G-CSF treatment (ρ=0,43, p-value <0,01). Neither the premobilization levels, the CRP increase nor the serum CRP levels after G-CSF treatment showed significant associations with any of the SNPs (data not shown).

The IL-6R SNPs rs2228145, rs4845618 and rs4379670 recipient genotype have significant effects on pretransplant levels of CRP and sIL-6R in allotransplant recipients

The recipients' pretransplant levels of IL-6, sIL-6R, sgp130 and CRP are presented in Table 3. The two IL-6R SNPs rs2228145 and rs4845618 showed significant associations with the pretransplant CRP serum levels, and higher levels were then observed for patients homozygous for the major alleles (Figure 2). In contrast, both these SNPs also showed significant associations with the pretransplant sIL-6R

levels, but lower sIL-6R levels were then seen for patients homozygous for the major alleles. The two SNPs had no effects on sgp130 serum levels or IL-6 levels.

The recipient IL6-R SNP rs4379670 also had an effect on the CRP levels with significantly lower CRP levels in patients being homozygous for the minor allele.

None of the other recipient or donor SNPs showed any influence on pretransplant serum levels of CRP, sIL-6R or sgp130 (data not shown), not even the SNP rs1800975 in the IL-6 promoter region that has been associated with altered IL-6 levels during inflammation [6, 39, 40].

The effects of the IL-6R SNPs rs2228145 and rs4845618 recipient genotype on sIL-6R levels are maintained after stem cell transplantation

Serum levels of CRP, sIL-6R, IL6 and sgp130 after transplantation were available for 69 of the patients with a median time of 182 days posttransplant (range 83-372 days, IQR 248 days); the median levels are given in Table 3. These patients represent a selected subset of patients with a stable clinical situation (median age 44 with range 15-70 years; 44 men and 30 women) and therefore being able to travel to the transplantation center for clinical evaluation and standardized blood sampling and handling of the serum samples. At the time of posttransplant sampling the majority of patients had a ECOG performance status 0-1, no signs of severe intercurrent infections and no signs of relapse. When analyzing the overall results, we could still observe a significant effects of the two IL-6R SNPs rs2228145 and rs4845618 on serum levels of sIL-6R (Figure 2) after the transplantation, but we could not detect any significant effect on CRP (Figure 2) or sgp130 levels (data not shown).

The IL-6R SNP rs4845618 donor genotype is associated with increased incidence of acute GVHD

The overall cumulative incidence of severe acute GVHD (aGVHD) requiring high-dose steroid treatment was 45.5 % (Figure 3a). Transplantation with a non-sibling donor was significantly associated with an increased risk of aGVHD (p-value <0,01, (subdistribution hazard ratios (SHR) 3,32, 95% confidence interval (CI) 1,58-6,96), while pretransplant CRP levels, age, female donor to male recipient and CMV positive donor to CMV negative recipient were not associated with a higher risk of aGVHD in our cohort.

Only the IL-6R SNP rs4845618 donor genotype was associated with an increased incidence of aGVHD (p-value 0.04, SHR 1.79, 1.01-3.2). This donor genotype was still associated with aGVHD requiring high-dose steroid treatment in an adjusted model including age, sibling vs non-sibling and donor rs4845618 genotype (p-value 0,05 SHR 1,75 95% CI 1,00-3,08) (Table 3). Finally, neither the donor nor the recipient genotype of the other IL-6R SNPs had any significant influence on the risk of aGVHD, and this was also true for the IL-6 promoter SNP rs1800975.

IL-6/IL-6R SNPs did not influence the risk of classic chronic GVHD

To evaluate the risk of chronic GVHD (cGVHD) a landmark analysis was performed. At day +100 post-transplant 46 of the original 101 patients were either dead, experienced relapse or had aGVHD still requiring continued high-dose steroid treatment on day +100. Twenty-five of the remaining 55 patients could subsequently wean off systemic immunosuppression as planned without any experience of cGVHD whereas 9 patients had a relapse when they were still on immunosuppression but without any manifestations of cGVHD. The last 21 patients experienced chronic GVHD that required either additional/new or prolonged systemic immunosuppression. Thus, the cumulative incidence of cGVHD was 38.3 % (Figure 3b). Because none of these 21 patients were previously diagnosed with acute GVHD, we ensured that we only included patients with classic chronic GVHD without any chronic overlap [37]. and we could use a simple and reliable readout by defining “cGVHD requiring additional systemic immunosuppression” as a clinical picture of chronic GVHD requiring either an additional or new immunosuppressive drug/treatment or prolonged ciclosporin A treatment.

Due to the small number of patients with cGVHD calculations were only possible for a minority of the selected SNPS (rs2228145, rs48456187 and rs120835357). As described above, the donor rs2228145 genotype had a significant impact on both pre- and posttransplant sIL-6R serum levels. However, patients carrying the rs2228145 had only a nonsignificant trend of higher frequency of cGVHD compared with patients homozygous for the ancestral allele, whereas patient age showed a significant effect. In an adjusted model both age and rs2228145 patient genotype had a significant effect (Table 4).

The recipient genotype of the IL-6R SNP rs4329505 influences non-relapse mortality 2 years post-transplant

Kaplan Meier plots for the overall survival and the cumulative incidence of transplant-related morality (TRM) are given in Figure 3c and 3d. Survival was 81.8 % at day +100 posttransplant and 55,2 % 2-years posttransplant, whereas the cumulative incidences of transplant-related mortalities at the same time points were 16.0 % and 27.8 %, respectively.

Pretransplant CRP levels were associated with an inferior outcome in univariate analysis whereas conditioning regimen, age and female donor/male recipient were not. The donor genotype for the IL-6 promoter SNP rs1800795 was also associated with decreased survival, whereas neither the donor nor the recipient genotype of the IL-6R SNPs were associated with outcome at day +100 post-transplant. The rs1800795 donor genotype still had a significant effect on early TRM in an adjusted model also including age and pretransplant CRP level.

Factors that were associated with an increase in the 2 year TRM in the univariate analysis, were donor type (sibling vs non-sibling), age and pretransplant CRP levels. Although the donor genotype for the IL-6 SNP rs1800795 was associated with adverse prognosis at day +100 (see above), a similar effect was not observed for the 2 years survival. However, the recipient genotype rs4329505 was associated with higher 2-years non-relapse mortality, and the effect of this SNP was highly significant also in the multivariate analysis. (Table 5)

DISCUSSION

Both experimental evidence and clinical studies suggest that IL-6 has a role in the regulation of inflammation and immunity in allotransplant recipients [25, 41, 42]. Although the effects of SNPs in the IL-6 gene on outcome after ASCT have been investigated previously [7-10], the possible importance of SNPs in the IL-6R gene has not been examined in detail. In our present pilot study we therefore investigated a panel of IL-6R SNPs, and observed an association between specific SNPs, non-relapse-mortality and systemic sIL-6R/CRP levels.

IL-6 is the main driver of CRP production; systemic IL-6 levels are therefore strongly correlated with CRP levels and can explain approximately 40-50% of intra-individual CRP variations [1, 43]. Previous studies have identified four different IL-6R SNPs (rs2228145 previously reported as rs8192284, rs12083537 previously reported as rs1386821, rs4329505 and rs484561) that independently influence sIL-6R levels [4, 24, 44]. These SNPs may then influence the levels of sIL-6R and mIL-6R and thereby the IL-6 functions and finally the CRP release. The biological consequences of this genetic variation have been best characterized for rs2228145 and rs4845617. While rs2228145 alters the proteolytic shedding of IL-6R from cell membrane, the rs4845617 is located in the IL-6R gene promoter region and influences IL-6R mRNA transcription [44]. rs2228145 seems to have the strongest effect on IL-6 signaling and explains approximately 30% of the intra-individual variations in sIL-6R levels [4, 24]. The effect of rs12083537 and rs4329505 on sIL-6R level are generally weaker, i.e. in the range of 0.4-1.1 % [4]. In the present study we observed significant associations between several IL-6R SNPs and the pretransplant levels of both CRP and sIL-6R.

Serum samples included in the current study were collected at scheduled visits at 3, 6, 9 or 12 months posttransplant. However, a significant number of patients with severe GVHD, complicating infections and other disabling diseases were not able to attend all of these visits at the transplantation center since patients were recruited from a large geographical area. This has several implications for the interpretation of the results. Firstly, this explains the reduced number of patients analyzed posttransplant and the large variation for the time point for the post-ASCT serum samples. Secondly, that patients included in the analysis should be regarded as a selected group of patients, with a higher performance status, without any disabling infections or inflammatory condition at the time of serum sampling. The relatively low CRP levels observed for these patients probably reflect this patient selection, and the influence of inflammatory transplant related-factors (mainly infections or GVHD) on serum levels of sIL-6R and CRP was thereby minimized. To summarize, our intention with the posttransplant analyses of mediator levels was to (i) ensure standardized sampling and sample preparation (i.e. collect samples at the transplantation center), (ii) analyze samples collected after hematopoietic stem cell reconstitution (i.e. 2-3 months after transplantation) but during the first year posttransplant (iii) for patients being in a stable clinical situation and being able to travel to the transplantation center (i.e. most patients having

ECOG performance status 0-1; no complications that could initiate an acute phase reaction or influence serum mediator levels). These three criteria were our priority, and to fulfill them we had to compromise and accept a variation in time of sampling. However, we have to emphasize that the variation in sampling time may influence our results, e.g. we expect the immune reconstitution to differ between patients examined 3 and 12 months posttransplant.

The biological context of the IL-6 system differs between healthy individuals/controls (i.e. stem cell donors) and allotransplant recipients. We observed significant associations between IL-6R SNPs and CRP as well as sIL-6R levels only for the recipients but not their donor; these observations suggest that the biological context (patients vs healthy individuals) actually is important and responsible for this increased IL-6 effect on the acute phase response (i.e. a marker of inflammation) in allotransplant recipients. However, the strong influence of rs2228145 is similar for allotransplant recipients and other patients and suggests a similar impact of altered proteolytic shedding when a proinflammatory context is present.

An effect of rs2228145 could not be observed for posttransplant CRP levels. However, the patients available for posttransplant evaluation represent a selected subset characterized by high performance status as well as no signs of severe infections, relapse or severe GVHD. As expected their serum CRP levels showed only a minor variation (CRP levels < 6 mg/L for most of these patients).

Several previous studies have shown that high pretransplant CRP levels are associated with high transplant-related mortality but without any apparent effect on the rate of GVHD [1, 45-49]. The rs4329505 IL-6R SNP had a significant and independent effect on transplant-related mortality. This effect was detected only after 2 years but not at day +100 post-transplant. Immunological reconstitution after allotransplantation in adult patients usually takes several months [50], and a possible explanation for detecting this effect only after 2 years may thus be a differences in the immunological context at these time two points possibly caused by the delayed immune reconstitution. This SNP has previously been reported to influence the response to IL-6 inhibition and has also been linked to the severity of chronic inflammatory disease [33, 51]. Unfortunately, due to the low allele frequency for this SNP an analysis of its possible effect on cGVHD was not possible.

The SNP rs4845618 in the IL-6R gene has been used as a tagging SNP. One study described an effect of this SNP on the risk of joint destructions in patients with rheumatoid arthritis independent of other SNPs in the IL-6R gene [29]. Allotransplant recipients have a unique overall biological context including several abnormalities of immunocompetent cells early after transplantation, and in this setting rs4845618 seems to influence both the IL-6 network (increased sIL-6R levels), inflammatory regulation (altered CRP levels) and the clinical course (risk of acute GVHD).

To the best of our knowledge the effects of different SNPs in the IL-6R gene have only been examined in one previous heterogeneous cohort of allotransplant recipients [30, 31]. In these two studies the effects on various endpoints of 256 different SNPs in various cytokine and cytokine receptor genes were examined; 8 of these SNPs being in the IL-6R gene [30, 31]. However, this study did not examine the effects of SNPs known to functional effects on the IL-6 system, and they only observed an association between rs4845617 and ocular chronic GVHD [31]. We could not detect any associations between any SNP and chronic GVHD, but it should be emphasized that we only examined a subset of the patients with classic chronic GVHD.

One major limitation of studies investigating outcome after ASCT is the recipient heterogeneity and differences in transplant related procedures that influence the risk of early and late complications. The most important risk factors for GVHD are disease status, patient comorbidity, graft source, use of anti-thymocyte globulin (ATG) and conditioning therapy [52, 53]. The different transplant related factors are often significantly intertwined (e.g. age and type of conditioning regime) so that the effects of single factors is often difficult to estimate/demonstrate. Studies investigating prognostic parameters in allotransplant recipients should therefore be based on well-characterized and if possible unselected patient cohorts. We did not exclude any patients to reduce the heterogeneity of our cohort for most of our analyses; as discussed above the only exceptions being the analyses of chronic GVHD (to have simple and reliable criteria for outcome) and posttransplant serum levels (to avoid masking of SNP effects by ongoing inflammation). For this reason we investigated the effects of IL-6/IL6R SNPs in a group of unselected (i.e. population-based) but well-characterized and relatively homogeneous group of patients. The large majority of our patients were adult Caucasians transplanted due to hematological malignancies, most patients received busulfan-based myeloablative conditioning and the same GVHD

prophylaxis (cyclosporine A plus methotrexate), and they were transplanted with G-CSF mobilized stem cell grafts derived from HLA-matched family donors. We regard our patient cohort to be representative for this particular subset of allotransplant recipients. Even though our cohort is relatively small, our results suggest that the IL-6 network is important for the biological characteristics and the clinical outcome of these patients. Other independent studies should clarify whether our observations are relevant also for other subsets of allotransplant recipients.

To conclude, SNPs in the IL-6R gene seem to influence both pre- and post-transplant biological characteristics as well as the long-term survival of allotransplant recipients, and the associations with sIL-6R levels indicate that IL-6 trans-activation is involved in these effects. Although none of the evaluated IL-6R SNPs significantly influenced the risk of acute or chronic GVHD, they seemed to influence both pre- and posttransplant IL-6 trans signaling. Future studies should therefore investigate whether IL-6 has similar effects also for other subsets of allotransplant recipients, because we investigated only a relatively small and selected subset of patients with classic chronic GVHD. However, our studies suggest that the immunoregulation varies during the posttransplant period and the timing of IL-6 targeting therapy may therefore be essential. Future studies exploring inhibition of Il-6 signaling in ASCT should include analysis of the SNPs shown to influence IL-6 trans signaling.

AUTHORSHIP

THAT and ØB conceived the idea and designed the study, recruited the patients and wrote the manuscript, THAT performed the statistical analyses, RH helped designing the study, handling DNA samples and interpretation of data, GT help collecting and validating data on survival and GVHD, KPR performed the Luminex essays, TGD and ABA helped collecting clinical data. All participants read and accepted the final manuscript.

DISCLOSURES

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CONFLICTS OF INTEREST

The authors report no conflicts of interest**.**

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Table 1. The characteristics of the allotransplant recipients and their donors included in the analysis.

RECIPIENTS (n=101)	
Age, median and range (Years)	48 (15-70)
Caucasian/non-Caucasian (number)	96/5
Diagnosis (number)	
AML, *de novo*	42
Myelodysplastic syndrome-AML	17
Myelodysplastic syndrome, high-risk	4
Acute lymphoblastic leukemia	20
Chronic myeloid leukemia	2
Myelofibrosis	4
Chronic myelomonocytic leukemia	2
Myeloproliferative neoplasia, unspecified	2
Aplastic anemia	5
Chronic lymphocytic leukemia	2
Hodgkin's lymphoma	1
Leukemia patients not in remission at transplantation (number)	1
aGVHD requiring high dose steroid treatment (number) [1]	49
Conditioning regimes (number)	
Busulfan + cyclophosphamide (myeloablative condition)	74
Fludarabine + busulfan (reduced intensity conditioning)	18
Antithymocyte globulin + cyclophosphamide	4
Others	5
GVHD prophylaxis (number)	
Cyclosporine A + methotrexate	98
Cyclosporine A + mycophenolate mofetil	1
Cyclosporine A + methotrexate + antithymocyte globulin	2
Stem cell source (number)	
Peripheral blood mobilized stem cells	96
Bone marrow grafts	5
DONORS (n=101)	
Sibling/ Parent/ Other	93/8/0
Female/ Male	40/61
Female donor to male recipient	22
Number of CMV positive recipient	66
CMV positive donor to CMV negative recipient	22

[1] The criteria for high-dose steroid treatment were acute GVHD grade II with gastrointestinal involvement or Grade III/IV acute GVHD.

Table 2. The investigated SNPs and their frequencies. The tables summarizes the SNPs examined, number of successfully genotyped patients, expected and observed minor allele frequency (MAF) of the patients, and patients being either heterozygous or homozygous for major (H-major) or minor (H-minor) alleles. The p-value indicates if there was any significant deviation from the Hardy-Weinberg equilibrium (HWE). The expected frequencies refer to previously published data [26].

Gene	SNP	Alleles (nucleotide)		N[1]	Expected MAF	Observed MAF	Genotype (number of patients)			p-value HWE
		Ancestral	Minor				H-Major	Hetero[2]	H-Minor	
IL-6	rs1800795	G	C	101	0,14	0,49	30	40	31	0,054
	rs1800797	G	A	101	0,13	0,50	31	40	30	0,053
IL-6R	rs2228145	A	C	101	0,29	0,43	34	49	18	1,00
	rs4845617	G	A	99	0,38	0,40	36	46	17	0,7435
	rs4845374	T	A	100	0,18	0,15	73	24	3	0,7285
	rs4845618	G	T	100	0,49	0,44	20	47	33	0,8527
	rs4453032	A	G	100	0,30	0,43	33	49	18	1,00
	rs4379670	A	T	99	0,20	0,24	62	27	10	0,0369
	rs12083537	A	G	101	0,20	0,22	60	38	4	0,8292
	rs4329505	C	T	101	0,22	0,15	74	24	3	0,7416
	rs4129267	A	G	101	0,29	0,43	60	38	4	1,00

[1]The number of patients that were successfully genotyped
[2] Hetero, heterozygous

Table 3. Systemic serum levels of soluble mediators in allotransplant recipients; a summary of the pre- and posttransplant CRP, IL-6, sIL-6R and sgp130 levels in the 101 allotransplant recipients. The median time of posttransplant sampling was 182 days (range 83-372) after transplantation.

Mediator	Pretransplant			Posttransplant		
	Median	**Range**	**IQR**	**Median**	**Range**	**IQR**
CRP (mg/L)	6	(1-120)	12	2	1-150	3
IL-6 (pg/ ml)	12.6	(0.92–581)	19.6	4,8	1,54-484,1	9,54
sIL-6R (pg/ ml)	11580	(609–42666)	1072	7209	2416-16815	2267
sgp130 (pg/ ml)	54808	(8286–226166)	60005	54789,4	20829-133777,3	17239

IQR: Interquartile range

Table 4. Risk of acute and chronic GVHD in our 101 allotransplant recipients; crude and adjusted subdistribution hazard ratios (SHR) for acute GVHD (upper part) and landmark analysis for chronic GVHD (lower part).

ACUTE GVHD							
Covariate	**Reference**	**Crude**			**Adjusted**		
		p-value	**SHR**	**95 % CI**	**p-value**	**SHR**	**95 % CI**
Age	Per decade	0,84	1,02	0,83-1,25	0,58	1,06	0,86-1,31
Non-sibling Donor	sibling	<0,01	3,32	1,59-6,97	<0,01	3,45	1,76-6,75
rs4845618	TT vs TG/GG	0,04	1,80	1,01-3,2	0,04	1,77	1,01-3,09
LANDMARK ANALYSIS CHRONIC GVHD							
Age	Per decade	0,01	1,72	1,11-2,66	0,01	1,76	1,10-2,80
Donor rs2228145	AA vs AC/CC	0,07	0,46.	0,20-1,09	0,04	0,43	0,19-0,98

SHR: Subdistribution hazard ratio
CI: Confidence interval

Table 5. Risk of treatment-related mortality for our 101 allotransplant recipients: Crude and adjusted subdistribution hazard ratios (SHR) for treatment related mortality for the 101 allotransplant recipients at day +100 days and 2 years posttransplant.

THERAPY RELATED MORTALITY AT DAY 100 POST-TRANSPLANT							
Covariate	**Reference**	**Crude**			**Adjusted**		
		p-value	**SHR**	**95 % CI**	**p-value**	**SHR**	**95 % CI**
Age	Per decade	0,672	1,06	0,79-1,43	0,971	1,00	0,97-1,03
CRP level	Continuous variable	<0,01	1,02	1,01-1,03	<0,01	1,02	1,01-1,04
Non-sibling	Sibling	0,09	2,64	0,85-8,16	0,309	1,98	0,53-1,03
Recipient rs1800795	GG vs GC/CC	0,02	2,98	1,16-7,68	0,03	3,64	1,11-11,94
TREATMENT RELATED MORTALITY AT 2 YEARS POST-TRANSPLANT							
Age	Per decade	0,14	1,21	0,94-1,58	0,01	1,38	1,08-1,79
CRP level	Continuous variable	0,06	1,01	0,99-1,03	<0,01	1,01	1,01-1,03
Non-sibling	Sibling	0,01	3,12	1,26-7,71	<0,01	4,92	2,16-11,21
Recipient rs4329505	CC vs CT/TT	0,03	2,21	1,07-4,58	0,01	2,72	1,25-5,91

Figure Legends:

Figure 1.

The IL-6R SNPs investigated in the study: The relative chromosome position (upper part) of the different SNP in the IL-6R (chromosome 1) and linkage disequilibrium expressed by r^2 for the analyzed SNP (lower panel) are presented in the figure.

Figure 2. The effects of the IL-6R SNPs rs2228145 (a) and rs4845618 (b) on pretransplant and posttransplant levels of CRP and sIL-6R.

Figure 3. The outcome after allogeneic stem cell transplantation. The figure shows the cumulative incidence of acute GVHD (A) and (B) chronic GHVD for the entire cohort, the overall survival for the entire period (C) and the cumulative incidence of TRM (D).