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Divergent roles of interleukin-12p40 and interleukin-18 in severe fibrosing chronic graft-*versus*-host-disease, immunity, and post-transplant outcomes

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IL-12p40 and IL-18 in Fibrosing cGvHD

Declarations

Authors' contributions

SO performed and designed research, acquired data, performed statistical analysis of the data, discussed data and wrote the paper. LP, MQ, RH, CP and TG performed research, acquired data, discussed data, wrote the paper. LA and MR performed statistical analysis of the data, discussed data,

wrote the paper. HB discussed data and wrote the paper. CMT and PD performed clinical research, discussed data, and wrote the paper. TL designed and organized the project, collected patient samples, performed and designed research, analyzed and discussed data, and wrote the paper. Prior to submission, all individuals acknowledged herein were informed of their inclusion and confirmed the accuracy of their contributions. All authors consented to the publication of the manuscript.

Conflict-of-interest statement

The authors have declared that no competing financial interests to this work exist.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Abstract

Chronic graft-versus-host disease (cGvHD) is a major cause of late morbidity after allogeneic hematopoietic stem-cell transplantation (alloHSCT), particularly in severe fibrosing forms. Although IFNγ-related cytokines have been implicated in cGvHD, their contributions to disease progression, survival, relapse, and response to immunosuppression remain unclear. In this retrospective study, 552 alloHSCT recipients with serum samples collected at cGvHD onset (or day 180 in controls) were analyzed for IFNγ-related cytokines. Among 14 cytokines, IL-12p40 and IL-18 emerged as divergent signatures. Both were elevated in patients who later developed severe fibrosing cGvHD, but their clinical associations differed markedly. IL-12p40 correlated with reduced relapse (cause-specific HR 0.88, 95% CI 0.84–0.93; p<0.001), improved overall survival (HR 0.91, 95% CI 0.87–0.95; p<0.001), and enhanced hematopoietic reconstitution. In contrast, IL-18 was associated with increased non-relapse mortality (cause-specific HR 1.75, 95% CI 1.47–2.09; p<0.001), systemic inflammation, and endothelial stress. Antithymocyte globulin prophylaxis was associated with sustained suppression of both cytokines and reduced severe fibrosing cGvHD incidence (5.1% vs 20.3% without ATG; p<0.001). Conversely, cumulative prednisolone exposure until day 100 selectively suppressed IL-12p40 without affecting IL-18 or fibrosing cGvHD incidence. In vitro, IL-12p40 secretion was corticosteroid-sensitive, whereas IL-18 remained largely resistant. These findings identify IL-12p40 as a marker of protective immune reconstitution and graft-versus-leukemia activity, while IL-18 defines a steroid-resistant inflammatory axis linked to endothelial injury and non-relapse mortality, supporting pathway-specific immunomodulatory strategies.

Introduction

The curative potential of allogeneic hematopoietic stem cell transplantation (alloHSCT) depends on effective immune responses against residual malignant cells - responses that remain incompletely understood but frequently coincide with chronic graft-versus-host disease (cGvHD). cGvHD is a late immune-mediated complication of alloHSCT with overlapping antitumor activity, as reflected by reduced post-transplant relapse rates (1-6). However, a subset of patients progresses to severe fibrosing cGvHD with sclerodermatous changes and/or fasciitis, or to cGvHD of the lungs, which is associated with high morbidity and mortality (7-9).

The concept of graft-versus-leukemia (GvL) encompasses clinical observations of curative immune responses, most notably during immunosuppression tapering or after donor leukocyte infusions (4, 10, 11). Rather than relying on antigen-specific tumor recognition, GvL appears to reflect broad alloreactivity directed against hematopoietic targets. Differences in the requirement for full donor chimerism suggest that GvL mechanisms in acute leukemia may differ from those in lymphoma or myeloma (12).

Immune mechanisms involved in GvL may differ between early and late post-transplant phases, similar to the diverging mechanisms associated with acute and chronic GvHD. In contrast to cGvHD, the contribution of acute GvHD (aGvHD) to disease control remains unclear and has been questioned (1, 3, 4). Prophylactic administration of ATG on days –3 to –1 has been shown to reduce cGvHD incidence and may also attenuate severe aGvHD, depending on the conditioning regimen and study context (13-15). Methotrexate on days 1, 3, and 6 reduces aGvHD incidence but does not improve GvHD-free, relapse-free survival (16), again pointing toward distinct early and late immune pathways (13).

Although cGvHD is clearly linked to GvL, its therapeutic value remains ambiguous. In mild to moderate forms, cGvHD is associated with improved overall survival (OS), primarily due to reduced relapse rates (1, 3, 4). In contrast, severe fibrosing cGvHD - particularly with involvement of the skin or lungs - can also lower relapse risk but is frequently accompanied by debilitating, lifelong morbidity and increased

non-relapse mortality (NRM), raising concerns about the net benefit of alloHSCT in affected patients (8, 9). Balancing effective disease control with the prevention of severe fibrosing cGvHD and its complications remains a major clinical challenge.

In this retrospective study we explored an IFNγ cytokine network at cGvHD onset (and on day 180 for controls) after alloHSCT to identify immunologic features associated with severe fibrosing cGvHD and clinical outcomes. Building on prior work by our group and others demonstrating that CXCL9 levels correlate with overall cGvHD severity, and that IL-18 is elevated in patients with severe fibrosing cGvHD (10, 17-21), we further investigated the cytokine network linked to severe fibrosing cGvHD. Among the cytokines analyzed, IL-12p40 and IL-18 emerged as opposing signatures - both were elevated in patients with severe fibrosing cGvHD, but diverged in their relationship to relapse, survival, and lymphoid reconstitution. IL-12p40 was linked to improved lymphoid recovery and reduced relapse, while IL-18 marked inflammatory complications and higher NRM. These cytokines also differed in their sensitivity to corticosteroids, suggesting a potential basis for steroid-refractory fibrosing cGvHD.

Methods

Patient population

This retrospective observational study included consecutive patients who underwent allogeneic hematopoietic stem-cell transplantation (alloHSCT) at our institution between October 2002 and July 2021, survived beyond day 180 post-transplant, and had available serum samples collected either within 7 days before cGvHD onset (median day 178, IQR 137-225) or, for controls without cGvHD, within ±7 days of day 180 post-transplant (median day 180, IQR 177-182). Day 180 was selected as the control landmark because it approximated the cohort's median time to cGvHD onset. Patients who died before day 180, relapsed before day 100, or developed steroid-refractory aGvHD were excluded (**supplemental Figure 1**). GvHD prophylaxis included antithymocyte globulin (ATG-Fresenius or Grafalon, 10 mg/kg/day on days −3 to −1 for unrelated donors), methotrexate on days 1, 3, and 6 or

mycophenolate mofetil from day 0 to 28, and a calcineurin inhibitor (cyclosporine or tacrolimus), tapered after day 60 for related donors and after day 100 for unrelated donors (**Table 1**).

Diagnosis and grading of cGvHD

Clinical data, cGvHD manifestations, treatments, and outcomes were retrospectively extracted from electronic medical records. cGvHD diagnosis and grading followed the 2005 National Institutes of Health (NIH) consensus criteria (17, 22). Severe cGvHD was defined as an NIH organ score of 3 in any organ or a lung score ≥2. Fibrosing manifestations included not only sclerotic skin changes but also ocular, oral, genital, musculoskeletal, and pulmonary involvement consistent with fibrosing disease. Severe gastrointestinal cGvHD was defined as >15% body-weight loss irrespective of upper or lower GI involvement. Non-severe cGvHD comprised all remaining cases (**supplemental Table 1 and 2)**. Disease onset was defined as the first appearance of a diagnostic or distinctive manifestation.

Assessment of cytokine serum levels

Serum samples were routinely collected weekly after alloHSCT, transferred into cryotubes, and stored at −80 °C until analysis. Samples were processed in randomized batches including internal controls. Cytokine concentrations were measured by ELISA (DuoSet, R&D Systems, UK) using the Tecan Sunrise Remote Elisa Microplate Reader (Tecan, Austria). The analyzed cytokines included IFNγ, CXCL9, CXCL10, IL-12p40, IL-12EBI3, IL-17, IL-18, IL-23, lymphocyte growth hormone, TNFα, IFNα, IL-1β, activin, and follistatin.

Statistical analysis

Associations between cytokines and severe fibrosing cGvHD were analyzed using univariable logistic regression with log2-transformed cytokine concentrations. Overall survival was evaluated using Weibull proportional-hazards models, while relapse and non-relapse mortality (NRM) were analyzed

as competing risks using separate cause-specific Weibull and Cox proportional-hazards models adjusted for prespecified covariates (**supplemental Methods**). Group comparisons used t-tests, Fisher's exact tests, Wilcoxon rank-sum tests, and Spearman correlations where appropriate. Multiple testing was corrected using the Benjamini–Hochberg false-discovery-rate method. Analyses were performed in R v4.3.3. All models were exploratory and used to assess associations rather than prediction.

Ethics approval and consent to participate

The study was approved by the ethics committee of the University of Heidelberg (S120/2002). All participants provided written informed consent for the collection, storage, and analysis of blood samples and clinical data in accordance with the Declaration of Helsinki and institutional guidelines.

Results

Patient characteristics

Clinical characteristics of the 552 patients are presented in **Table 1**. Following a change in our in-house policies starting in January 2010, 491 (88.9 %) consecutive patients received endothelial prophylaxis with pravastatin (20 mg/d) and ursodeoxycholic acid (UDCA) (750 mg/d) from the start of conditioning therapy.

A total of 294 patients (53.3%) developed initial signs of cGvHD after a median of 5.8 months (interquartile range (IQR) 4.4–8.8), while 258 patients (46.7%) remained free of cGvHD. Among affected patients, the median time to onset of severe fibrosing cGvHD was 14.3 months (IQR 9.3–24.1). Over a median follow-up of 33.6 months (IQR 18.3–53.6), 52 of the 294 patients with cGvHD (17.7%), corresponding to 9.4% of the total cohort, progressed to severe fibrosing cGvHD. An additional 16 patients (5.4% of cGvHD cases; 2.9% of the total cohort) developed isolated severe gastrointestinal cGvHD, with a median onset of 11.3 months (IQR 9.7–26.1).

IFNγ cytokine profiling identifies immune correlates of severe fibrosing cGvHD and mortality

To identify early immune signatures associated with progression to severe fibrosing cGvHD, we analyzed serum samples collected within 7 days before onset of cGvHD or at day 180 for controls (median day 180, IQR 169–188, **see Methods**). This sampling strategy was chosen to align immune profiling with the time point immediately preceding formal cGvHD diagnosis, while using day 180 post-transplant as a comparable reference landmark for patients who did not develop cGvHD. Secondly, by excluding patients with prior relapse or steroid-refractory acute GvHD, we minimized immune perturbations and reduced potential confounding from antineoplastic therapy, high-dose immunosuppression, and systemic inflammation. We assessed potential temporal bias arising from changes in clinical care between 2002 and 2021, as well as variable sampling time points in the cGvHD group. No significant associations between sampling time and cytokine concentrations were observed, indicating that cytokine levels were not primarily driven by timing of sample collection (**supplemental Figure 2**).

We profiled 14 inflammatory cytokines involved in IFNγ-related signaling pathways (IFNγ, CXCL9, CXCL10, IL-12p40, EBI3, L-GH, IL-17, IL-18, and IL-23) and innate immune activation (TNFα, IFNα, IL-1β, activin, and follistatin). Spearman's rho analysis showed that all tested cytokines were positively correlated with both CXCL9 and CXCL10, consistent with activation of the IFNγ pathway. As expected, IL-17 correlated with IL-23, whereas no significant correlation was observed between IL-12p40 and IL-23 (**Figure 1A**). In univariable logistic regression, IL-12p40 (OR per concentration doubling 1.19, CI 1.07-1.33, p=0.002), CXCL9 (OR per concentration doubling 1.20, CI 1.07-1.38, p=0.005) and CXCL10 (OR per concentration doubling 1.53, CI 1.18-2.02, p=0.002) were significantly associated with subsequent development of severe fibrosing cGvHD after onset cGvHD (**Figure 1B).** Although IL-18 did not reach statistical significance in this model, its levels were significantly higher in patients with severe

fibrosing cGvHD than in those with mild disease (p<0.05, **supplemental Figure 3**), supporting an association with fibrosing disease severity.

While CXCL9, CXCL10 and IL-18 have previously been linked to cGvHD (10, 17-21) , the role of IL-12p40 in human cGvHD remains largely unexplored. Serum IL-12p40 levels were significantly elevated in patients with mild or severe fibrosing cGvHD compared to patients without cGvHD (p<0.001, **Figure 1C**). In univariable parametric Weibull regression analyses IL-12p40 (HR per concentration doubling 0.89, CI 0.85–0.94, p<0.001) and IL-18 (HR per concentration doubling 1.41, CI 1.22–1.63, p<0.001) were the only cytokines significantly associated with overall mortality after day 180 (**Figure 1D**). The protective effect of IL-12p40 on survival was further reflected in its association with reduced relapse rates (csHR per concentration doubling 0.89, CI 0.83–0.94, p<0.001, **Figure 1F**) and NRM (csHR per concentration doubling 0.87, CI 0.81–0.93, p=0.002, **Figure 1E**). Conversely, IL-18 was linked to an increased risk of NRM (csHR per concentration doubling is 1.79, CI 1.47–2.19, p<0.001, **Figure 1E**).

IL-12p40 and IL-18 on day 180 associate with opposite patient outcomes after alloHSCT

The association of IL-12p40 and IL-18 on at cGvHD onset or day 180 for control patients with overall mortality was confirmed in a multivariable Cox regression model of the total cohort (n=552) age, disease stage, disease type, donor type, year of alloHSCT, MTX prophylaxis, ATG prophylaxis, endothelial prophylaxis with pravastatin and UDCA, donor and recipient sex, and cGvHD as a time-dependent covariate (**Figure 2A**). IL-12p40 was associated with reduced overall mortality (HR per log2 increase 0.91, CI 0.87-0.95, p<0.001), a decreased hazard of NRM (csHR per log2 increase 0.87, CI 0.82-0.93, p<0.001, **Figure 2B**) and relapse (csHR 0.88, CI 0.84-0.93, p<0.001, **Figure 2C**). Elevated IL-18 correlated with increased overall mortality (HR per cytokine doubling 1.4, CI 1.23-1.59, p<0.001, **Figure 2A**) and an increased hazard of NRM (csHR per cytokine doubling 1.75, CI 1.47-2.09, p<0.001, **Figure 2B**). Expected clinical covariates also contributed to outcomes: age and HLA mismatch were associated

with increased mortality (HR per log2 increase 1.03, CI 1.02-1.04, p=0.007 and 1.63, CI 1.16-2.3, p=0.005) and NRM (csHR 1.02, CI 1.01-1.05, p=0.004 and 1.86, CI 1.18–2.94, p=0.008, **Figure 2A-B**), while cGvHD diagnosis was associated with reduced overall mortality (HR 0.60, CI 0.44–0.82, p=0.002, **Figure 2A**) and relapse (csHR 0.62, CI 0.45–0.87, p=0.005, **Figure 2C**). Unexpectedly, ATG was associated with reduced relapse risk in this model (csHR 0.6, CI 0.41-0.87, p=0.007, **Figure 2C**). However, post-hoc cause-specific Cox model comparisons showed that adding IL-12p40 significantly improved model fit over ATG alone ($\Delta\chi^2$=22.34) and substantially altered the ATG coefficient (83.7% relative change in β=log[csHR]), suggesting that the apparent ATG–relapse association was largely confounded by IL-12p40 rather than reflecting an independent anti-relapse effect of ATG (**supplemental Figure 4**). To assess the robustness of these findings and address potential sources of bias, we performed additional sensitivity analyses accounting for variability in sampling time within the cGvHD arm. We repeated the multivariable Cox regression analyses after stratifying cGvHD patients according to sampling time, including cGvHD patients sampled between days 150 and 210 post-transplant and, separately, patients sampled outside this interval (<day 150 or >day 210; **supplemental Figure 5**). In all sensitivity analyses, IL-12p40 and IL-18 retained their significant associations with patient outcomes, supporting the robustness of the observed cytokine-outcome relationships.

To better understand the divergent associations of IL-12p40 and IL-18 with patient outcomes, we examined clinical laboratory parameters on day 180 and over a 100-day follow-up period (+25, +50, +100 days after blood sampling). IL-12p40 and IL-18 levels positively correlated with Glutamate-Pyruvate-Transaminase levels (p<0.001). In addition, IL-18 was significantly associated with higher EASIX (35.9%, 24.1-48.9%, p<0.0001) and CRP levels (37.5%, 21.5-55.6%, p<0.0001) as well as lower Albumin levels (-8.6%, -11.4 to -5.8%, p<0.0001) per SD increase in cytokine concentration, independent of the time-dependent variation of these parameters during follow-up (**Figure 2D**).

IL-12p40 correlates with expanded immune cell compartments

To explore the association of IL-12p40 and IL-18 with cellular immune phenotypes, we analyzed PBMC samples from 200 patients from the total cohort, collected at the same time point as the serum samples used for IFNγ–related cytokine measurements. Patients were stratified according to IL-12p40 and IL-18 levels to ensure balanced representation across the IL-12p40 and IL-18 expression ranges. Immune profiling was performed using a 23-marker flow cytometry panel designed to assess activation, proliferation, trafficking, and lineage-specific subsets enabling the identification of 75 distinct immunophenotypes. Of the 200 analyzed samples, 186 met quality control criteria and were included in the final analysis, in which absolute cell counts for each immunophenotype were correlated with IL-12p40 and IL-18 levels.

Spearman's correlation analysis revealed clearly distinct cellular immune signatures associated with IL-12p40 and IL-18 levels (**Figure 3A**). IL-12p40 exhibited broad and predominantly positive correlations across multiple T-cell compartments, including activated and memory CD4+ and CD8+ subsets, γδ T cells, as well as regulatory and checkpoint-expressing phenotypes, consistent with an association with enhanced T-cell reconstitution. The strongest correlations were observed for activated monocytes and CD4+ and CD8+ T-cell subsets, several of which remained significant after FDR correction (FDR ≤ 0.1). Strikingly, IL-12p40 significantly correlated with 50 of 75 immune populations, underscoring its extensive immunological footprint. Only two of these associations were negative, whereas the vast majority demonstrated positive correlations, corresponding to higher counts of multiple T-cell and myeloid subsets in the setting of elevated IL-12p40 levels. In contrast, IL-18 displayed a markedly more restricted and partially divergent correlation profile. Only 6 of 75 populations were significantly associated with IL-18. Moreover, IL-18 exhibited a mixed distribution of positive and negative correlations, including an inverse association with dendritic cells, while only selected subsets, such as PD-1+ CD8+ T cells and CXCR4+ T helper cells, showed moderate positive correlations. Collectively, these findings indicate that IL-12p40 demonstrates a substantially broader and more coherent

association with immune reconstitution than IL-18, supporting the concept of distinct immunobiological roles for these cytokines after alloHSCT.

Exploratory associations between immune cell subsets and clinical outcomes

To assess whether specific immune cell populations might contribute to the beneficial associations of IL-12p40 with patient outcomes, we performed univariable analyses for 16 major populations in relation to severe fibrosing cGvHD, relapse, and NRM (**Figure 3B-D**). Naïve CD4+ T cells and central memory CD4+ T cells were significantly associated with an increased risk of progression to severe fibrosing cGvHD (HR 1.24, 95% CI 1.07-1.44, p=0.01 and HR 1.47, 95% CI 1.11-1.93, p=0.03, **Figure 3B**). In contrast, quantitative immune reconstitution across multiple lymphoid and myeloid compartments was associated with a reduced risk of relapse (**Figure 3D**). The strongest protective association was observed for γδ T cells (csHR 0.77, 95% CI 0.66–0.89, p<0.001); however, several additional immune subsets, including central and effector memory CD8+ T cells, CD4+ effector memory cells, CD8+ CM and EM subsets, as well as monocytes and dendritic cells, also showed significant inverse associations with relapse risk. None of the analyzed immune populations were significantly associated with NRM (**Figure 3C**).

ATG prophylaxis modulates IL-12p40– and IL-18–associated immune reconstitution

To determine whether therapeutic immune modulation influences the identified cytokine signatures, we examined the association of ATG prophylaxis with IL-12p40 and IL-18 levels. ATG prophylaxis, administered from day –3 to –1 prior to alloHSCT, was associated with a markedly reduced incidence of severe fibrosing cGvHD: only 20 of 394 patients (5.1%) who received ATG developed severe fibrosing cGvHD, compared to 32 of 158 (20.3%) without ATG (p<0.0001; **Table 2**).

Patients receiving ATG prophylaxis exhibited significantly lower IL-18 levels at day 180, both among those without cGvHD (p=0.008) and in those who subsequently developed cGvHD (p=0.012; **Figure 4B**). In contrast, IL-12p40 levels were reduced in ATG-treated patients who later developed cGvHD (p=0.001), whereas patients without cGvHD exhibited uniformly low IL-12p40 levels irrespective of ATG exposure (**Figure 4A**).

ATG prophylaxis was further associated with a marked reduction in circulating B cells, naïve CD8+ T cells, and across all CD4+ T-cell compartments, including naïve, central memory, effector memory, and TEMRA subsets (**Figure 4C**). Notably, the abundance of naïve and central memory CD4+ T cells was independently associated with progression to severe fibrosing cGvHD (**Figure 3B**), suggesting that ATG-mediated modulation of these subsets may contribute to the reduced risk of severe fibrosing cGvHD.

Early prednisolone selectively suppresses IL-12p40 but not IL-18

We next evaluated the impact of early prednisolone exposure, defined as the cumulative dose administered from day 0 to day 100 after alloHSCT, on IL-12p40 and IL-18 levels as well as immune reconstitution. Patients were stratified into low- (0–100 mg), intermediate- (101–500 mg), and high-dose (>500 mg) groups. In contrast to ATG prophylaxis, high-dose corticosteroid exposure was not associated with a reduced incidence of severe fibrosing cGvHD (**Table 2**). The rationale for this analysis was to determine whether early corticosteroid-mediated immunosuppression, within a similar biologically relevant post-transplant window affected by ATG, influences later severe fibrosing cGvHD development and IL-12p40 or IL-18 expression.

In patients without cGvHD, early prednisolone exposure did not significantly affect IL-12p40 or IL-18 levels (**Figure 5A–B**). Among patients who developed cGvHD, however, high cumulative corticosteroid exposure (>500 mg prednisolone by day 100) was associated with selective suppression of IL-12p40 (p=0.028), whereas IL-18 levels remained unaffected. High-dose prednisolone exposure was also

associated with reduced counts of NKT cells and dendritic cells, the latter being independently associated with a lower risk of relapse in our cohort ($p<0.01$, **Figure 3D**).

To explore whether selective suppression of IL-12p40 reflected direct, differential cytokine sensitivity to glucocorticoids, we stimulated THP-1 cells and primary CD14+ PBMCs with LPS and dexamethasone. In both systems, IL-12p40 was strongly suppressed at low nanomolar concentrations (1–10 nM), with near-complete inhibition at 100 nM dexamethasone ($p<0.001$). In contrast, IL-18 secretion was only modestly affected, with residual levels ≥43.5% of baseline even at high dexamethasone doses (**Figure 6**). These findings support partial glucocorticoid resistance of IL-18 secretion, consistent with our clinical observations.

Discussion

The established contribution of the IFNγ pathway, particularly CXCL9 and IL-18, to cGvHD pathogenesis (10, 17-21, 23) prompted us to broaden our analysis of the cytokine network related to this pathway. Among the 14 IFNγ-associated cytokines studied, IL-12p40, CXCL9, and CXCL10 at onset of cGvHD were associated with increased risk of severe fibrosing cGvHD. The main findings were that IL-12p40 and IL-18 showed opposing outcome associations, with IL-12p40 linked to reduced relapse risk, improved survival, and broad immune reconstitution, whereas IL-18 correlated with increased NRM. Early cumulative steroid exposure selectively reduced IL-12p40 at cGvHD onset, while ATG prophylaxis suppressed both IL-12p40 and IL-18. Accordingly, ATG, but not steroid exposure, was associated with reduced fibrosing cGvHD incidence.

IL-12p40 and IL-18 showed significant correlations with CXCL9 and CXCL10, consistent with their role as IFNγ-inducible cytokines. As expected, CXCL9 strongly correlated with CXCL10 (24), and IL-23 with IL-17 (25); however, no correlation was observed between IL-12p40 and IL-23 or IL-17, suggesting that IL-12p40 in this context reflects IL-12p70 (p35+p40) rather than IL-23 (p19+p40) activity (26).

IL-12p40 is primarily produced by antigen-presenting cells via CD40 ligand and JAK-stimulated pathways (27). In contrast, IL-18 is produced by various cell types, including monocytes, macrophages, and keratinocytes, and is implicated in autoimmune diseases (28, 29). Across the cohort, IL-18 concentrations were broadly elevated and showed substantial overlap between patients with and without severe fibrosing cGvHD. Consistent with this observation, IL-18 levels were highest in patients without cGvHD who had not received ATG. Accordingly, IL-18 levels strongly correlated with the EASIX score and CRP (30, 31), and were robustly associated with NRM and overall mortality (32). We have further demonstrated relative glucocorticoid resistance of IL-18 in vivo and in vitro, consistent with inflammasome-dependent regulation and broader cellular sources. Together, these findings support IL-18 as a biomarker of systemic inflammatory activity and endothelial injury after alloHSCT rather than a disease-specific mediator of fibrosing cGvHD (33-35).

Historically, IL-12 has been regarded as a pro-inflammatory driver of GvHD (36-40), leading to clinical trials focused on IL-12p40 inhibition as a therapeutic strategy (41). Our study challenges this simplified view, showing that IL-12p40 exhibits a dual role: while it is associated with severe fibrosing cGvHD, it is also linked to improved survival and enhanced hematopoietic recovery. The observed correlation between IL-12p40 and immune reconstitution aligns with reports implicating IL-12p40 in lympho-hematopoiesis (42) and its prevention of thymic involution in aged mice (43). Additionally, in primate models, low-dose IL-12 enhanced peripheral hematopoiesis, engraftment, and mitigated myelosuppression (44-46). In our cohort, IL-12p40 tracked with recovery across multiple innate and adaptive cell compartments. Among IL-12p40-associated populations γδ T cells showed the strongest inverse association with relapse risk, supporting a relationship between immune reconstitution and GvL activity (47).

Although cytokines were measured within 7 days before initial cGvHD onset, severe fibrosing cGvHD developed months later. We therefore interpret IL-12p40 and IL-18 not as direct markers of established fibrosing cGvHD, but as early immunological markers that may shape subsequent cGvHD

trajectories. In this framework, IL-18 reflects a systemic inflammatory and endothelial injury axis, whereas IL-12p40 is linked to hematopoietic recovery and graft-versus-leukemia activity.

The translational relevance of these cytokine signatures is underscored by their differential responses to immunosuppressive modulation. ATG prophylaxis was associated with reduced IL-12p40 and IL-18 levels, a markedly lower incidence of severe fibrosing cGvHD, and broad reductions across multiple immune compartments. In contrast, early cumulative prednisolone exposure selectively suppressed IL-12p40 at cGvHD onset (48) without affecting IL-18 levels or fibrosing cGvHD incidence, and had only modest effects on circulating immune cell counts compared to ATG prophylaxis. Together, these observations support the interpretation of IL-12p40 as a biomarker reflecting the degree of immunosuppressive exposure, the integrity of post-transplant immune recovery and GvL.

These results are hypothesis-generating and support prospective validation of IL-12p40 and IL-18 within multi-parameter models for risk stratification and treatment monitoring, including formal assessment of predictive performance and added clinical value in independent multicenter cohorts. While these findings argue for caution with broad IL-12p40-targeting strategies (49, 50), they also underscore the need to distinguish pathogenic inflammatory signals from immune-reconstitution-associated cytokine patterns after alloHSCT.

Several limitations should be considered. This retrospective, single-center study reflects center-specific practices, including predominant peripheral blood stem cell graft use, limited bone marrow, haploidentical, and PTCY-based transplants, and routine pravastatin/UDCA prophylaxis in most patients. The overall incidence of cGvHD was relatively high, which may in part reflect center-specific immunosuppression tapering strategies and the clinical acceptance of mild to moderate cGvHD as a manifestation of graft-versus-leukemia alloreactivity and sample availability–based inclusion criteria. However, the incidence of severe fibrosing cGvHD, the main clinical endpoint of this study, was 9.4% of the total cohort and therefore within the range reported in previous studies (13-15). Statin/UDCA exposure was associated with reduced IL-12p40 but not IL-18. Non-exposed patients were few and

transplanted before in-house implementation in 2010, limiting direct comparisons. The long study period also introduced potential heterogeneity from evolving supportive care, GvHD prophylaxis, and newer therapies such as ruxolitinib, although adjustment for transplant year did not materially alter the main findings.

Together, these findings support a model in which IL-12p40 reflects beneficial immune reconstitution and graft-versus-leukemia activity, whereas IL-18 defines a steroid-resistant inflammatory axis associated with endothelial injury, thereby providing a potential mechanistic framework for divergent cGvHD trajectories after alloHSCT.

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Tables

Table 1. Patient characteristics.

	No cGvHD	Mild cGvHD	Severe fibrosing cGvHD	Severe gastrointestinal cGvHD	p-value
n	258 (100)	226 (100)	52 (100)	16 (100)	n/a
Year of alloHSCT	2003-21	2002-21	2003-21	2006-21	0.382
Median age [range], years	56 [18-75]	52 [18-74]	54 [21-73]	50 [19-73]	0.0018
Male sex	151 (58.5)	145 (64.2)	31 (59.6)	9 (56.3)	0.6
Underlying hematological disease:					
Acute myeloid leukemia	106 (41.1)	95 (42.0)	18 (34.6)	7 (43.8)	0.802
Lymphatic/lymphoblastic neoplasms	74 (28.7)	64 (28.3)	15 (28.8)	4 (25.0)	1
Myeloproliferative/myelodysplastic disorders	63 (24.4)	49 (21.7)	15 (28.8)	3 (18.8)	0.676
Multiple myeloma	15 (5.8)	18 (8.0)	4 (7.7)	2 (12.5)	0.493
Disease stage before alloHSCT:					
0	121 (46.9)	109 (48.2)	28 (53.8)	9 (56.3)	0.747
1	67 (27.9)	63 (27.9)	12 (23.1)	2 (12.5)	0.587
2	70 (23.9)	54 (23.9)	12 (23.1)	5 (31.3)	0.768
Donor type:					
Matched related donor	40 (15.5)	66 (29.2)	33 (63.5)	4 (25.0)	<0.001
matched unrelated donor	165 (64.0)	126 (55.8)	17 (32.7)	7 (43.8)	<0.001
mismatched unrelated donor	41 (15.9)	28 (12.4)	2 (3.8)	4 (25.0)	0.043
haploidentical donor	12 (4.7)	6 (2.7)	0 (0.0)	1 (6.3)	0.243
Donor sex male	183 (70.9)	148 (65.5)	29 (55.8)	8 (50.0)	0.069
Stem cell source:					
mobilized peripheral blood	240 (93.0)	218 (96.5)	50 (96.2)	15 (93.8)	0.307
bone marrow	18 (7.0)	8 (3.8)	2 (3.8)	1 (6.3)	0.312
Conditioning regimen:					
reduced intensity	197 (76.4)	165 (73.0)	41 (78.8)	13 (81.3)	0.743
myeloablative	61 (23.6)	61 (27.0)	11 (21.2)	3 (18.8)	0.746
First-line immunosuppression:					
anti-thymocyte globulin	216 (83.7)	147 (65.0)	20 (38.5)	11 (68.8)	<0.001
methotrexate days 1,3,6	146 (56.6)	129 (57.1)	30 (57.7)	7 (43.8)	0.776
Statin+UDCA prophylaxis	230 (89.1)	200 (88.5)	49 (94.2)	12 (75.0)	0.194
Acute GvHD:					
no aGvHD	137 (53.1)	119 (52.7)	28 (53.8)	9 (56.3)	0.997
°I-II aGvHD	77 (29.8)	69 (30.5)	17 (32.7)	4 (25.0)	0.953
°III-IV aGvHD	44 (17.1)	38 (16.8)	7 (13.5)	3 (18.8)	0.92

Table 2. Effects of prednisolone and ATG on the risk of severe fibrosing cGvHD

	0-99 mg Prednisolone	**100-499 mg Prednisolone**	**>500 mg Prednisolone**
No severe fibrosing cGvHD	41 (91.1%)	72 (84.7%)	104 (86.0%)
Severe fibrosing cGvHD	4 (8.9%)	13 (15.3%)	17 (14.0%)
	No ATG prophylaxis	**ATG prophylaxis 10 mg/kg/d d-3 to d-1**	
No severe fibrosing cGvHD	126 (79.7%)	374 (94.9%)	
Severe fibrosing cGvHD	32 (20.3%)	20 (5.1%)	

Abbreviations

aGvHD, acute graft-versus-host disease

alloHSCT, allogeneic hematopoietic stem cell transplantation

ATCC, American Type Culture Collection

ATG, anti-thymocyte globulin

BD, Becton Dickinson

cGvHD, chronic graft-versus-host disease

CD, cluster of differentiation

CI, confidence interval

CRP, C-reactive protein

csHR, cause-specific hazard ratio

DC, dendritic cell

DN, double-negative T cell

DP, double-positive T cell

EASIX, Endothelial Activation and Stress Index

ELISA, enzyme-linked immunosorbent assay

EM, effector memory T cell

EMRA, effector memory T cell re-expressing CD45RA

FBS, fetal bovine serum

FDR, false discovery rate

GvL, graft-versus-leukemia

HLA, human leukocyte antigen

HR, hazard ratio

IFNα, interferon alpha

IFNγ, interferon gamma

IL, interleukin

IQR, interquartile range

L-GH, lymphocyte growth hormone

LDH, lactate dehydrogenase

LPS, lipopolysaccharide

MTX, methotrexate

NIH, National Institutes of Health

NK, natural killer

NRM, non-relapse mortality

OR, odds ratio

OS, overall survival

PBMC, peripheral blood mononuclear cell

PBS, phosphate-buffered saline

Pen-Strep, penicillin-streptomycin

Suppl., supplemental

Tac, tacrolimus

UDCA, ursodeoxycholic acid

Figure legends

Figure 1. IFNγ cytokine profiling identifies immune correlates of severe fibrosing cGvHD and mortality

(A) Heatmap showing pairwise correlations between cytokines, with hierarchical clustering identifying distinct correlation blocks. Color intensity indicates the strength and direction of correlation (red = positive, blue = negative). Black boxes highlight clusters of highly correlated cytokines. Asterisks denote statistical significance. (B) Forest plot showing odds ratios (OR) and 95% confidence intervals (CI) for the association between cytokine levels at onset cGvHD or day 180 for controls (n = 552) and subsequent development of severe fibrosing chronic GvHD (n = 52). Cytokine levels were log2-transformed; ORs represent the effect per doubling in cytokine concentration. (C) IL-12p40 levels (pg/ml) in no (n = 258), mild (n = 226), and severe fibrosing cGvHD (n=52) measured at day 180 post-transplant for none and onset cGvHD for mild and severe fibrosing cGvHD groups. (D-F) Forest plots displaying hazard ratios (HR) cause-specific hazard ratios (csHR) and 95% confidence intervals (CI) for each cytokine, modeled per log2 increase. Shown are associations with overall mortality (n = 349, events = 102), relapse with NRM as competing risk (n = 349, events = 60), and NRM with relapse as competing risk (n = 349, events = 61). Statistically significant associations are marked: °p<0.1, *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 2. IL-12p40 and IL-18 associate with opposite patient outcomes after alloHSCT

(A-C) Multivariable Cox regression analyses in the main cohort (n =552) assessing associations between cytokine levels at day 180 post-alloHSCT (log2-transformed) and (left to right) overall mortality (A; events = 146), non-relapse mortality (B; NRM; events = 75), and relapse (C; events =113). Elevated IL-12p40 levels were independently associated with reduced overall mortality, relapse, and NRM, while high IL-18 was linked to increased risk of mortality and NRM. Models were adjusted for age, disease type and stage, HLA mismatch, donor–recipient sex mismatch, stem cell source, GvHD prophylaxis, and

time-dependent chronic GvHD. Competing risks were accounted for in NRM and relapse models. (D) Association of baseline IL-12p40 and IL-18 with longitudinal laboratory parameters. Linear mixed-effects models were used to estimate the association between baseline cytokine levels and repeated laboratory measurements (days 0, 25, 50, and 100 after blood sampling). The plot shows the percent change in each laboratory marker per 1 standard deviation (SD) increase in IL-12p40 or IL-18, adjusted for time since blood sampling and within-patient correlation. Points represent model estimates and horizontal bars indicate 95% confidence intervals. Statistical significance is indicated as: °p<0.1, *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 3. IL-12p40–associated immune expansion correlates with reduced relapse risk after alloHSCT

(A) Circular bar plots illustrating Spearman correlation coefficients (ρ) between serum IL-12p40 (right) and IL-18 (left) at day 180 post-alloHSCT and the absolute counts of 75 peripheral blood mononuclear cell subsets in 186 patients. Positive correlations are shown in red tones and negative correlations in blue tones, with darker shades indicating stronger correlations and FDR-adjusted significance (p_adj ≤ 0.1). Grey bars denote non-significant associations (p_adj > 0.1). Bar length corresponds to the Spearman correlation coefficient. (B-D) Cause-specific Cox regression assessing associations between immune cell counts of 16 major populations and risk of severe fibrosing chronic GvHD (severe fibrosing cGvHD; n = 184, events = 19), non-relapse mortality (NRM; n =186, events =20), and relapse (n =186, events =50). Cause-specific Hazard ratios (csHRs are shown per log2 doubling of cell counts. Increased γδ T cells (Tγδ), monocytes and dendritic cells (DC) were significantly associated with reduced relapse risk, while naïve and central memory CD4+ T cell subsets showed significant associations with severe fibrosing cGvHD. Statistical significance: °p<0.1, *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4. ATG prophylaxis correlates with decreased IL-12p40/IL-18 and reduced CD4+ compartments

(A–B) Longitudinal serum concentrations of IL-12p40 (A) and IL-18 (B) measured at days −14, 3, 12, 28, 100, and 180 relative to alloHSCT. Mean values are shown with shaded areas representing standard deviation. Data are stratified by ATG prophylaxis (ATG vs no ATG) and further separated according to subsequent development of chronic GvHD (cGvHD vs no cGvHD) (n = 349). (C) Absolute counts of 16 major immune cell populations at blood sampling, log10-transformed, comparing patients with and without ATG prophylaxis. Box plots indicate median and interquartile range; individual data points are shown. Statistical significance: °p<0.1, *p < 0.05, **p < 0.01, ***p < 0.001.

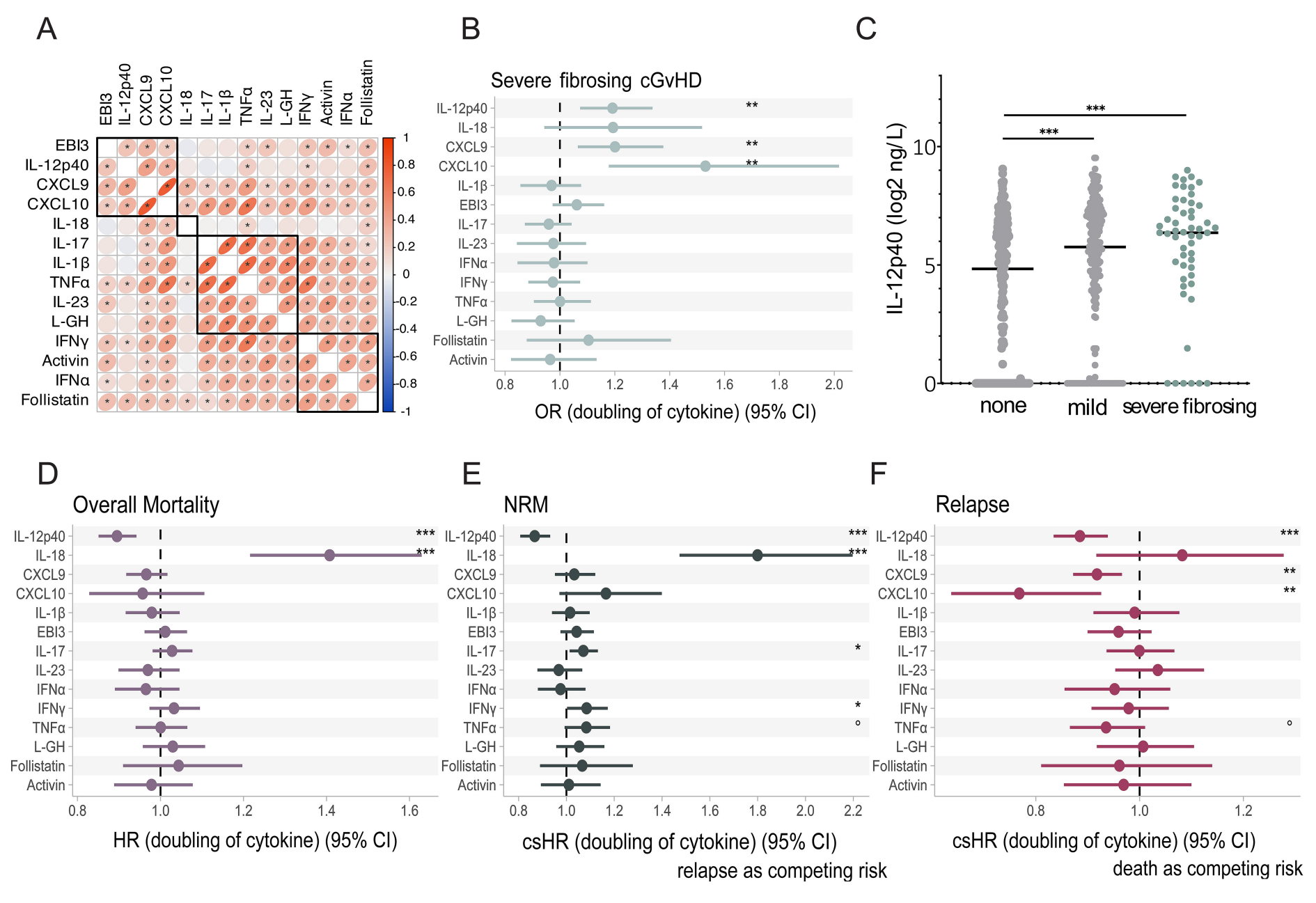
Figure 5. Early prednisolone selectively suppresses IL-12p40 but not IL-18

(A–B) Longitudinal serum concentrations of IL-12p40 (A) and IL-18 (B) measured at days −14, 3, 12, 28, 100, and 180 relative to alloHSCT. Mean values are shown with shaded areas representing standard deviation. Data are stratified by cumulative prednisolone intake (days 0–100; n = 251), grouped into low (0–100 mg), intermediate (101–500 mg), and high (>500 mg) exposure, and further stratified by cGvHD status. (C) Absolute counts of 16 major immune cell populations at blood sampling, log10-transformed, comparing patients with low, intermediate and high prednisolone exposure. Box plots indicate median and interquartile range; individual data points are shown.

Figure 6. Differential suppression of cytokine production by dexamethasone in THP-1 cells and primary $CD14^+$ monocytes

(A) Dose–response analysis of dexamethasone-mediated cytokine suppression in THP-1 cells (left) and primary CD14+ PBMC-derived monocytes (right). Cells were stimulated with LPS (1 µg/mL) and treated with increasing concentrations of dexamethasone (10^0–10^4 nM). Cytokine suppression was measured by ELISA and is shown as percentage relative to stimulated, untreated controls. Data represent

mean ± SEM from three biological replicates. Statistical significance: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.


A
EBI3
IL-12p40
CXCL9
CXCL10
IL-18
IL-17
IL-1β
TNFα
IL-23
L-GH
IFNγ
Activin
IFNα
Follistatin
1
0.8
0.6
0.4
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0
-0.2
-0.4
-0.6
-0.8
-1
B
Severe fibrosing cGvHD
IL-12p40
IL-18
CXCL9
CXCL10
IL-1β
EBI3
IL-17
IL-23
IFNα
IFNγ
TNFα
L-GH
Follistatin
Activin
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**
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0.8
1.0
1.2
1.4
1.6
1.8
2.0
OR (doubling of cytokine) (95% CI)
C
IL-12p40 (log2 ng/L)
10
5
0
***
***
none
mild
severe fibrosing
D
Overall Mortality
IL-12p40
IL-18
CXCL9
CXCL10
IL-1β
EBI3
IL-17
IL-23
IFNα
IFNγ
TNFα
L-GH
Follistatin
Activin
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0.8
1.0
1.2
1.4
1.6
HR (doubling of cytokine) (95% CI)
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NRM
IL-12p40
IL-18
CXCL9
CXCL10
IL-1β
EBI3
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IL-23
IFNα
IFNγ
TNFα
L-GH
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°
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
csHR (doubling of cytokine) (95% CI)
relapse as competing risk
F
Relapse
IL-12p40
IL-18
CXCL9
CXCL10
IL-1β
EBI3
IL-17
IL-23
IFNα
IFNγ
TNFα
L-GH
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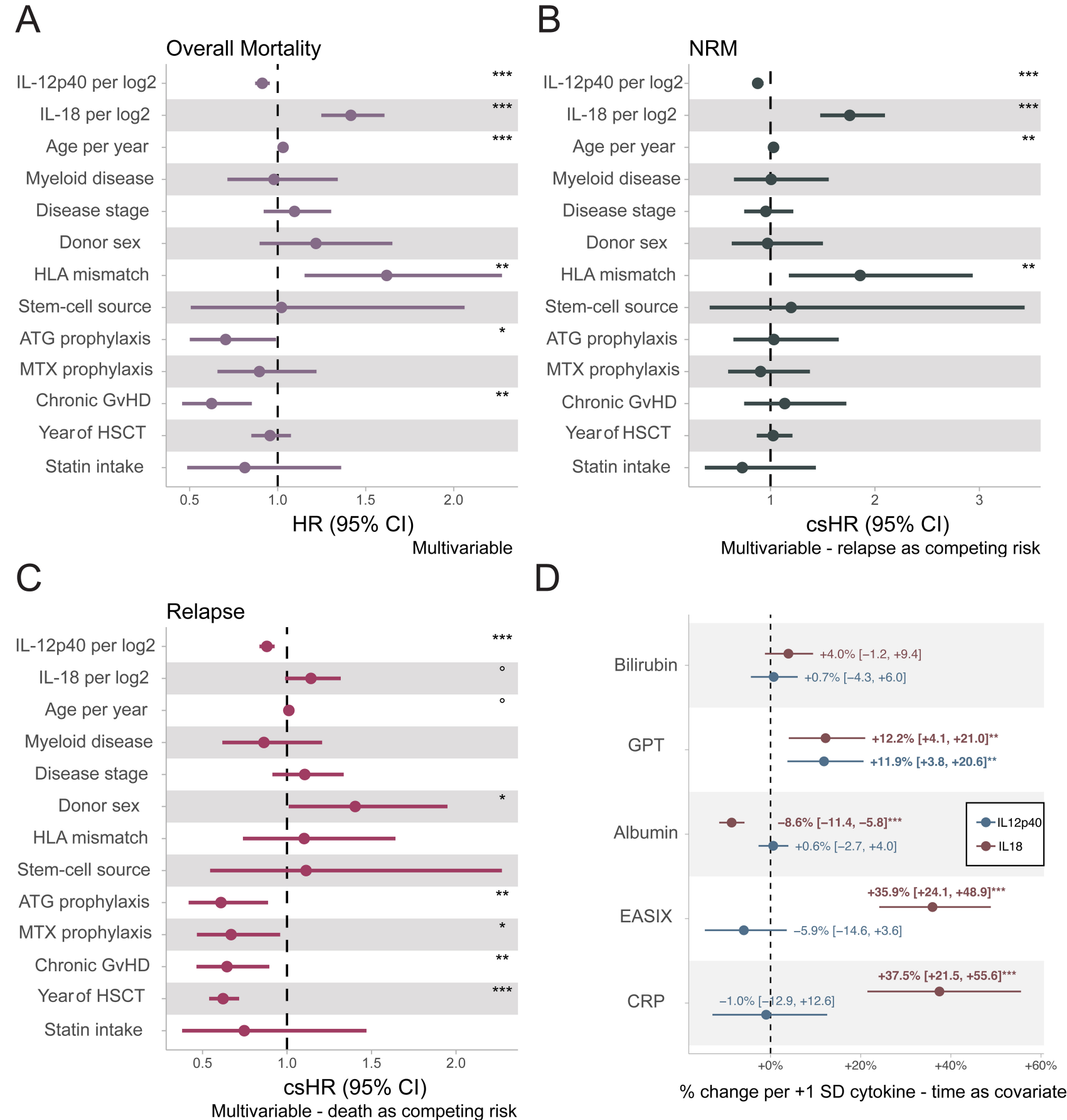
csHR (doubling of cytokine) (95% CI)
death as competing risk


A
Overall Mortality
IL-12p40 per log2
IL-18 per log2
Age per year
Myeloid disease
Disease stage
Donor sex
HLA mismatch
Stem-cell source
ATG prophylaxis
MTX prophylaxis
Chronic GvHD
Year of HSCT
Statin intake
***
***
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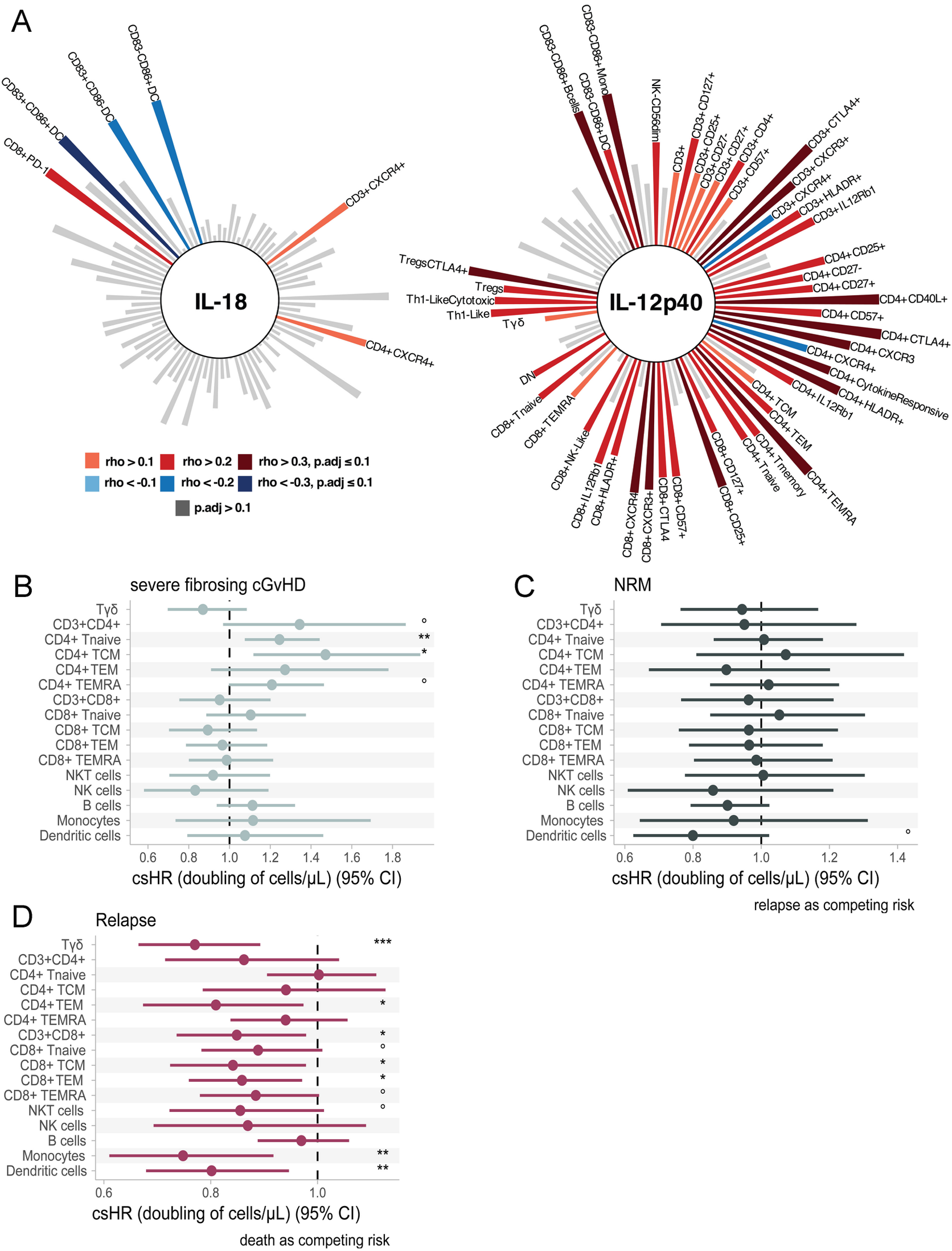
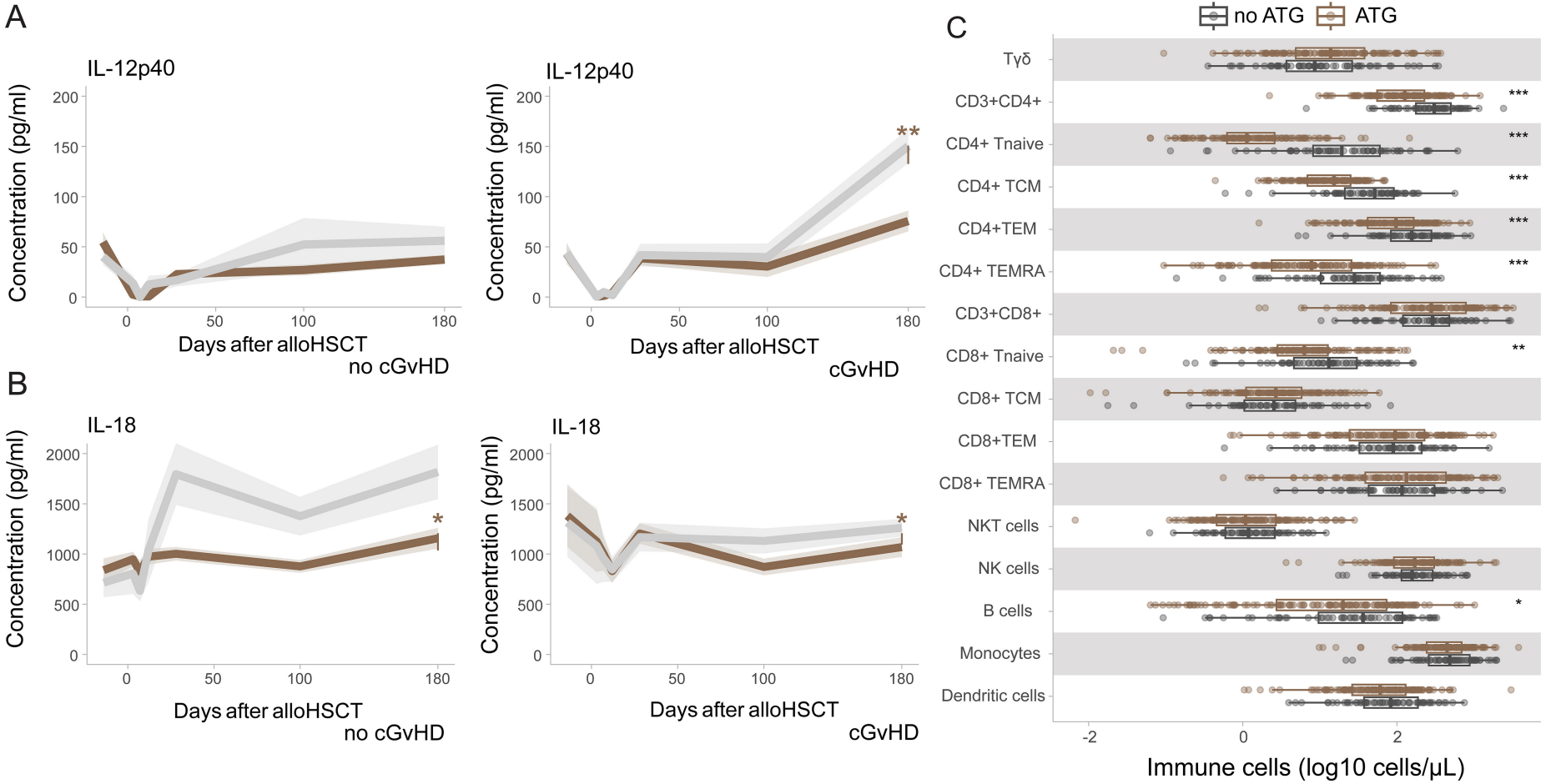
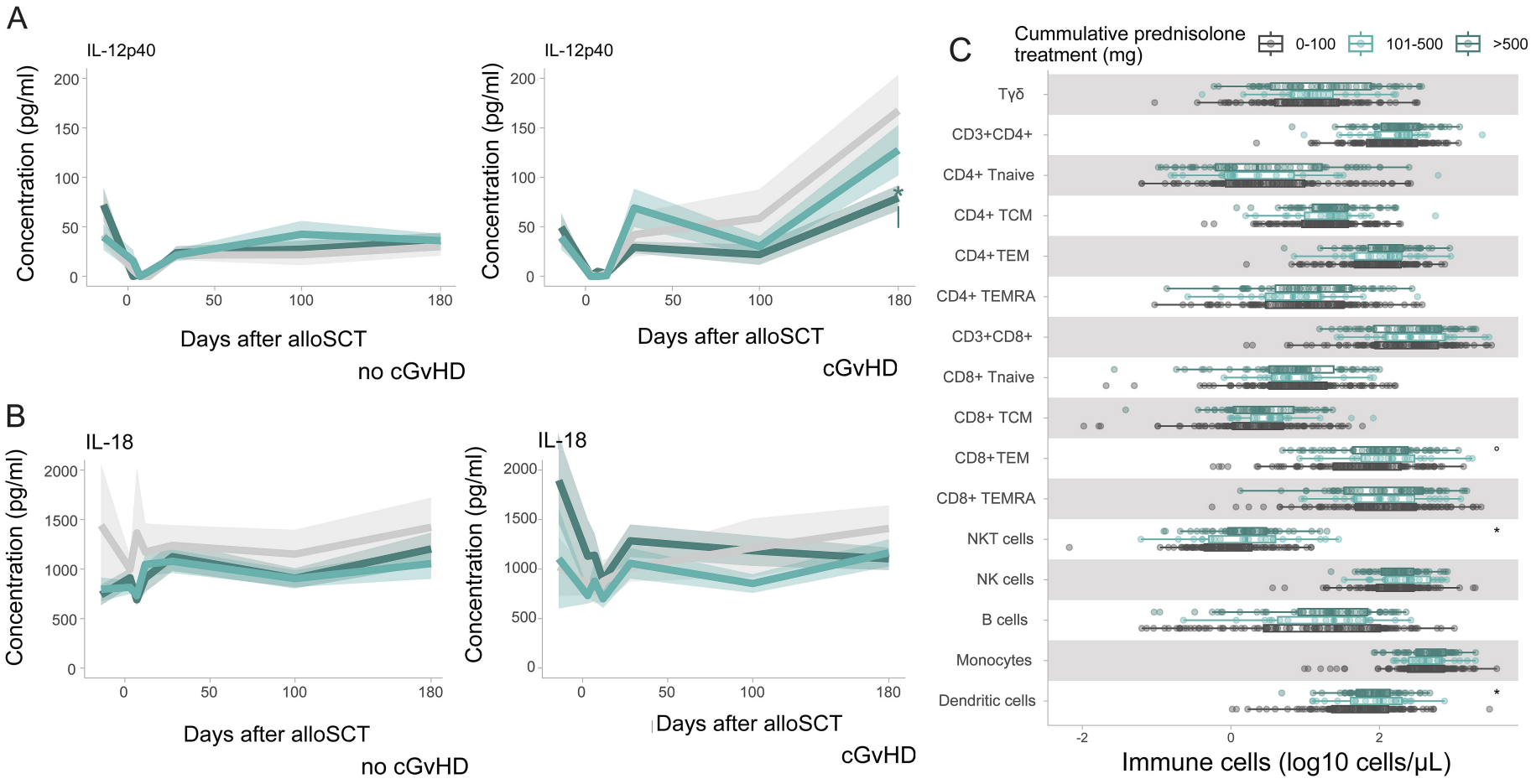
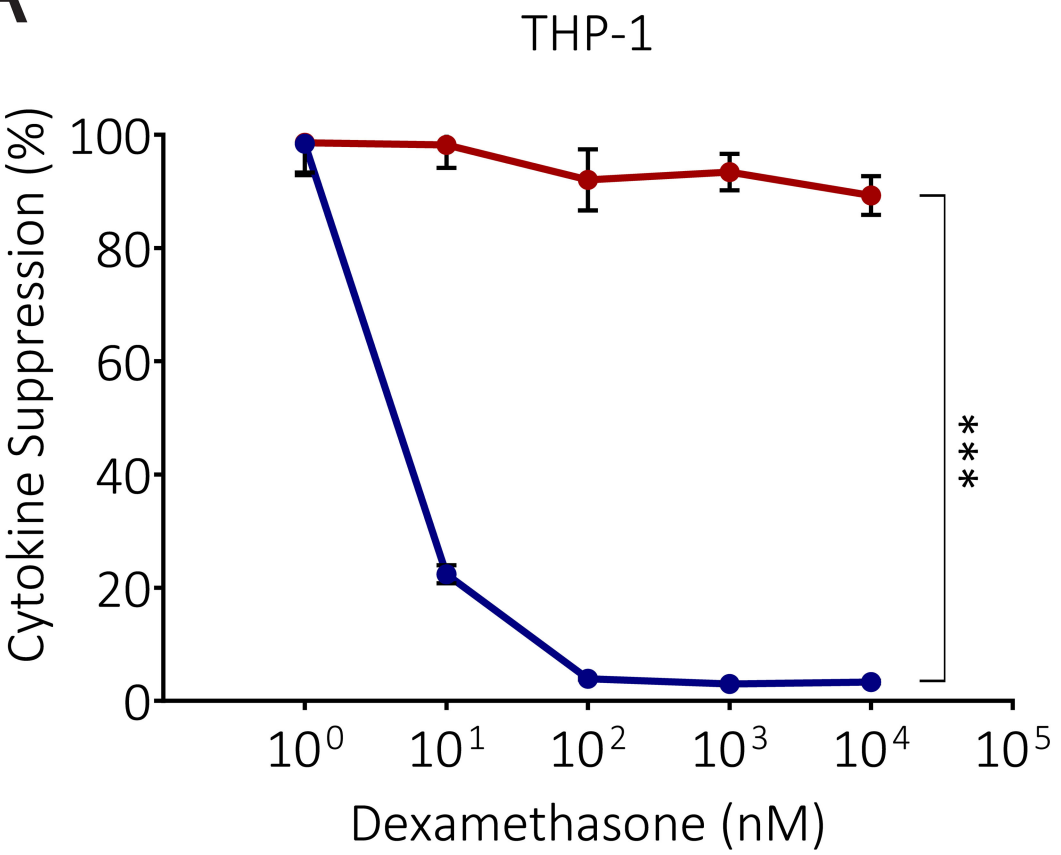
*
**
0.5
1.0
1.5
2.0
HR (95% CI)
Multivariable
B
NRM
***
***
**
**
1
2
3
csHR (95% CI)
Multivariable - relapse as competing risk
C
Relapse
***
°
°
*
**
*
**
***
csHR (95% CI)
Multivariable - death as competing risk
D
Bilirubin
+4.0% [−1.2, +9.4]
+0.7% [−4.3, +6.0]
GPT
+12.2% [+4.1, +21.0]**
+11.9% [+3.8, +20.6]**
Albumin
−8.6% [−11.4, −5.8]***
+0.6% [−2.7, +4.0]
IL12p40
IL18
EASIX
+35.9% [+24.1, +48.9]***
−5.9% [−14.6, +3.6]
CRP
+37.5% [+21.5, +55.6]***
−1.0% [−12.9, +12.6]
+0%
+20%
+40%
+60%
% change per +1 SD cytokine - time as covariate

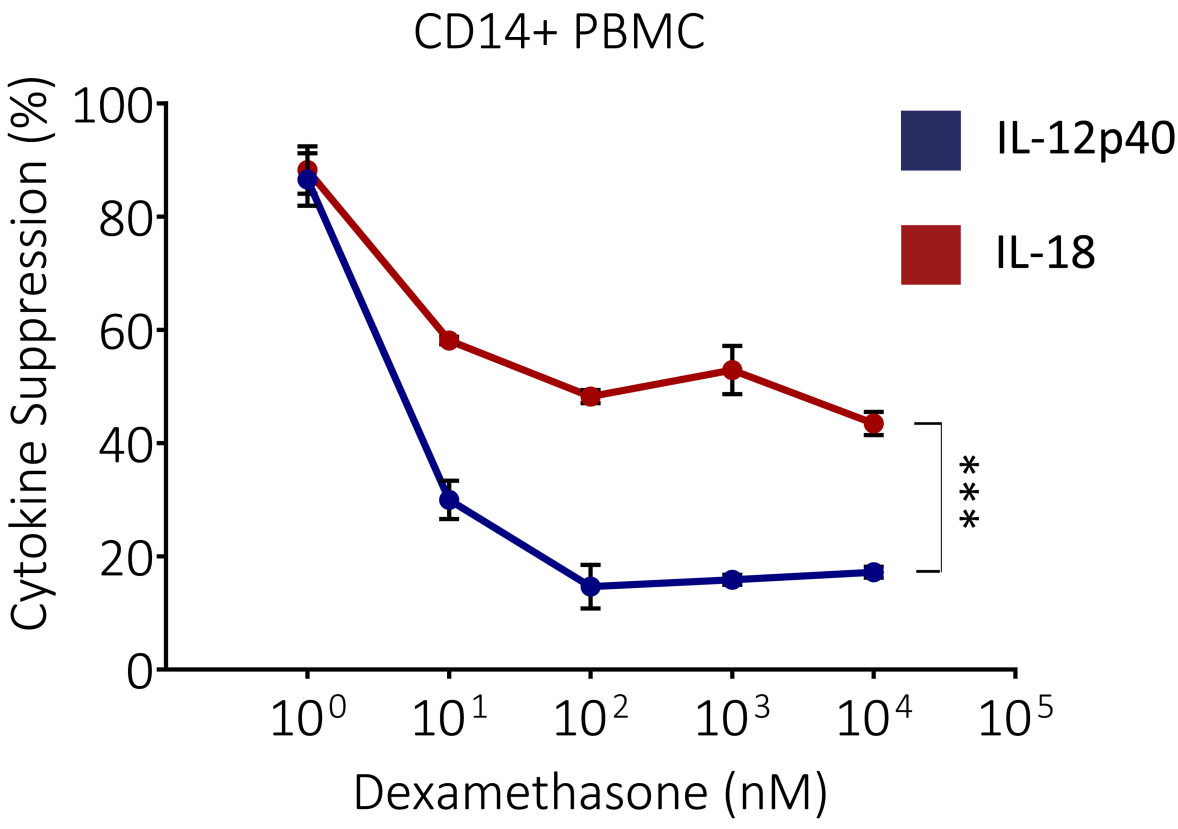

A
IL-18
CD83+CD86+DC
CD83+CD86-DC
CD83-CD86+DC
CD8+PD-1
CD3+CXCR4+
CD4+CXCR4+
IL-12p40
CD83-CD86+Bcells
CD83-CD86+Mono
CD83-CD86+DC
NK-CD56dim
CD3+
CD3+CD127+
CD3+CD25+
CD3+CD27-
CD3+CD27+
CD3+CD4+
CD3+CD57+
CD3+CTLA4+
CD3+CXCR3+
CD3+CXCR4+
CD3+HLADR+
CD3+IL12Rb1
TregsCTLA4+
Tregs
Th1-LikeCytotoxic
Th1-Like
Tγδ
DN
CD8+Tnaive
CD8+TEMRA
CD8+NK-Like
CD8+IL12Rb1
CD8+HLADR+
CD8+CXCR4
CD8+CXCR3+
CD8+CTLA4
CD8+CD57+
CD8+CD127+
CD8+CD25+
CD4+TCM
CD4+TEM
CD4+Tnaive
CD4+Tmemory
CD4+TEMRA
CD4+CD25+
CD4+CD27-
CD4+CD27+
CD4+CD40L+
CD4+CD57+
CD4+CTLA4+
CD4+CXCR3
CD4+CXCR4+
CD4+CytokineResponsive
CD4+IL12Rb1
CD4+HLADR+
rho > 0.1
rho > 0.2
rho > 0.3, p.adj ≤ 0.1
rho < -0.1
rho < -0.2
rho < -0.3, p.adj ≤ 0.1
p.adj > 0.1
B
severe fibrosing cGvHD
Tγδ
CD3+CD4+
CD4+ Tnaive
CD4+ TCM
CD4+TEM
CD4+ TEMRA
CD3+CD8+
CD8+ Tnaive
CD8+ TCM
CD8+TEM
CD8+ TEMRA
NKT cells
NK cells
B cells
Monocytes
Dendritic cells
°
**
*
°
0.6
0.8
1.0
1.2
1.4
1.6
1.8
csHR (doubling of cells/µL) (95% CI)
C
NRM
Tγδ
CD3+CD4+
CD4+ Tnaive
CD4+ TCM
CD4+TEM
CD4+ TEMRA
CD3+CD8+
CD8+ Tnaive
CD8+ TCM
CD8+TEM
CD8+ TEMRA
NKT cells
NK cells
B cells
Monocytes
Dendritic cells
°
0.6
0.8
1.0
1.2
1.4
csHR (doubling of cells/µL) (95% CI)
relapse as competing risk
D
Relapse
Tγδ
CD3+CD4+
CD4+ Tnaive
CD4+ TCM
CD4+TEM
CD4+ TEMRA
CD3+CD8+
CD8+ Tnaive
CD8+ TCM
CD8+TEM
CD8+ TEMRA
NKT cells
NK cells
B cells
Monocytes
Dendritic cells
***
*
*
°
*
*
°
°
**
**
0.6
0.8
1.0
csHR (doubling of cells/µL) (95% CI)
death as competing risk


A
IL-12p40
Concentration (pg/ml)
200
150
100
50
0
0
50
100
180
Days after alloHSCT
no cGvHD
IL-12p40
**
Concentration (pg/ml)
Days after alloHSCT
cGvHD
B
IL-18
Concentration (pg/ml)
2000
1500
1000
500
0
*
Days after alloHSCT
no cGvHD
IL-18
*
Concentration (pg/ml)
Days after alloHSCT
cGvHD
C
no ATG
ATG
Tγδ
CD3+CD4+
CD4+ Tnaive
CD4+ TCM
CD4+TEM
CD4+ TEMRA
CD3+CD8+
CD8+ Tnaive
CD8+ TCM
CD8+TEM
CD8+ TEMRA
NKT cells
NK cells
B cells
Monocytes
Dendritic cells
***
***
***
***
***
**
*
-2
0
2
Immune cells (log10 cells/µL)


A
IL-12p40
Concentration (pg/ml)
200
150
100
50
0
0
50
100
180
Days after alloSCT
no cGvHD
IL-12p40
Concentration (pg/ml)
200
150
100
50
0
0
50
100
180
*
Days after alloSCT
cGvHD
B
IL-18
Concentration (pg/ml)
2000
1500
1000
500
0
0
50
100
180
Days after alloSCT
no cGvHD
IL-18
Concentration (pg/ml)
2000
1500
1000
500
0
0
50
100
180
Days after alloSCT
cGvHD
C
Cummulative prednisolone treatment (mg)
0-100
101-500
>500
Tγδ
CD3+CD4+
CD4+ Tnaive
CD4+ TCM
CD4+TEM
CD4+ TEMRA
CD3+CD8+
CD8+ Tnaive
CD8+ TCM
CD8+TEM
°
CD8+ TEMRA
NKT cells
*
NK cells
B cells
Monocytes
Dendritic cells
*
-2
0
2
Immune cells (log10 cells/µL)

A


THP-1
Cytokine Suppression (%)
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
10^5
Dexamethasone (nM)
****


CD14+ PBMC
Cytokine Suppression (%)
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
10^5
Dexamethasone (nM)
IL-12p40
IL-18
****

Supplementary material

Divergent roles of interleukin-12p40 and interleukin-18 in severe fibrosing chronic graft-versus-host-disease, immunity, and post-transplant outcomes

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[3]Goethe University, Faculty of Medicine, Institute for Transfusion Medicine and Immunohematology, Frankfurt a.M., Germany.

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Running title:

IL-12p40 and IL-18 in Fibrosing cGvHD

Supplementary Methods:

Treatment of cGvHD

As this study was not conducted as a prospective interventional trial, treatment of cGvHD was not standardized. Mild cases were primarily managed with topical corticosteroids. Progressive or severe disease was treated with systemic corticosteroids (initial dose approximately 0.5 mg/kg), typically in combination with calcineurin inhibitors (cyclosporine or tacrolimus) to reduce steroid exposure. Organ-specific management included montelukast and azithromycin for severe pulmonary cGvHD, low-dose methotrexate (10 mg/week, subcutaneous) for sclerodermatous manifestations, and extracorporeal photopheresis in refractory cases.

Flow cytometry

We selected 200 PBMC samples collected at the same time point as the serum samples used for IFNγ–related cytokine measurements, equally representing IL-12p40/IL-18 expression above and below the median. PBMCs were collected weekly post-alloHSCT and enriched using Ficoll separation in parallel with the collection of serum samples described above. Samples were cryopreserved in liquid nitrogen until processing. After thawing, samples were washed in PBS, and 2×10^6 PBMCs per 100 µL were stained with a prepared antibody master mix, including TruStain FcX (Biolegend, Cat. No. 422302) and viability dye, for 30 minutes at 4 °C. Patient characteristics and antibody details are listed in **supplemental Table 3 and 4**. Flow cytometry was performed on a BD FACSymphony A3, and gating was conducted using Kaluza analysis software (Beckman Coulter, Inc.). The gating started by removing dead cells and selecting singlets; immune cells were then identified by their CD45 positivity. An overview of the gating strategy is shown in **supplemental Figure 6**. Electronically documented numbers of mononuclear cells per µL from day 180 were available and determined by differential leukocyte count. **Supplementary Figure and Table legends**

Cell culture and Stimulation

THP-1 cells (ATCC TIB-202) and CD14+ PBMC from healthy donors were cultured in RPMI-1640 medium with 10% FBS, 1% Pen-Strep, and 2 mM L-glutamine at 37 °C, 5% CO_2. PBMC were isolated using CD14+ Positive Selection Beads (Miltenyi Biotec) after standard Ficoll separation. Cells (0.5×10^6/mL) were plated in 24-well plates and stimulated with LPS (1 µg/mL, Sigma-Aldrich) for cytokine secretion. Dexamethasone (Sigma-Aldrich) was added at designated concentrations to assess cytokine suppression in response to corticosteroids. Controls received vehicle (PBS). After 24 hours, supernatants were collected for cytokine quantification by ELISA.

Statistical analysis (extended)

For univariable analyses, cytokine concentrations and immune cell counts were log2-transformed, effect estimates are therefore interpreted per doubling increase. Severe fibrosing cGvHD was modeled using logistic regression. Overall survival (all-cause mortality) was analyzed using parametric Weibull proportional-hazards models. Relapse and NRM were treated as competing risks using cause-specific Weibull proportional-hazards models. Cause-specific hazard ratios (csHR) were estimated, censoring individuals at the time of the competing event. An analogous analysis was performed with the immune cell populations.

Multivariable Cox proportional-hazards regression models were constructed for overall survival, relapse, and NRM. These models included IL-12p40 and IL-18 (log2-transformed) and were adjusted for predefined clinical covariates: age, disease stage, diagnosis, donor type, methotrexate exposure, anti-thymocyte globulin (ATG) prophylaxis, donor and recipient sex, stem-cell source, and cGvHD as a time-dependent covariate.

Multiple hypothesis testing was controlled using the Benjamini–Hochberg false discovery rate (FDR) method. All statistical analyses were performed in R version 4.3.3 using the packages SurvRegCensCov (v1.7), survival (v3.8), and tidyverse (v2.0).

Supplementary Figure 1. Study flowchart and cohort definition

1625 patients were assessed for eligibility. Of these, 1073 were excluded due to lack of available serum samples (n=669), death before day 180 (n=276), relapse before day 100 (n=64), steroid-refractory acute graft-versus-host disease (aGvHD; n=64), or loss to follow-up (n=29). The final study cohort comprised 552 patients with available serum samples within 7 days before cGvHD onset or within ±7 days 180 post-transplant. Patients were stratified into a cGvHD group (n=294) and a control group without cGvHD (n=258). Serum samples from all patients were analyzed by ELISA, and peripheral blood mononuclear cells (PBMCs) were available for flow cytometric analysis in a subset of patients (cGvHD: n=110; control: n=90).

Supplemental Figure 2. Association between cGvHD onset and year of alloHSCT timing with cytokine levels.

Correlation between time to cGvHD onset after alloHSCT (A) or year of alloHSCT (B) and serum concentrations of IL-18 and IL-12p40 in patients with cGvHD. Cytokine concentrations are shown on a log2 scale. Blue lines indicate linear regression fits with 95% confidence intervals. Spearman correlation coefficients (ρ) and corresponding p values are shown. No significant association was observed between time to cGvHD onset and either cytokine, suggesting that cytokine levels were not primarily driven by variability in sampling time.

Supplementary Figure 3. IL-12p40 and IL-18 levels according to severe fibrosing cGvHD status.

Comparison of serum IL-12p40 (left) and IL-18 (right) levels in patients with cGvHD, (n = 278) stratified according to the presence of severe fibrosing cGvHD (n = 52) versus mild cGvHD (n = 226).

Supplemental Figure 4. Cause-specific Cox model analysis of ATG and IL-12p40 associations with relapse risk

Forest plot showing hazard ratios and 95% confidence intervals for relapse in Cox models including ATG alone, ATG adjusted for IL-12p40, and IL-12p40 alone. Addition of IL-12p40 significantly improved model fit compared with the ATG-only model, as assessed by likelihood ratio testing (LRT $p<0.001$; $\Delta\chi^2=22.34$), and substantially changed the ATG regression coefficient ($\Delta\beta=-83.7\%$). These findings suggest that the apparent association between ATG and reduced relapse risk was model-dependent and largely explained by IL-12p40.

Supplemental Figure 5. Sensitivity analyses of IL-12p40 and IL-18 associations with patient outcomes according to cGvHD sampling time.

Multivariable Cox regression analyses assessing the association of IL-12p40 and IL-18 with overall mortality, non-relapse mortality (NRM), and relapse after stratification of patients with cGvHD according to sampling time. Analyses were performed separately for patients sampled between days

150 and 210 post-transplant (A-C) and for patients sampled outside this interval (<day 150 or >day 210, D-F). Models were adjusted for age, disease type, disease stage, donor sex, HLA mismatch, stem-cell source, ATG treatment, MTX treatment, cGvHD as a time-dependent covariate, year of alloHSCT, and pravastatin plus ursodeoxycholic acid (UDCA) prophylaxis. Hazard ratios (HR) are shown for overall mortality, and cause-specific hazard ratios (csHR) are shown for NRM and relapse, with relapse and death treated as competing risks, respectively. Error bars indicate 95% confidence intervals. Across both sampling strata, IL-12p40 and IL-18 retained the direction and significance of their main outcome associations, supporting the robustness of the cytokine-outcome relationships. P values are indicated as: P<0.05, *P<0.01, **P<0.001.

Supplementary Figure 6. Flow cytometry gating strategy for immune cell populations.

Gating strategy used to identify immune cell subsets. Dead cells were excluded and singlets were selected, followed by gating on CD45+ immune cells. Basic immune populations were identified, including T cells, B cells, NK cells, monocytes, and dendritic cells (DCs). Within the T-cell compartment, CD4+, CD8+, double-negative (DN), and double-positive (DP) T cells were defined, and T-cell differentiation states were further characterized as naïve, central memory, effector memory (EM), and effector memory CD45RA+ (EMRA). Absolute cell counts per µL were calculated by combining differential leukocyte counts with the relative frequency of each subset. These basic populations were analyzed in relation to clinical outcomes. In addition, an extended gating strategy comprising 75 immunophenotypes—primarily within the T-cell compartment—was applied to explore associations with immune reconstitution markers. These extended phenotypes were analyzed only in correlation with IL-12p40 and IL-18. The gating strategy for the basic populations and representative examples of the extended phenotypes are shown.

Supplementary Table 1. Criteria for severe cGvHD adapted from NIH consensus criteria.
Organ-specific criteria used to classify severe fibrosing and severe gastrointestinal cGvHD in this study. Severe cGvHD was defined according to NIH-based organ severity criteria, including sclerotic skin involvement, severe ocular, oral, genital, musculoskeletal, pulmonary or gastrointestinal manifestations. Severe gastrointestinal cGvHD was defined as weight loss >15% of body weight.

Supplementary Table 2. Detailed organ involvement among patients classified as having severe cGvHD.

Patient-level summary of organ involvement in all patients classified as having severe cGvHD (n=68), including severe fibrosing cGvHD and severe gastrointestinal cGvHD. The table lists organs involved during non-severe cGvHD and the organ manifestation defining progression to severe disease.

Supplementary Table 3. Clinical characteristics of flow cytometry cohorts.

Summary of clinical characteristics for patients in the flow cytometry analysis.

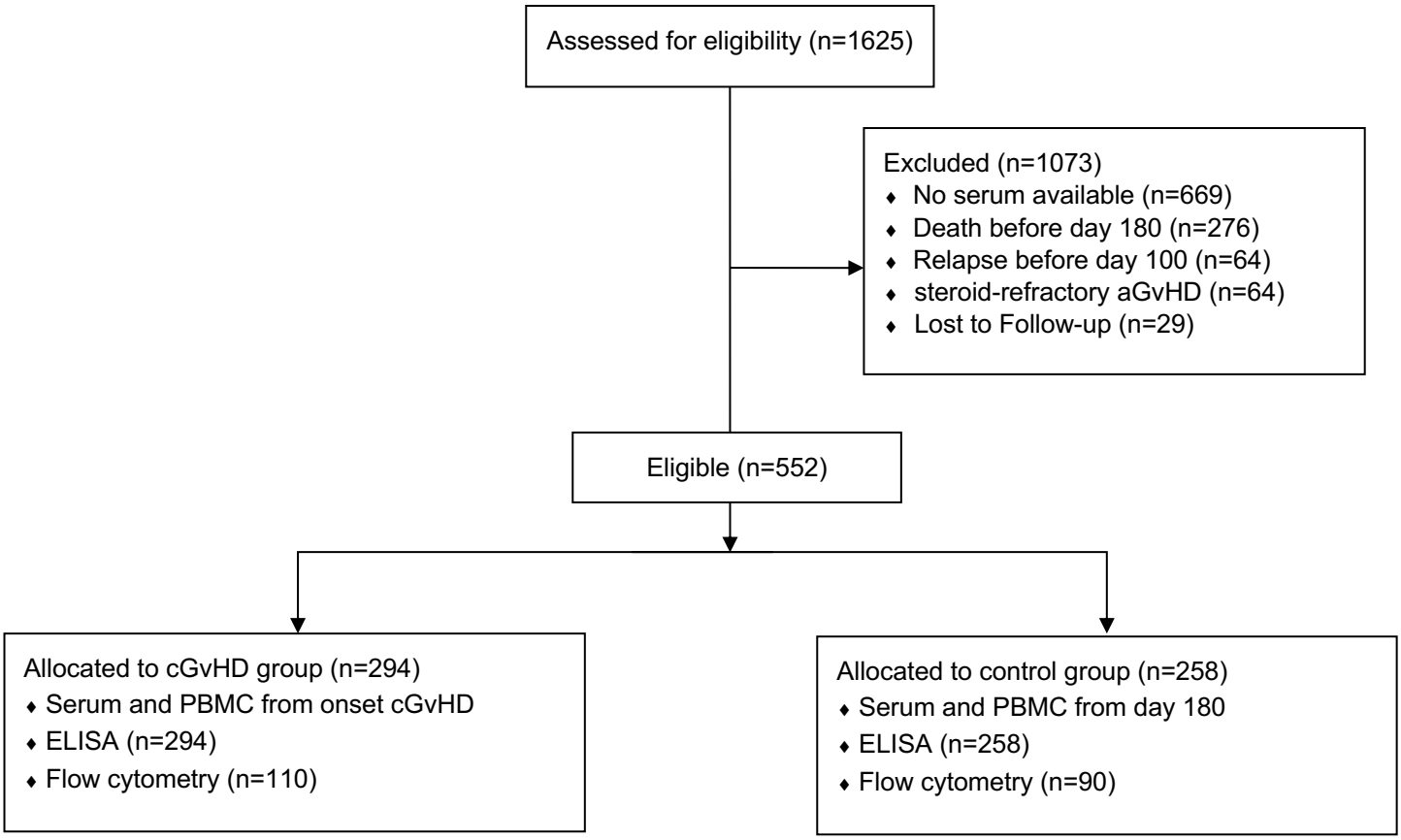
Supplementary Table 4. Antibody panel information.

Details of all antibodies used in the study, including target, clone, fluorochrome, and source.

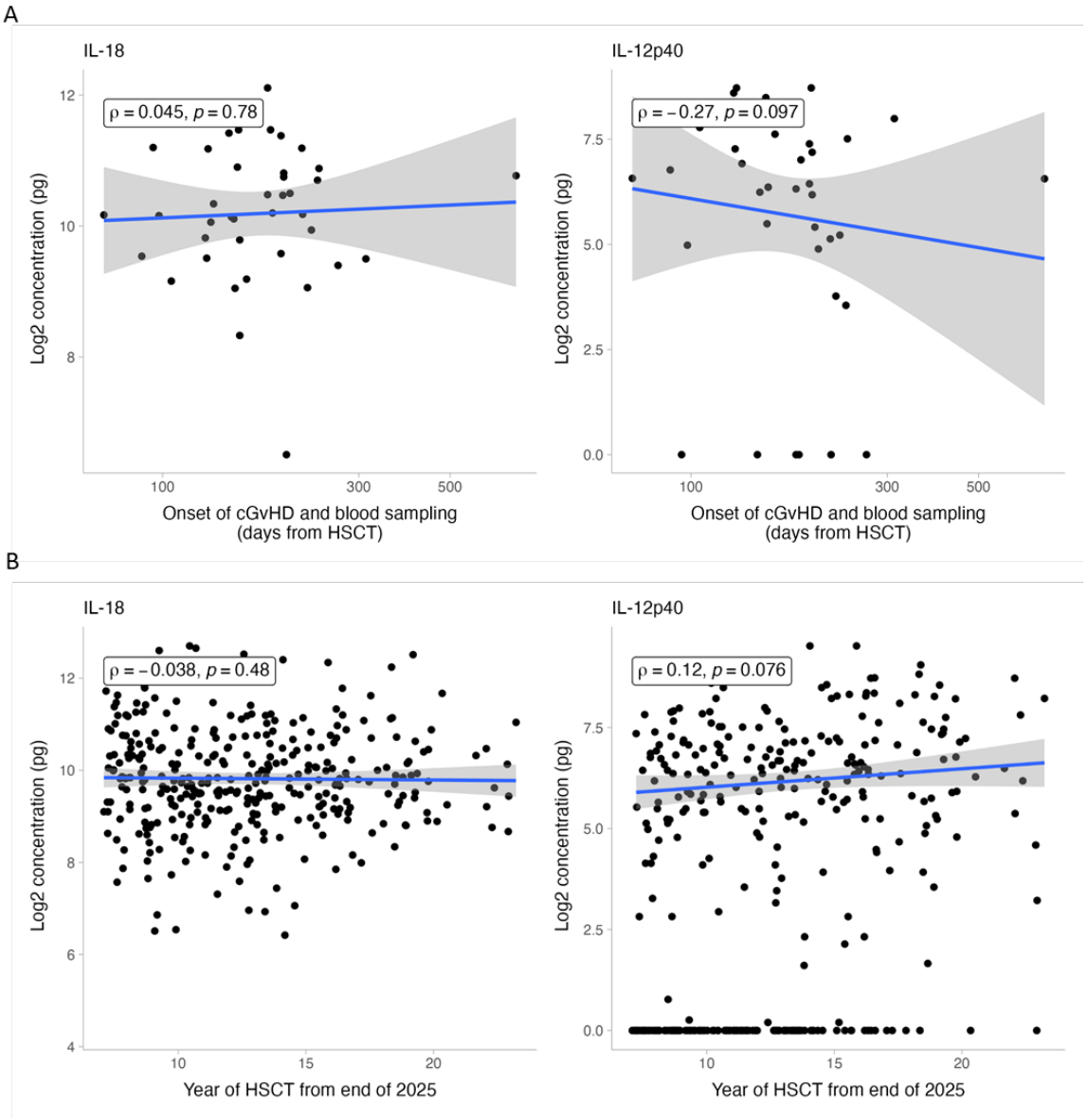
Supplementary Table 5. Patient numbers for cytokine measurements.

Availability of cytokine measurements across the full study cohort and according to the clinical end points (NRM, relapse, fibrosing cGVHD, and overall mortality) used for the analyses shown in this manuscript.

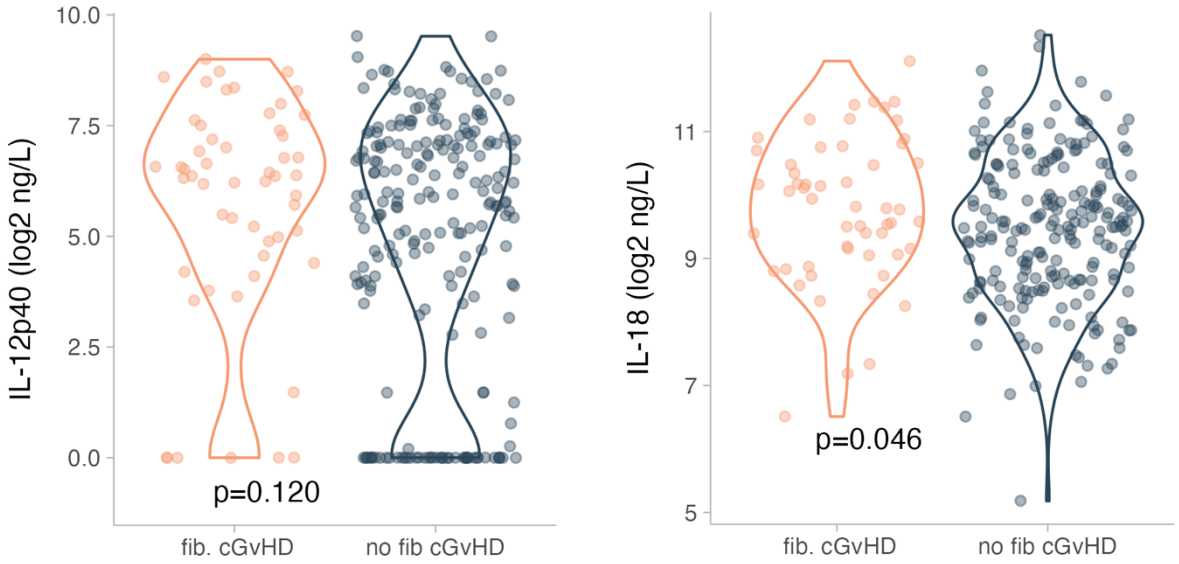
Supplementary Figure 1. Flow Diagram



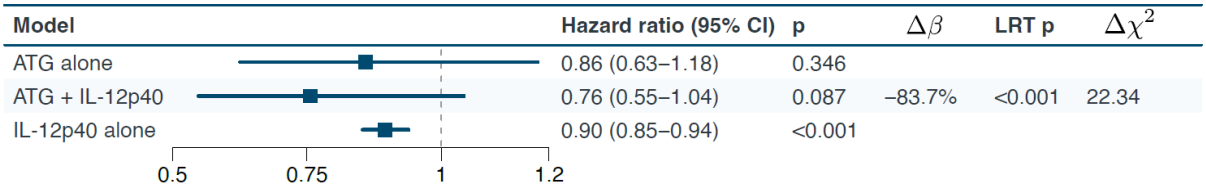
Supplementary Figure 2. Association between cGvHD onset and year of alloHSCT timing with cytokine levels.



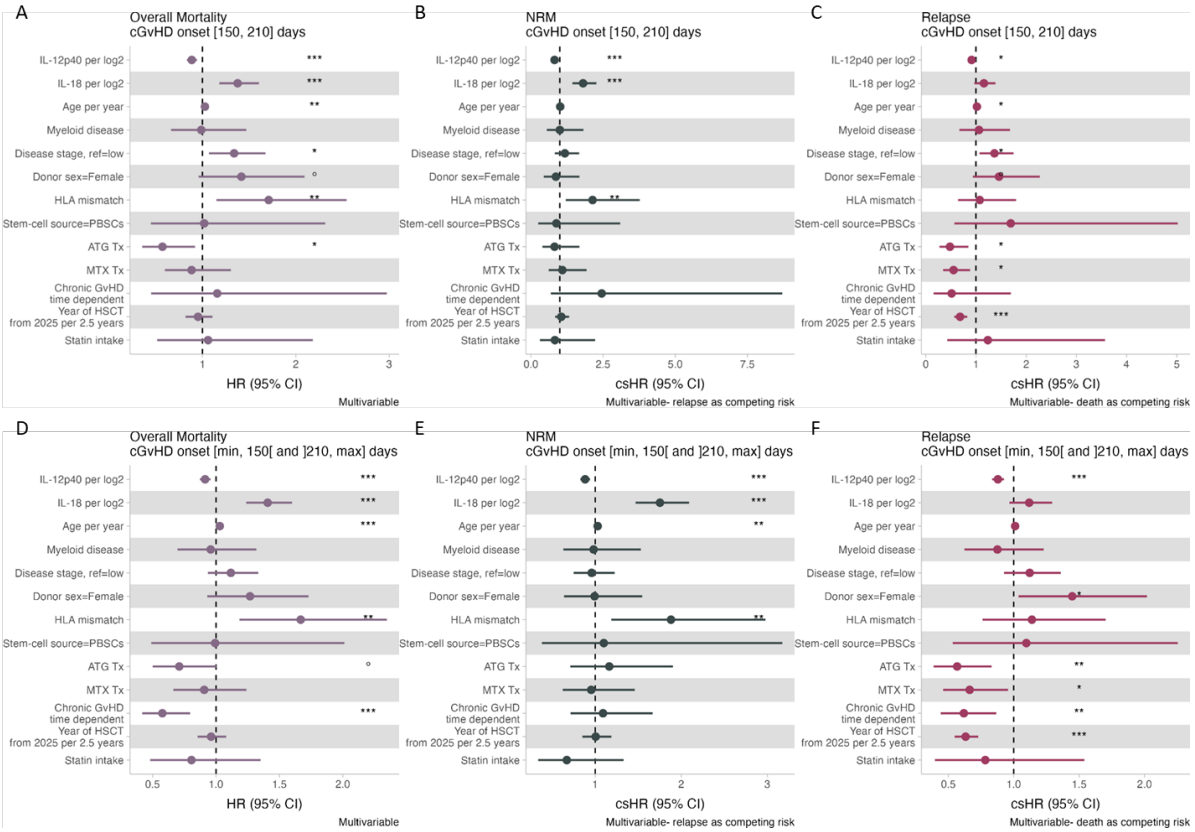
Supplementary Figure 3. IL-12p40 and IL-18 levels according to severe fibrosing cGvHD status.



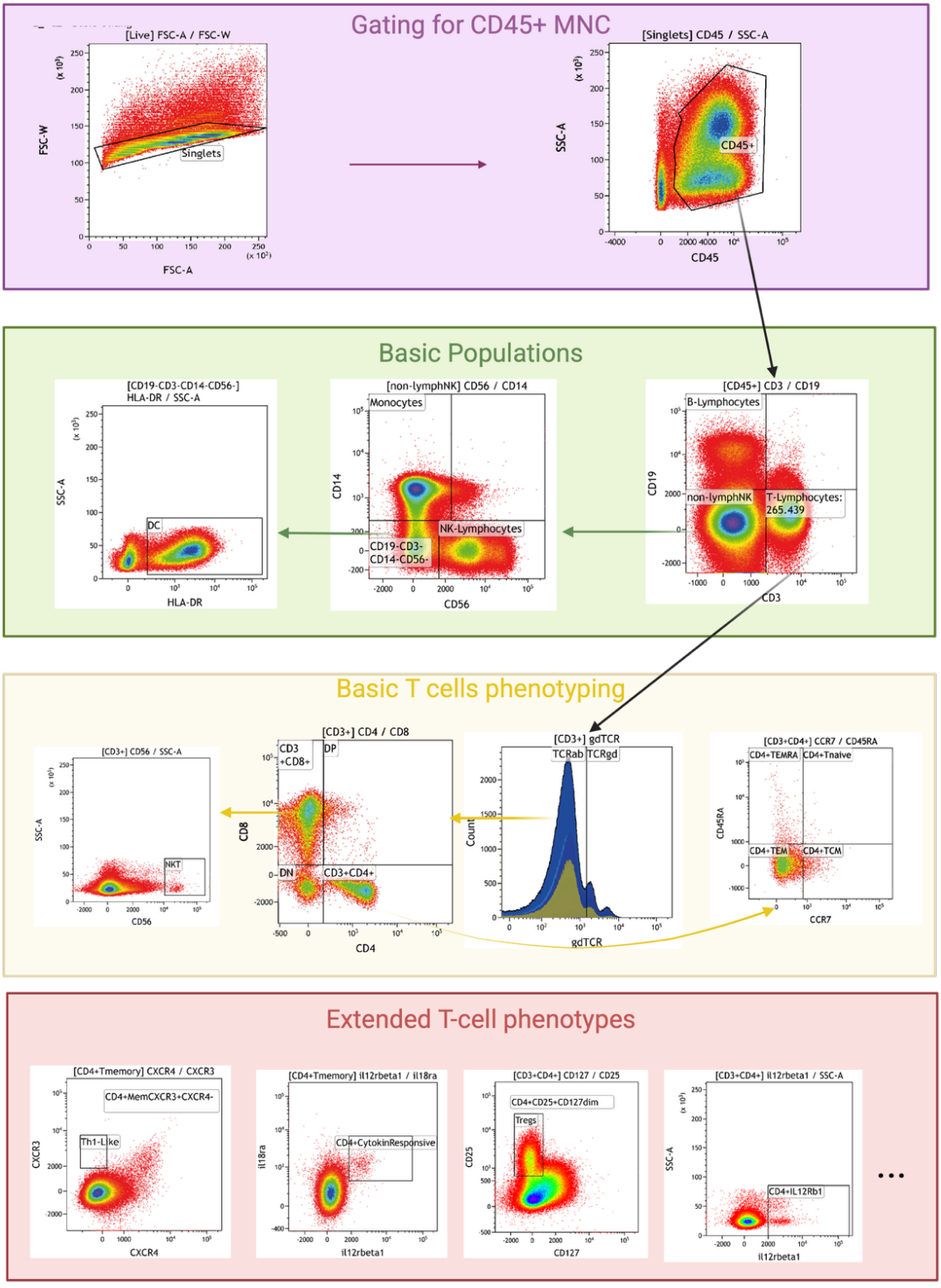
Supplementary Figure 4. Cause-specific Cox model analysis of ATG and IL-12p40 associations with relapse risk



Supplemental Figure 5. Sensitivity analyses of IL-12p40 and IL-18 associations with patient outcomes according to cGvHD sampling time.



Supplementary Figure 6. Gating strategy of flow cytometry analyses



Supplementary Table 1. Criteria of severe cGvHD adapted from the NIH consensus criteria.

Organ system	Criteria of severe cGvHD
Skin	Sclerosis
Eyes	Loss of vision, inability to work, requirement of special eye wear
Mouth	Major limitation of oral intake
Gastrointestinal tract	Weight loss > 15% of body weight
Lung	Dyspnea after walking of flat ground; FEV1<60%
Liver	Bilirubin or liver enzymes > 5xULN plus biopsy
Genitalia	Strictures, ulcerations and severe pain
Muscles/joints	Contractures

Abbreviations: ULN = upper limit of normal; FEV1 = forced expiratory volume in one second.

Supplementary Table 2. Detailed organ involvement of cGvHD cases with classified as severe (n=68)

Patient ID	cGVHD grade	organs in non-severe cGVHD	organ defining severity
1	severe fibrosing	e;l;ser	e;lung
2	severe fibrosing	e;l;m;s	s
3	severe fibrosing	lung;s	lung
4	severe fibrosing	lung;s	lung
5	severe fibrosing	l;lung	lung
6	severe fibrosing	l;m	m
7	severe fibrosing	l;s	s
8	severe fibrosing	s	s
9	severe fibrosing	s	s
10	severe fibrosing	j;l;lung;m;s	lung;m;s
11	severe fibrosing	lung	lung
12	severe fibrosing	l;lung	lung
13	severe fibrosing	e;s	s
14	severe fibrosing	g;lung;m	lung
15	severe fibrosing	e;gi;l	e;j
16	severe fibrosing	m	lung
17	severe fibrosing	e;gi;l;s	lung;s
18	severe fibrosing	e;l;m	s
19	severe fibrosing	s	s
20	severe fibrosing	lung;s	lung;s
21	severe fibrosing	l;s	lung;s
22	severe fibrosing	gi;l;lung	lung
23	severe fibrosing	gi;l	lung
24	severe fibrosing	l;m;s	m;s
25	severe fibrosing	e;l;m;s	s

26	severe fibrosing	g;m;s	g
27	severe fibrosing	l;lung;s	lung
28	severe fibrosing	m;s	s
29	severe fibrosing	m;s	s
30	severe fibrosing	e	lung
31	severe fibrosing	gi;m;s	s
32	severe fibrosing	m;s	s
33	severe fibrosing	m;s	s
34	severe fibrosing	l;s	s
35	severe fibrosing	m;s	s
36	severe fibrosing	gi;j;m;s	f;j;s
37	severe fibrosing	s	lung;s
38	severe fibrosing	e;l;lung;s	lung;s
39	severe fibrosing	e;lung	lung
40	severe fibrosing	e;gi;s	s
41	severe fibrosing	lung	lung
42	severe fibrosing	l;m	m
43	severe fibrosing	s	s
44	severe fibrosing	s	s
45	severe fibrosing	lung	lung
46	severe fibrosing	m;s	s
47	severe fibrosing	gi;l;lung;s	lung
48	severe fibrosing	m	m
49	severe fibrosing	l;lung;s	lung
50	severe fibrosing	gi;lung;m;s	lung
51	severe fibrosing	e;f;j;lung;m;s	s
52	severe fibrosing	f;j	j
53	severe gastrointestinal	m	gi
54	severe gastrointestinal	s	gi
55	severe gastrointestinal	m;s	gi
56	severe gastrointestinal	e;m;s	gi
57	severe gastrointestinal	e;m;s	gi
58	severe gastrointestinal	m;s	gi
59	severe gastrointestinal	l;m;s	gi
60	severe gastrointestinal	l;m;s	gi
61	severe gastrointestinal	gi	gi
62	severe gastrointestinal	gi	gi
63	severe gastrointestinal	gi;s	gi
64	severe gastrointestinal	gi;l;m	gi
65	severe gastrointestinal	l	gi
66	severe gastrointestinal	gi;m	gi
67	severe gastrointestinal	gi;s	gi
68	severe gastrointestinal	gi	gi

Abbreviations: e= eyes; m= mucosals; s= skin; l= liver; gi= gastrointestinal tract; j= joints/musculoskeletal; f= fasciae; cns= central nervous system; lung= lung; ser= serosal involvement; g= genital.

Supplementary Table 3. Patients' characteristics of flow cytometry cohorts.

	no. of patients: n = 200 (100%)
Median age [range], years	56.4 [19-75]
Male sex	118 (59.0)
Underlying hematological disease:	
Acute myeloid leukemia	88 (44.0)
Lymphatic/lymphoblastic neoplasias	46 (23.0)
Myeloproliferative/	48 (24.0)
myelodysplastic disorders	18 (9.0)
Multiple myeloma	
Donor type:	
matched related	50 (25.0)
matched unrelated	117 (58.5)
mismatched unrelated	23 (11.5)
haplo-identical	10 (5.0)
Donor sex male	136 (68.0)
Stem cell source:	
peripheral blood stem cells	189 (94.5)
bone marrow	11 (5.5)
Conditioning regimen:	
reduced intensity	146 (73.0)
myeloablative	54 (27.0)
First-line immunosuppression:	
anti-thymocyte globulin	133 (66.5)
methotrexate days 1,3,6	130 (65.0)
Statin+UDCA prophylaxis	197 (98.5)
Acute GVHD:	
no aGVHD	113 (56.5)
grade 1-2 aGVHD	81 (40.5)
grade 3-4 aGVHD	0 (0)
Chronic GVHD:	
no cGVHD	90 (45.0)
mild cGVHD	76 (38.0)
severe fibrosing cGVHD	34 (17.0)
severe gastrointestinal cGVHD	0 (0)

Abbreviations: UDCA – ursodeoxycholic acid

Supplementary Table 4. Antibodies.

Antigen	Conjugate	Clone	Cat. No.	Vendor
CCR7	BV421	150503	562555	BD Biosciences
CD40L	BV510	TRAP1	744114	BD Biosciences
Fixable Viability Stain 575V	575V		565694	BD Biosciences
CD14	BV605	M5E2	564055	BD Biosciences
CD56	BV650	NCAM16.2	564057	BD Biosciences
CD127	BV711	HIL-7R-M21	563165	BD Biosciences
CD3	BV750	SK7	747058	BD Biosciences
CD45RA	BV786	HI100	563670	BD Biosciences
CD57	BB515	NK-1	565285	BD Biosciences
TCR γδ	PE-CF594	B1	562511	BD Biosciences
CD25	PE-Cy7	M-A251	557741	BD Biosciences
CTLA-4	PE-Cy5	BNI3	555854	BD Biosciences
HLA-DR	APC-R700	G46-6	565127	BD Biosciences
CD8	BUV395	RPA-T8	563795	BD Biosciences
CD4	BUV496	SK3	612936	BD Biosciences
CD19	BUV563	SJ25C1	612916	BD Biosciences
IL12RB1	BUV615	2.4E6	752317	BD Biosciences
CXCR4	BUV563	12G5	741400	BD Biosciences
CXCR3	BUV661	1C6	741649	BD Biosciences
IL18R	APC	REA1095	130-118-582	Miltenyi Biotec
CD27	BUV737	L128	612829	BD Biosciences
CD45	BUV805	H30	612891	BD Biosciences
GPR65	PE	CG4.rMAb	567212	BD Biosciences
TCR δ1	APC-R700	11F2	657707	BD Biosciences
TCR Vδ2	BUV615	B6	751368	BD Biosciences
CD86	APC	2331	555660	BD Biosciences
CD83	PE-Cy5	HB15e	551058	BD Biosciences
TCR Vγ9	BV510	B3	744035	BD Biosciences

Supplementary Table 5. Patient numbers for cytokine measurements.

Variable	Outcome	N total	N events
IL12p40	NRM	549	75
	Relapse	549	113
	fcGVHD	544	52
	OM	546	146
IL-18	NRM	544	74
	Relapse	544	112
	fcGVHD	539	52
	OM	541	144
CXCL9	NRM	545	75
	Relapse	545	112
	fcGVHD	540	52
	OM	542	145
CXCL10	NRM	299	51
	Relapse	299	50
	fcGVHD	297	28
	OM	299	82
IFNγ	NRM	348	61
	Relapse	348	60
	fcGVHD	346	37
	OM	348	102
TNFα	NRM	348	61
	Relapse	348	60
	fcGVHD	346	37
	OM	348	102
IL-1β	NRM	348	61
	Relapse	348	60
	fcGVHD	346	37
	OM	348	102
EBI3	NRM	338	56
	Relapse	338	59
	fcGVHD	336	34
	OM	338	96
Activin	NRM	317	54
	Relapse	317	52
	fcGVHD	315	30
	OM	317	87
IL-17	NRM	308	51
	Relapse	308	52
	fcGVHD	306	30
	OM	308	84
L-GH	NRM	347	61
	Relapse	347	60
	fcGVHD	345	37

	OM	347	102
Follistatin	NRM	341	57
	Relapse	341	60
	fcGVHD	339	34
	OM	341	98
IFNα	NRM	337	57
	Relapse	337	59
	fcGVHD	335	35
	OM	337	97
IL-23	NRM	265	42
	Relapse	265	42
	fcGVHD	264	21
	OM	265	68