Anti-Apoptotic Molecule BCL2 Is a Therapeutic Target in Steroid-Refractory Graft-Versus-Host Disease

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Graft-versus-host disease (GVHD) is the leading cause of mortality after hematopoietic stem cell transplantation and primarily affects barrier organs such as the skin. One-third of cases are refractory to steroid treatment resulting in poor outcomes and the need for novel therapies. Longitudinal analysis of T-cell transcriptomes in patients before the appearance of GVHD symptoms revealed the upregulation of anti-apoptotic regulator B-cell lymphoma 2 (BCL2) at GVHD initiation. To determine the potential of BCL2 inhibition in active GVHD, we analyzed tissues of 88 patients with acute or chronic GVHD. BCL2 RNA was elevated in multiple organs affected by GVHD and expression correlated with transplant-related mortality and steroid-refractory GVHD. BCL2-expressing lymphocytes were present in skin lesions and peripheral blood of patients with acute and chronic GVHD. Inhibition of BCL2 increased the CD4 to CD8 ratio in allogeneic T cells in vitro and induced apoptosis of T cells from patients with steroid-pretreated chronic GVHD ex vivo. In addition, the higher ratio of regulatory to nonregulatory T cells upon blockage of BCL2 could add to the anti-inflammatory effect of BCL2 blockage. Collectively, our results highlight BCL2 as an important factor for GVHD development and introduce BCL2 inhibition as previously unreported and urgently needed targeted therapy in the treatment of steroid-refractory GVHD.

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is a potentially curative but high-risk therapy used to treat hematological malignancies (Chhabra et al., 2018). Depending on various factors (e.g., patient-donor HLA-compatibility), 30–80% of graft recipients develop acute graft-versus-host disease (aGVHD) or chronic graft-versus-host disease (cGVHD), leading to a mortality rate of up to 30% (Flowers et al., 2011; Zeiser et al., 2015). aGVHD is most pronounced in barrier tissues such as the skin and gastrointestinal (GI) tract, whereas cGVHD may also affect eyes, oral and genital mucosa, salivary glands, fascia, or lungs. In aGVHD, immunocompetent donor-derived T cells mediate tissue destruction in host barrier organs via the release of pro-inflammatory cytokines, including IL-1β, IL-6, IL-17, and IFNγ (Kumar et al., 2017; Wang et al., 2009; Yi et al., 2009). In cGVHD, alloreactive T cells and B cells are supposedly responsible for chronic inflammation and fibrosis (Srinivasan et al., 2012), resulting in highly polymorphic disease manifestations, most commonly presenting as lichenoid or sclerodermiform skin features.

When graft-versus-host disease (GVHD) develops despite prophylaxis with immunosuppressive reagents, systemic glucocorticosteroids are recommended as first-line therapy (Martin et al., 2012). However, about one-third of patients with aGVHD are refractory to initial treatment with steroids (steroid-refractory GVHD [SR-GVHD]), resulting in poor outcomes with an estimated 2-year overall survival of <20% (Munneke et al., 2016). Less than 50% of patients respond to the treatment with complete remission, and there is concern about broad immunosuppression risking infections and minimizing graft-versus-leukemia effects, raising the need for more specific alternatives.

In this study, we investigate expression profiles of T cells early in GVHD development to define new vulnerabilities exploitable for treatment. We explore the role of the anti-apoptotic protein BCL2 in skin and other organs affected by GVHD, providing a rationale for a clinical trial targeting BCL2 in high-risk patients with SR-GVHD.

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Abbreviations: aGVHD, acute graft-versus-host disease; BCL2, B-cell lymphoma 2; cGVHD, chronic graft-versus-host disease; GI, gastrointestinal; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; SR, steroid-refractory; Tconv, conventional T cells; Treg, regulatory T cells

Received 23 October 2019; revised 11 February 2020; accepted 23 February 2020; accepted manuscript published online 2 April 2020; corrected proof published online 28 April 2020
RESULTS
Distinct transcriptional profiles of T cells from patients developing GVHD after HSCT
T cells are instrumental for the development and exacerbation of GVHD (Brüggen et al., 2014). To investigate the transcriptional regulation of T cells longitudinally in the course of HSCT, we isolated peripheral blood T cells at four predefined time points before and after HSCT (cohort 1, Supplementary Figure S1) and performed RNA-sequencing (Figure 1a). We next stratified patients according to signs of aGVHD after day +14, GVHD developers and patients who did not show symptoms of GVHD (no GVHD). Despite low inter-donor variability (Supplementary Figure S2a), principal component analysis and hierarchical clustering with individual donors revealed the clustering of T cells from patients of the no GVHD and GVHD developer groups (Figure 1b). Mean expression values of donors allowed for clear distinction of GVHD developer and no GVHD T cells, both before GVHD development (day +14) and after GVHD treatment (day +100) (Supplementary Figure S2b). Clustering is explained by statistical differences in mean gene expression (by G-test) between those groups, 36 apoptosis-related genes with the highest log-fold change (log-fold change > 2 and false discovery rate < 0.05) are shown as a heatmap (Figure 1c). We next identified 10 genes with continuous upregulation in patients with GVHD at day +14 and day +100 (Figure 1d and e). Among those dysregulated genes, several belong to or interact with the BCL2 apoptosis pathway, including PMAIP1, CASP9, and anti-apoptotic regulator BCL2, which has recently been emerging as a therapeutic target in lymphoproliferative diseases (Valentin et al., 2018). Further analysis of time-course dependent regulation of pro- and anti-apoptotic genes revealed that several BCL2-pathway members were upregulated on the day of transplantation (day 0) and, BCL2 and PMAIP1 remained upregulated in patients developing GVHD (Supplementary Figure S2c).

BCL2 mRNA expression is elevated in tissue samples of patients with GVHD
To directly examine the levels of BCL2 RNA in GVHD-affected tissues, we performed qPCR analysis of organs with histopathologically confirmed aGVHD (cohort 2) and cGVHD (cohort 3) (Figure 2) (Supplementary Figure S2d). Because BCL2 RNA is ubiquitously expressed in noninflamed human barrier tissues and by PBMCs (Thul et al., 2017; Uhlén et al., 2015), we compared mRNA levels with noninflamed controls of the respective organ. Expression of BCL2 mRNA was significantly elevated in PBMCs of patients with GVHD, as well as in aGVHD and cGVHD of the skin and aGVHD of the GI tract; a trend of elevated BCL2 RNA was also found in cGVHD of liver and lung (Figures 2a and b) (Supplementary Figure S2d). To further assess the specificity of BCL2 expression in GVHD, we compared mRNA levels of histologically confirmed GVHD of the GI tract with non-GVHD-related inflammation after HSCT (chemical-toxic GI inflammation or infections). We found significantly higher levels of BCL2 in GI GVHD compared with non-GVHD inflammation of the GI tract (Figure 2c, S2d), indicating that measurement of BCL2 mRNA level could be helpful in the diagnosis of gastrointestinal inflammation after HSCT.

High BCL2 expression at the time of diagnosis is associated with poor prognosis
To determine the impact of our findings on the clinical course of GVHD, we correlated BCL2 mRNA levels to patient characteristics, response to steroid treatment, and overall outcome of cohorts 2 and 3 (Figure 2d–i) (Supplementary Figures S3 and S4). Expression levels of BCL2 did not depend on the type of conditioning, underlying disease, GVHD grade, or time to GVHD onset, and we observed no significant differences depending on age (Supplementary Figure S3). Interestingly, high BCL2 mRNA levels in cutaneous GVHD at the time point of diagnosis coincided with SR-GVHD (Figure 2f). The trend of BCL2 RNA expression correlating with SR-GVHD was also observed in intestinal and PBMC samples but did not reach a level of significance (Figures 2d and g). However, evaluating BCL2 on protein level revealed higher frequencies of BCL2+ leukocytes and BCL2+ CD8+ T cells in the blood of patients with SR-GVHD (Figure 2e). No difference between responder groups was observed in BCL2+ total T cells and BCL2+ CD4+ T cells (Supplementary Figures S4c and d). We further observed a negative association of BCL2 expression in PBMC of patients from cohorts 2 and 3 to survival after HSCT (Figure 2h). Patients who died of GVHD-related complications had higher levels of BCL2 at the time of GVHD occurrence than patients in whom GVHD eventually healed. The receiver operating characteristic—analysis yielded an area under the curve of 0.925 with a 95% DeLong confidence interval (0.7614, 0.9251). With a cutoff of 0.91 for BCL2 expression in PBMC, specificity was 1 (all eight survivors were classified correctly) and sensitivity was 0.8, with 1 of 5 deceased patients misclassified (Supplementary Figures S4a and b). Two patients were excluded from analysis because of leukemia relapse. Kaplan-Meier curves were plotted for patients with levels above and below the cutoff, and a log-rank test was performed, identifying a higher hazard for patients with elevated BCL2 expression (P = 0.042) (Figure 2i). Our data indicate that a low BCL2 expression level at the onset of GVHD is predictive of a better outcome and patient survival in this limited patient cohort.

BCL2+ circulating leukocytes are expanded in the peripheral blood of patients with GVHD
In concordance with previous studies on PBMC at GVHD diagnosis (Zeiser and Blazar, 2017), we found a relative increase of T cells in aGVHD and T and B lymphocytes in cGVHD compared with individuals that did not develop GVHD (Figures 3a and b) (Supplementary Figure S4e). Notably, we detected higher percentages of BCL2+ total leukocytes in patients with aGVHD versus individuals who were GVHD-free after HSCT (Figure 3c). The upregulation of BCL2 in total PBMC was due to increased frequencies of BCL2+ cells among CD4+ (cGVHD) and CD8+ T cells and NK T cells (aGVHD). In contrast, BCL2-expressing B cells, NK cells, and monocytes of the peripheral blood from patients with GVHD were found in similar or even lower amounts compared with patients without GVHD (Figure 3c).

Exclusive upregulation of BCL2 protein in cells mediating acute and chronic cutaneous GVHD
To study the pathogenicity of BCL2-expressing cells for GVHD-affected tissue, we performed an analysis of lesional skin biopsies. Immunostaining of frozen skin sections...
Figure 1. Distinct transcriptional profiles of T cells from patients with and without GVHD development. (a) Study design: patients of cohort 1 stratified in two groups: GVHDdev (n = 5), the appearance of GVHD symptoms after day +14 and no GVHD (n = 6). (b) Principal component analysis and hierarchical clustering of T cells from GVHDdev (n = 5) and no GVHD patients (n = 6) at days 14 and 100 after HSCT. Each data point represents one patient time point (mean values; Supplementary Figure S2a). (c) Heat map of 36 apoptosis-related genes with mean expression >2-fold (false discovery rate [FDR] P < 0.05) in GVHDdev (n = 5) versus no GVHD (n = 6). (d, e) Scatterplot of genes from (c) according to the expression on day +14 days (d) and day +100 (e). Genes highlighted in red are continuously upregulated in GVHDdev at both timepoints. GVHD, graft-versus-host disease; GVHDdev, GVHD developers; HSCT, hematopoietic stem cell transplantation.
revealed the epidermal and dermal infiltrate to be dominated by CD3⁺ T cells (Figures 4a and b). Although NK T cells and NK cells occurred in aGVHD and cGVHD lesions, B cells were only found in the dermis of cGVHD in sizable numbers (Figure 4b) (Supplementary Table S2d). Generally, BCL2-expressing cells were not elevated in GVHD skin lesions compared with healthy donors (Figure 4c) (Supplementary Figure S5a). Similar levels of BCL2-expressing pan-keratin⁺ cells of the epidermis (keratinocytes) and dermis (glandular cells) were found in cutaneous GVHD and skin from healthy controls (Figure 4c) (Supplementary Figure S5b). However, by quantification of BCL2⁺ leukocyte subsets, we
found significantly more BCL2-expressing T cells, B cells, and NK cells in aGVHD and cGVHD skin lesions (Figures 4b and c) (Supplementary Figures S5a and c). To dissect roles of T cells promoting and regulating GVHD, we next compared levels on BCL2 in regulatory T cells (Treg) to conventional T cells (Tconv). Previous work showed reduced levels of BCL2 in peripheral blood Tregs of patients with non-GVHD after HSCT, arguing that Tregs are more susceptible to apoptosis (Alho et al., 2016). We detected comparable numbers of Treg in GVHD-affected skin and healthy controls (Figure 5a).
Figure 4. Infiltration of BCL2⁺ lymphocytes in aGVHD and cGVHD skin lesions. (a) Cell subsets in cutaneous GVHD lesions of cohorts 2 (n = 18) and 3 (n = 13) and healthy controls (n = 12) as identified by IF of skin cryosections. Data are shown as the mean percentage of DAPI⁺ cells. (b) Representative image of BCL2 and lymphocytes in aGVHD (left) and cGVHD (right) (*, subepidermal blister, Bar [overview] = 100 μm, Bar [detail] = 10 μm). (c) Percentage of BCL2⁺ cells among all cells, pan-cytokeratin⁺ keratinocytes, T cells, B cells, NK cells, NK T cells as identified by IF. One-way ANOVA and unpaired t tests were
Although the absolute number of BCL2-expressing Treg was elevated in GVHD lesions (Figures 5b and d), the proportion of BCL2-expressing Treg was not increased (Figure 5c). We conclude that both pro-inflammatory and regulatory lymphocytes express abundant levels of BCL2 in aGVHD and cGVHD and may be targeted to a similar extent by BCL2 inhibition.

Increased BCL2 to BCL2L1 ratio in GVHD tissue indicates susceptibility to BCL-2 inhibition
Recent reports demonstrate that the BCL2 to BCL2L1 ratio may serve as a predictor of sensitivity to venetoclax superior to BCL2 expression alone (Carrington et al., 2017; Touzeau et al., 2014). Therefore, we quantified BCL2, BCL2L1, and β2M mRNA in previously untreated GVHD-affected tissue. aGVHD skin clearly showed an increased BCL2 to BCL2L1 ratio compared with controls (Figure 6a), supporting the likelihood of successful induction of apoptosis by BCL2 inhibition in GVHD.

BCL2 inhibition reduces cytotoxic T cells in vitro by induction of apoptosis
To test the effect of systemic BCL2 inhibition on alloreactive T-cell responses, we established 6-day active mixed leukocyte reactions of unrelated healthy donors. We then introduced treatment with the BCL2 small molecule inhibitor ABT-199 (venetoclax) after 5 days (Figure 6b). We assessed the dose-dependent effects of ABT-199 treatment in mixed leukocyte reaction and found higher numbers of apoptotic T cells with increasing dosages (Figure 6c). Strikingly, upon treatment with ABT-199 for 24 hours, the CD8⁺ T-cell proportion was significantly reduced in favor of CD4⁺ T cells (Figure 6d). Before ABT-199 treatment, only 20% of CD4⁺ T cells expressed BCL2, while roughly 70% of CD8⁺ T cells showed a positive BCL2 signal (Figure 6e), indicating increased sensitivity of CD8⁺ T cells because of differential BCL2-levels. Thus, BCL2 inhibition may reduce numbers of cytotoxic effector cells in an acute allogeneic setting directly by the induction of apoptosis in cells expressing high levels of BCL2.

Effect of BCL2 inhibition on T cells of patients with chronic SR-GVHD under systemic corticosteroid treatment
Given the importance of steroid response in treatment stratification of GVHD, we compared the effect of BCL2 inhibition in patients with and without response to steroid treatment (cohort 4). We sampled peripheral blood of patients in GVHD remission with the response to steroid treatment (post-GVHD, non-SR) and patients with chronic SR-GVHD receiving systemic corticosteroids at the time of sampling (SR). Interestingly, apoptosis was induced by BCL2-inhibition in both CD4⁺ and CD8⁺ T cells of patients with SR, whereas no changes were observed in healthy non-SR T cells (Figure 6f). Differential response to BCL2 inhibition of those patient subgroups may be explained by higher BCL2 levels in SR-GVHD (Figure 6e). Importantly, BCL2-inhibition also led to an increase in the ratio of Treg to Tconv, commonly considered favorable in the treatment of GVHD (Figure 6g). Therefore, in vitro inhibition of BCL2 exhibits an additive anti-inflammatory effect to steroid treatment in patients with SR-cGVHD.

DISCUSSION
This study reveals the dysregulation of the BCL2 pathway early in GVHD development. We found elevated BCL2 expression in GVHD-affected organs and immune cell
**Figure 6.** In vitro BCL2-inhibition reduces inflammatory alloreactive T cells by apoptosis induction. (a) The ratio of BCL2 to BCL2L1 mRNA expression (normalized to β2M) in cohort 2 skin samples (n = 11) and healthy skin (n = 13). (b) Graphical overview of MLR setup. (c) Percentage of AnnexinV+ cells among CD3+ lymphocytes in 6-day MLR (n = 8) after treatment with ABT-199. (d) Representative scatterplots and the ratio of CD4+ to CD8+ T cells in MLR after ABT-199. (e) Percentage of BCL2+ T cells in day 5 MLR. (f) Apoptotic CD4+ and CD8+ cells of cohort 4 (SR, n = 9; non-SR, n = 10) upon ABT-199; (g) Ratio of Treg to Tconv in cells of cohort 4 (n = 19) after ABT-199. Statistical testing with one-way ANOVA and paired t-test, unpaired t-test for (a, e). Dots represent the mean values of two independent measurements. Data are shown as mean ± SEM. MLR, mixed leukocyte reaction; SR, steroid-refractory; Tconv, conventional T cells; Treg, regulatory T cells;
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subsets to be associated with poor prognosis and SR-GVHD. Moreover, BCL2-inhibition can induce preferential apoptosis in nonregulatory alloreactive T cells of patients with SR-GVHD.

Although several genes were dysregulated preceding GVHD development in our patient cohort, only a few remained upregulated after HSCT. Among those were genes associated with T-cell activation in GVHD (TNFR, LMNA) (Krezdorn et al., 2019; Voskoboinik et al., 2015) and, interestingly, genes regulating apoptosis (including genes of the BCL2 pathway [BCL2, PMAIP1]).

BCL2 is the most prominent member of the BCL2 family and expressed in all human organs, with the highest mRNA and protein levels in barrier tissues and immune cells (Hockenberg et al., 1993; Uhlén et al., 2015). BCL2 is critically involved in immune regulation of the peripheral blood and skin, and its inhibition leads to apoptosis of lymphocytes (Weder et al., 2018). Upregulation of BCL2 expression has been linked to different types of cancers, including B-cell and T-cell malignancies (Otake et al., 2007) and malignant melanoma (D’Aguanno et al., 2018), and the selective BCL2 inhibitor venetoclax (ABT-199) is currently approved as a treatment of chronic lymphocytic and acute myeloid leukemia (DiNardo et al., 2019; Roberts et al., 2016). Furthermore, murine studies are elucidating the effectiveness of BCL2-inhibition in autoimmune conditions (Ottina et al., 2015; Tian et al., 2017; Weder et al., 2018).

In our patient cohorts, BCL2 mRNA expression in PBMC was highly associated with SR-GVHD and death from GVHD, whereas survival probability in patients with BCL2-levels below healthy controls (log-fold change < 0.91) was at 100%. We found differences between BCL2-expressing lymphocyte subsets in acute and chronic cutaneous GVHD and distinct BCL2 mRNA levels in gastrointestinal-GVHD samples and non-GVHD inflammation of the GI tract, pointing toward an application of BCL2 immuno-phenotyping in GVHD diagnosis. Confirmation of these striking observations in a larger cohort will be necessary to determine the value of BCL2 as a diagnostic and prognostic marker.

Circulating T lymphocytes showed upregulation of BCL2 RNA upon conditioning treatment and HSCT. Factors leading to BCL2-upregulation likely include the activation of BCL2-promotor NF-κB (Catz and Johnson, 2001). Preceding NF-κB activation via the canonical pathway, the conserved helix-loop-helix ubiquitous kinase phosphorylates NF-κB-inhibitory IκB proteins. We detected CHUK alongside BCL2 among the top differentially expressed genes in patients developing GVHD, arguing for a driving role of the classical NF-κB pathway in BCL2 upregulation after HSCT. BCL2-dysregulation seems to be of particular importance in the developmental stage of GVHD. We found upregulation in T cells before the appearance of clinical signs, and a recent publication describes beneficial outcomes in murine HSCT after pretreatment inhibition of BCL2 in murine NK cells (Jiao et al., 2019). In conjunction with our findings in human GVHD-affected tissues, BCL2 inhibition may serve both as a conditioning agent reducing activated radioresistant lymphocytes and as therapeutic strategy dampening alloreactive T-cell responses in SR-GVHD.

We first evaluated the ratio of BCL2 to MCL1 in GVHD lesions to estimate response to BCL2 inhibition (Carrington et al., 2017; Touzeau et al., 2014) before illustrating the dose-dependent effect of ABT-199 on alloreactive T cells. Importantly, the effects were already reached at doses lower than plasma levels after standard oral administration (1–30 μM) (FDA, 2016; Jones et al., 2016; Salem et al., 2017). Furthermore, we detected an increase in the ratio of Treg to Tconv after ABT-199 treatment in T cells of patients pretreated with steroid, likely because of lower BCL2-expression in the Treg subset (Alho et al., 2016).

To summarize, we characterize expanded BCL2+ lymphocyte subsets and demonstrate the potential relevance of BCL2 in the diagnosis of aGVHD and cGVHD as a predictive marker of response to first-line treatment and as a target in preventing or treating GVHD. Overall, our findings highlight BCL2-signaling as a promising target to reduce morbidity and mortality in patients with SR-GVHD after HSCT.

MATERIALS AND METHODS

Samples

We sampled adult patients who underwent allogeneic HSCT in four cohorts (Supplementary Figure S1). Punch biopsies of skin were taken and cryopreserved as previously described (Brüggen et al., 2014) or directly digested to single-cell suspension for cell sorting (cohort 1). Lung, liver, and GI tract GVHD samples were obtained during diagnostic testing and were formalin-fixed, and paraffin-embedded (cohorts 2 and 3). The severity of aGVHD and cGVHD were graded according to modified Glucksberg and National Institutes of Health consensus criteria, respectively (Cahn et al., 2005; Moon et al., 2014; Przepiorka et al., 1995; Vigorito et al., 2009). SR-GVHD was determined by nonresponsiveness to glucocorticoid treatment within 2 weeks of diagnosis and initiation of additional treatment (Supplementary Table S1). GI tract—inflammation biopsies were obtained from patients after HSCT presenting with clinical signs of GI tract GVHD in which histopathologic and infectious disease workup revealed chemical-toxic inflammation, viral (human cytomegalovirus), or bacterial (Clostridium difficile) infection. Healthy tissues were obtained from skin reduction procedures and solid tumor resections > 3 cm distant from tumor border, and blood was donated by healthy volunteers. The study was approved by the Ethics Committee of the Medical University of Vienna, Vienna, Austria (ECS 1087/2016) and was performed in accordance with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from individuals participating in the study.

Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described using TRIZol-Reagent (ThermoFisher, Waltham, MA) and TaqMan Gene Expression primers (Applied Biosystems, Waltham, MA) (Stoffel et al., 2017). Expression levels were normalized to human β2-microglobulin. The Δ-CT method was used to calculate fold-change to respective healthy control material.

Flow Cytometry and cell sorting

Isolation of live PBMC was performed under sterile conditions with Ficoll-Paque reagent (Sigma Aldrich, St. Louis, MO). Flow cytometric analysis of single-cell suspensions was performed by surface and intracellular marker staining as previously described (Brüggen et al., 2015) using antibodies shown in Supplementary Table S3. Nonpermeabilized T cells were sorted into lysis buffer for RNA-sequencing.
RNA-sequencing and bioinformatic analysis of sequencing data

The sequencing of low-input samples (100 cells) was performed using a SMART-seq2 protocol. RNA isolation and cDNA synthesis were performed, followed by enrichment, as previously described (Picelli et al., 2014). Libraries were sequenced using an Illumina HiSeq (San Diego, CA) 3000/4000 platform and a 50 base pair single-read configuration.

Single-end reads were trimmed for removal of low-quality reads using Read Tools (v.1.0.0) (Gómez-Sánchez and Schlüter, 2018). Trimmed reads were mapped to the Homo sapiens genome using STAR mapper (v.2.5.3a) (Dobin et al., 2013). Reads mapped in multiple genomic locations were eliminated using Sambolos (v.1.4) (Li et al., 2009). Read counts for exons were generated using feature counts function of the Subread package (v.1.22.1) (Liao et al., 2014). Genes with < 10 reads were eliminated from further analysis in all samples. Gene expression analysis was done with DESeq2 (v.3.22.3) (Love et al., 2014) using the Ensembl Known Gene models (version GRCh38.92) as reference annotations. Normalized gene expression ($log_{2}$) obtained by DESeq2 was used to perform the G-test in R between GVHD developers and patients without GVHD and selected 36 BCL2 family genes (with fold-change $\geq 2$ and false discovery rate $P < 0.05$ at either time point). Heatmap for these 36 genes was created in R by using the ComplexHeatmap package (Gu et al., 2016). Principal component analysis and hierarchical clustering were also performed with the same genes using the normalized gene expression from DESeq2.

Immunofluorescence

Quadruple immunofluorescence stainings of skin cryosections were performed with directly and indirectly labeled monoclonal antibodies (Supplementary Table S3). In brief, after incubation with the primary antibodies overnight, an appropriate secondary fluorescence-labeled antibody was applied for 30 minutes at room temperature, followed counterstaining with DAPI. Immunostainings were controlled with isotype-matched conjugates (Supplementary Figure S5d). For evaluation of immunofluorescence results, images were acquired at room temperature using a Z1 Axio Observer microscope equipped with an LD Plan-Neofluar ×20/0.4 objective (Zeiss, Oberkochen, Germany) and quantified using TissueFAXS and/or TissueQUEST image analysis software (TissueGnostics, Vienna, Austria).

Mixed leukocyte reactions

Isolated PBMC from healthy donors (n = 7) were FACS-sorted for pure T-cell (CD45$^-$/CD3$^+$), and monocyte (CD45$^-$/CD14$^+$) populations, CFSE-labeled and HLA-mismatched T-cell and monocyte cultures (5:1) were established. Cultures were incubated at 37 °C for 5 days before the addition of BLC2-inhibitor AB-199. After 24 hours, read-out was performed by flow cytometric analysis.

In vitro treatment of patient-derived leukocytes

PBMC of SR-cGVHD patients (n = 9) and non-SR controls (n = 10) were freshly isolated 2–4 hours after prednisone intake (daily oral administration $\geq 3$ weeks). Cells were incubated in cell media with 0 or 10 nM of ABT-199 at 37 °C for 24 hours.

Statistics

Flow cytometric samples were acquired using FACsaria III cell sorter (BD Biosciences, San Diego, CA) using FACSDiva software and analyzed with FlowJo software (FlowJo, Ashland, OR). Statistical testing was performed using GraphPad Prism (version 7) and SPSS software. Data were tested for normal distribution by the Shapiro-Wilk test. In cases of normal distribution, parametric one-way ANOVA analysis with Tukey's pairwise comparisons was used to compare more than two groups and a nonparametric t-test for two groups. For analysis of patient outcome, patients were stratified for clinical endpoints: death from GVHD (n = 5), death from relapse (n = 2), survival with persistent chronic GVHD (n = 2), and survival without symptoms of GVHD (n = 6). Patients with relapse were excluded from further survival curve analysis. Receiver operating characteristic curve analysis was used to detect a hazard cutoff. Validation was performed using the DeLong bootstrap confidence interval. A log-rank test was performed for comparison of survival curves in a Kaplan-Meier plot. $P < 0.05$ were considered statistically significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

Data availability

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE146495 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146495).

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CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by funds of the Austrian Central Bank Anniversary Fund (project no. 1178), by the German Foundation of Dermatology (Deutsche Stiftung Dermatologie) and the Innovation Fund of the Austrian Academy of Sciences (project no. IF_2017_29). JS is a recipient of a DOCMed Fellowship of the Austrian Academy of Sciences. The authors thank Andreas Villunger, Matthias Farlik, and Georg Stingl for their valuable scientific input.

AUTHOR CONTRIBUTIONS

Conceptualization: GH, GS; Data Curation: RVP; Formal Analysis: JS, RVP, CK; Funding Acquisition: GS; Investigation: JS, LB, BR; Methodology: TK, RVP, JS, CB, GS; Project Administration: GS; Resources: MH, PW, MM, PK, WR, GS; Supervision: CB, GH, MM, WR, CB, GS; Visualization: JS, RVP, NB; Writing - Original Draft Preparation: JS, GS; Writing - Review and Editing: RVP, TK, CB, GH, GS

SUPPLEMENTAL MATERIAL

Supplemental material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.02.029.

REFERENCES


