Summary

Acute graft-versus-host disease (GVHD) remains a major complication after allogeneic haematopoietic stem cell transplantation (allo-HSCT). The emergence of different immuno-prophylaxis strategies, such as post-transplant cyclophosphamide or anti-thymocytoglobin, has reduced the incidence of acute GVHD in recent years. The biology of the acute GVHD we observe in the clinic may change due to the use of novel immuno-stimulatory agents, including immune checkpoint inhibitors or anti-neoplastic immune-modifiers, like lenalidomide, given before or after allo-HSCT. Here we discuss the recent advances in our understanding of acute GVHD with a focus on early events of the disease, including tissue damaging factors, innate immune cells, costimulatory pathways, immune cell signalling, immuno-regulatory cell types, biomarkers of GVHD and regenerative approaches. New insight in the pathogenesis of acute GVHD has revealed the role of pro-inflammatory intracellular signalling, defects in intestinal tissue regeneration and anti-bacterial defence, as well as a reduced diversity of the microbiome, which will be the basis for the development of novel therapies.

Keywords: GVHD, stem cell transplantation, immunopathology.

Early tissue damage and innate immune cells in GVHD

Allogeneic haematopoietic stem cell transplantation (allo-HSCT) is a potentially curative treatment for various haematological malignancies. Besides the cytotoxic effect of the chemotherapy given before infusion of haematopoietic stem cells and immune cells, the donor immune system is responsible for the elimination of the malignant cells, termed as the graft-versus leukaemia (GVL) effect (Kolb et al, 1990). Since allo-HSCT requires conditioning, tissue damaging events will occur in all patients undergoing this procedure.

The first line of defence when tissue damage occurs are the innate immune cells, including neutrophil granulocytes (neutrophils), macrophages and monocytes. Neutrophils reach the injured tissue first. Neutrophils are the most abundant leucocyte subtype and essential to the innate immune response against invading pathogens via the release of superoxide radicals and other secondarily-derived reactive oxygen species (ROS) and granules containing antimicrobial peptides, proteins and degradative enzymes as well as phagocytosis. In contrast to adaptive immune responses, the neutrophil-mediated elimination of microorganisms is non-specific and independent of previous exposure to microorganisms. Neutrophils also cause monocyte and macrophage recruitment to the injured tissue. Macrophages and monocytes phagocytose invading bacteria and present antigens to T cells.

Several lines of evidence have shown a role for neutrophils in the early phase of GVHD (Socié et al, 2004; Giroux et al., 2011; Schwab et al, 2014; Hülsdünker et al., 2018). Initial studies showed that high frequencies of granulocytes were connected to an unfavourable outcome after allo-HSCT in patients (Socié et al, 2004). More functional studies in pre-clinical models then showed that neutrophils contribute directly to tissue damage by the release of ROS, which damages the gastrointestinal (GI) tract (Schwab et al, 2014). The activation of neutrophils was caused by bacteria that penetrated through the intestinal wall while germ-free mice were protected from the neutrophil influx (Schwab et al., 2014). The tracking of neutrophils in living animals was facilitated by in vivo myeloperoxidase imaging (Hülsdünker & Zeiser, 2016). ROS production was dependent on intact nicotinamide adenine dinucleotide phosphate hydrate (NADPH) oxidase while neutrophils with mutant gp91phox caused less tissue damage and therefore less acute GVHD (Schwab et al., 2014). This role of ROS production is in agreement with a low GVHD rate in chronic granulomatous disease patients.
with defective neutrophil ROS production undergoing allo-HSCT (Martinez et al, 2012; Gungor et al, 2014). More recently it was shown that neutrophils not only cause tissue damage, but can also migrate from the inflamed ileum to the mesenteric lymph nodes, where they are involved in antigen presentation (Huelsdünker et al, 2018). By using a light inducible photo-converter system it was shown that neutrophils that were photo-converted in the terminal ileum were later detected in the mesenteric lymph nodes. Granulocyte-macrophage colony-stimulating factor drives GVHD pathology by licensing donor-derived myeloid phagocytic cells to produce inflammatory mediators, such as interleukin (IL) 1β and ROS and promote GVHD (Tugues et al., 2018).

Besides neutrophils, monocytes that are activated by danger-associated molecular patterns (DAMPS) (Wilhelm et al, 2010) contribute to GVHD (Klämbt et al, 2015). DAMPS are molecules that can cause and perpetuate a non-inflammatory inflammatory response. DAMPS can be nuclear or cytosolic proteins that are released from injured or stressed cells and indicate to the immune system that cell damage has occurred. In contrast to DAMPS, pathogen-associated molecular patterns (PAMPS) initiate and perpetuate the infectious pathogen-induced inflammatory response.

The innate immune cells involved in GVHD are not homogeneous but comprise pro- and anti-inflammatory populations. Therefore, neutrophils and monocytes may also reduce GVHD severity depending on local tissue factors, the time point after allo-HSCT and their plasticity. For example, it was shown that certain neutrophils are phenotypically indistinguishable from myeloid-derived suppressor cells (MDSC), which reduce GVHD (Highfill et al, 2010). However the immunosuppressive potential of MDSC is abrogated when the tissue microenvironment is pro-inflammatory (Koehn et al, 2015). Granulocyte colony-stimulating factor-mobilized regulatory monocytes reduce acute GVHD (D’Aveni et al, 2015).

Besides the classical PAMPS that activate neutrophils, dendritic cells and inflammatory monocytes, the release of DAMPS promotes the disease. DAMPS are molecules that are normally not found in the extracellular space but released when GVHD evolves. GVHD can be enhanced by extracellular adenosine triphosphate (ATP) activating P2X7R (Wilhelm et al, 2010). P2X7 is expressed on antigen-presenting cells (APCs), which prime the incoming T cells and promote their contribution to GVHD. Under normal conditions ATP levels are kept under control by CD39, which mediates conversion into adenosine monophosphate which is then metabolized by CD73 into anti-inflammatory adenosine. When this system is disturbed the severity of GVHD is enhanced in mice because they lack CD73 (Tsukamoto et al, 2012). DAMPS that were shown to play a role in the pathogenesis of GVHD include uric acid, heparan sulfate, siglec, mitochondrial components, HMGB-1 or IL33, among others [reviewed in Zeiser and Blazar (2017)]. Both ATP and uric acid activate the nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing (NLRP) 3 inflammasome, which leads to the production of pro-inflammatory IL1β. Consistent with the role of uric acid, its depletion reduced GVHD-related death in preclinical models (Jankovic et al, 2013). Activation of the NLRP3 inflammasome in the recipient was shown to promote GVHD (Jankovic et al, 2013; Chen et al, 2015). Conversely, NLRP6 exacerbated GVHD, independent of gut microbial composition (Toubai et al, 2019). The different steps of DAMP- and PAMP-mediated immune activation are depicted in Figure 1.

Multiple PAMPs were analysed with respect to their role in GVHD [reviewed in Zeiser and Blazar (2017)]. While bacterial transmigration may trigger GVHD, the antibacterial effects of antibiotics have an unfavourable impact on patient outcome post-alloHSCT, as microbiota-derived metabolites promote the regeneration of intestinal stem cells (Mathewson et al, 2016). A lack of the microbiota-derived metabolites will lead to less regeneration of intestinal stem cells. The regeneration of intestinal stem cells is beneficial for the HSCT recipient because it promotes the intestinal barrier function, which reduces the translocation of intestinal bacteria into the submucosa. Butyrate, secreted by GI bacteria, is an histone deacetylase (HDAC) inhibitor, shown to potently reduce GVHD by modulating indoleamine-2,3-dioxygenase-dependent innate immune and allogeneic-stimulating APC functions in a STAT-3-dependent manner (Mathewson et al, 2016). Consistently, broad-spectrum antibiotics were shown to increased GVHD-related mortality in preclinical models and in patients (Jenq et al, 2012; Shono et al, 2016). Bacterial colonization of the classical GVHD target organs, skin and intestinal tract, as well as liver draining the lymphoid fluid from the intestinal tract has led to the hypothesis that bacterial transmigration is essential for the disease. In other organs affected by GVHD, such as the gonads, lungs, central nervous system and retina cells (Hartrampf et al, 2013; Kalyaperumal et al, 2014; Mirza et al, 2016; Grønningsæter et al, 2017) other pathomechanisms, that are not related to microbiota, may be active.

Besides bacteria, a role for fungi and viruses was recently reported in the setting of GVHD. Cytomegalovirus reactivation frequently precedes acute GVHD (Palaniyandi et al, 2013) and a recent study analysed the longitudinal gut virome in patients undergoing allo-HSCT using metagenomics (Legoff et al, 2017). The authors reported a viral ‘bloom’ following allo-HSCT which may reflect the immunodeficiency of the recipients (Legoff et al, 2017). Also the study identified picornaviruses as predictive of the occurrence of severe enteric GVHD (Legoff et al, 2017).

Costimulatory pathways, chemokines and cytokines in acute GVHD

The role of multiple costimulatory pathways has been studied in acute GVHD including the positive regulatory molecules promoting T-cell activation-like (Blazar et al, 1999),
inducible costimulator (ICOS) (Taylor et al., 2005); Tumour necrosis factor receptor (TNFR)-superfamily receptors (CD40LG, OX40, 4-1BB) and negative regulatory molecules like cytotoxic T cell lymphocyte antigen (CTLA)-4, Programmed death receptor (PD)-1/PD-L1 and PD-L2 [reviewed in (Zeiser & Blazar, 2017)]. In the clinical setting of hyperacute steroid-refractory GVHD following haploidentical HSCT costimulatory blockade with abatacept was tested (Jaiswal et al., 2016). Five patients were treated with a combination of T cell costimulation blockade with abatacept, and etanercept and basiliximab. The overall response at days 29 and 56 were 100% and 40% and the two patients achieving a complete remission were long-term disease-free survivors off immunosuppression (Jaiswal et al., 2016).

While costimulatory blockade can reduce GVHD, the blockade of immune checkpoint molecules can enhance GVHD. A recent meta-analysis reported on 283 patients treated with immune checkpoint inhibitors before or after allo-HSCT (Ijaz et al., 2019). Of the patients treated before and after allo-HSCT, 56% and 14%, respectively, developed acute GVHD. For pre- and post-alloHSCT treatment, the authors reported 20 and 40 deaths, respectively; 28% and 60%, respectively, were GVHD-related.

In a study on anti-PD1 checkpoint inhibition for Hodgkin lymphoma, Haverkos et al. (2017) reported that 55% of the patients developed treatment-emergent GVHD and only 2 of 17 patients achieved complete response to GVHD treatment.

A major step in GVHD pathogenesis is the production of cytokines and chemokines, such as IL1, T-helper 1 (Th1) cytokines (IFN-γ, IL2, and TNF), IL6, IL11, IL12, IL15, IL17, IL18, IL21 and IL33 amongst others [reviewed in (Zeiser & Blazar, 2017)]. Not all cytokines exert an exclusively pro-inflammatory effect, for example the common gamma chain cytokine, IL2, promotes the expansion of anti-inflammatory T-regulatory cells (Tregs) (Zeiser et al., 2006; Zeiser & Negrin, 2008; Shin et al., 2011). This has clinical implications as the major backbone of most immunosuppressive regimens contain calcineurin inhibitors which reduce IL2 production. In contrast to calcineurin inhibitors, mTOR inhibitors preferentially block the expansion of conventional T cells (Zeiser et al., 2008). The connection between IL2 and Treg expansion was used in clinical trials in which ultra-low dose IL2 (Kennedy-Nasser et al., 2014) was given as prophylaxis. Besides expanding Tregs in patients, IL2 improved chronic GVHD, e.g. in a first study on 23 patients, 12 had major responses of their chronic GVHD manifestations (Koreth et al., 2011). Another common gamma chain cytokine that was studied in the context of GVHD was IL15 (Blaser et al., 2005), which is known to promote GVL effects (Mathew et al., 2018).

Cytokines cause the activation of CD4⁺ and CD8⁺ T cells, which then eliminate epithelial cells via Fas ligand expression.
or via the release of cytotoxic molecules, including perforin and granzyme B (Blazar et al., 1997). In mouse models where only major histocompatibility complex (MHC) class I is mismatched, CD4+ T cells alone are sufficient to induce GVHD. Furthermore, cytokines can directly exert cytotoxicity, like TNF-α that induced apoptosis in epithelial cells that are in close proximity. Multiple cell types contribute to T cell-mediated tissue damage. Priming of naïve T cells takes place via interaction with recipient-derived dendritic cells (DCs) and donor-derived CD103+ DCs that are derived from the colon (Koyama et al., 2015). A selection of studies performed in the mouse model and their clinical correlates are listed in Table I.

**Biomarkers in acute GVHD**

It would be desirable to predict which patients are at a high risk to develop acute GVHD, how they will respond to corticosteroids and what is their risk for non-relapse mortality (NRM). To address these questions multiple biomarkers have been determined and correlated with clinical outcome. Multiple biomarkers including regenerating islet-derived protein (REG) 3α, TNFR1, IL2Rα, cytokeratins, BAFF and CXCL10 (Ahmed et al., 2015) have been studied in the context of acute GVHD, sometimes with specific predictive value for certain target organs of GVHD. An example is the antimicrobial peptide REG3α, which has predictive value for intestinal acute GVHD (Ferrara et al., 2011). The combination of the 4 biomarkers, suppressor of tumorigenicity 2 (ST2), TNFR1, IL2Rα and REG3α, was shown to be predictive for lethal acute GVHD (Hartwell et al., 2017). By modelling 6-month NRM in an independent test set and validation set, a 2-biomarker model using ST2 and REG3α concentrations identified patients with a cumulative incidence of 6-month NRM of 28% in the high-risk group and 7% in the low-risk group (Hartwell et al., 2017). Besides soluble factors in the blood of the GVHD patients, micro-RNAs, which determine the transcription of multiple target genes were evaluated after allo-HSCT [reviewed in (Zeiser & Blazar, 2017)]. Functional studies in preclinical models showed that several miRs promoted or inhibited GVHD, such as miR-155 (Ranganathan et al., 2012; Chen et al., 2015), miR100 (Leonhardt et al., 2013), miR-146a (Stickel et al., 2014; Stickel et al., 2017). The pro-inflammatory miR-155 was required for CXCR4-dependent donor T-cell migration (Ranganathan et al., 2012) and NLRP3 inflammasome activation in dendritic cells (Chen et al., 2015).

**Cellular and microbiota-based therapy**

The transfer of a tolerogenic immune cells could, ideally, lead to the achievement of long-term tolerance and prevent or treat GVHD as shown for Tregs in mice (Cohen et al., 2002; Hoffmann et al., 2002; Taylor et al., 2002). Based on the strong immunoregulatory effect of Tregs in mice, clinical studies using Treg transfer in the prophylactic setting were performed (Brunstein et al., 2011; Di Ianni et al., 2011; Martelli et al., 2014; Brunstein et al., 2016). Treg transfer was found to be connected to low acute GVHD rates and normal to enhanced immune reconstitution (Brunstein et al., 2011; Di Ianni et al., 2011).

Another immune regulatory cell type is mesenchymal stroma cells (MSCs) that were shown to reduce GVHD severity in a humanized mouse model of T cell activation (Maitra et al., 2004). The role of MSC is still controversial, as some clinical studies using MSCs showed activity against acute GVHD while a randomized clinical trial failed to show improvement of GVHD-related mortality (Le Blanc et al., 2004; Kordelas et al., 2014). Based on the increasing understanding of the role of a diverse microbiome, fecal microbiota transplants (FMTs) have been studied as therapy in patients suffering from steroid-resistant acute GVHD (Kakihana et al., 2016). In this study, FMT allowed reduction of the steroid dose by 69% (Kakihana et al., 2016). Other studies had analysed the impact of FMT as GVHD prophylaxis, for example a prospective open-label pilot study reported the results using third-party FMT capsules in patients undergoing allo-HSCT (DeFilipp et al., 2018). This strategy led to a higher microbiome diversity after FMT and to the expansion of stool-donor taxa (DeFilipp et al., 2018). FMT is still considered an experimental treatment approach and needs validation in carefully designed prospective clinical trials.

**Kinase inhibitors and outlook**

Based on our understanding of cytokines in the pathogenesis of GVHD, inhibitors targeting signalling pathways downstream of multiple cytokine receptors became an interesting treatment option. This was made possible because small molecules that were designed to inhibit signalling caused by oncogenic mutations lead to constant activation of a receptor tyrosine kinase. Tyrosine kinase inhibitors target mutations that are frequently found in solid tumours. One example is a BRAF inhibitor that targets oncogenic BRAF in melanoma or hairy cell leukaemia, or inhibition of MEK to interfere with the BRAF/MEK/ERK pathway. Interestingly, multiple oncogenic mutations occur downstream of signalling pathways that are key for cytokine or growth factor signalling. These pathways include, besides many others, mitogen-activated protein kinase kinase (MEK), aurora kinase A (AURKA), Janus activates kinase (JAK) 1/2, CDK2/5 and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), and were tested in preclinical GVHD models [reviewed in (Zeiser & Blazar, 2016; Zeiser & Blazar, 2017)]. Naïve T cells cause GVHD and, in contrast to other T-cell subtypes, these exhibited a strong activation of the RAS/MEK/ERK pathway (Shindo et al., 2013). Blocking this pathway by MEK inhibition reduced acute GVHD and blocked alloreactive T-cell expansion (Shindo et al., 2013). A gene expression profile of T cells isolated from nonhuman primates (NHP) during acute
| Main conclusion from the preclinical model of GVHD (year) | Reference(s) | Main conclusion from the clinical trials (year) | Reference(s) |
|----------------------------------------------------------|--------------|-----------------------------------------------|--------------|
| CyA and MTX reduce GVHD in the canine GVHD model (1982). | Deeg et al (1982) | MTX and CyA is superior to CyA alone for GVHD prophylaxis (1986). Phase III prospective, randomized trial. | Storb et al (1986) |
| GVHD is reduced in some but not all mouse models of GVHD when IL1 is blocked (1991). | McCarthy et al (1991) | No significant protection by IL1 against GVHD (prophylaxis setting) (2002). Phase III prospective placebo-controlled study. | Antin et al (2002) |
| Psoralen and UVA radiation reduces GVHD in the mouse model (1991). | Ullrich (1991) | ECP has effects against acute GVHD (2000, 2015). Pilot study( 2000). Analysis of multiple prospective ECP studies (2015). | Greinix et al (2000) |
| CTLA4 antagonism reduces GVHD in the mouse model (1994). | Blazar et al (1994) | CD28:CD80/86 co-stimulation blockade (abatacept) leads to low GVHD rates (2013). Single-arm feasibility study. | Koura et al (2013) |
| Blocking TNF-α decreased GVHD in mice (1999, 2003). | Hill et al (1999); Schmaltz et al (2003) | Infliximab and corticosteroids are effective as initial treatment of GVHD (2009). Prospective phase III study, retrospective analysis (2011). | Couriel et al (2009); Bager et al (2011) |
| Anti-CCR5 antibody treatment protects against aGVHD-related mortality (1999). | Murai et al (1999) | CCR5 inhibition prevents acute GVHD of liver and gut before day 100 (2012). Phase 1/2 single institution trial. | Reshef et al (2012) |
| Bortezomib reduces acute GVHD in mice (2004). | Sun et al (2004) | Short-course, bortezomib-based GVHD prophylaxis yields low acute GVHD rates (2009, 2012). Phase 1 trial (2009). Prospective phase I/II trial (2012). | Koreth et al (2012) |
| IL6 inhibition decreased acute GVHD in mice (2009). | Chen et al (2009); Tawara et al (2011) | Early IL6 inhibition with tocilizumab leads to a low risk of acute GVHD (2014). Phase 1/2 single institution trial. | Kennedy et al (2014) |
| The sphingosine 1-phosphate receptor agonist FTY720 reduces GVHD (2003). | Kim et al (2003) | Active clinical study on KRP203 in patients undergoing allo-HSCT (2016). Randomized, Open-label Phase 1/2 study. | Clinicaltrials.gov: NCT01830010 |
| Memory CD4+ T cells cause less GVHD (2003). | Anderson et al (2003) | Naïve T cell-depleted stem cell graft transfer is connected to a low GVHD incidence (2015). Single-arm, 2 site clinical trial. | Bleakley et al (2015) |
| Statins reduce GVHD severity (2007). | Zeiser et al (2007) | Statin intake is connected to reduced GVHD incidence in patients (2010, 2010, 2013). Retrospective analysis (2010). Prospective phase II trial, donor and recipient treatment (2013). | Rotta et al (2010) |
| HDAC inhibition reduced GVHD severity in mice (2008). | Reddy et al (2008) | HDAC blockade together with standard GVHD prophylaxis is associated with a low incidence of severe acute GVHD (2014). Phase 1/2 trial. | Choi et al (2014) |
| JAK1/2 inhibition reduces acute GVHD (2012, 2014, 2015). | Choi et al (2012); Spoerl et al (2014) | JAK1/2 inhibition reduces acute GVHD in patients (2015). Retrospective analysis. Prospective phase III trial (NCT02913261) has recruited, analysis is ongoing (2019). | Zeiser et al (2015) |

Allo-HSCT, allogeneic haematopoietic stem cell transplantation; CyA, ciclosporin; ECP, extracorporeal photopheresis; GVHD, graft-versus-host disease; HDAC, histone deacetylase; IL, interleukin; MTX, methotrexate; UVA, ultraviolet A.
GVHD identified AURKA as a kinase target (Furlan et al., 2015). Functional studies in mouse models of GVHD showed that AURKA inhibition reduced GVHD severity (Furlan et al., 2015). JAK1/2 inhibition was also shown to reduce GVHD when given systemically in mice (Spoerl et al., 2014) and a retrospective study suggested a benefit in patients with steroid-resistant GVHD (Zeiser et al., 2015). Preclinical studies showed that JAK1/2 inhibition had also a profound effect on DCs and neutrophils in the setting of acute GVHD. JAK1/2 blockade reduced expression of the transcription factor CIITA which activates the MHC class II promoter in DCs (Stickel et al., 2017) and reduced the migration of recipient neutrophils from the ileum to mesenteric lymph nodes (Hülsdünker et al., 2018). In May 2019, the JAK1/2 inhibitor ruxolitinib was approved by the US Food and Drug Administration (FDA) for the treatment of acute steroid-refractory GVHD (FDA, 2019) based on an open-label, single-arm, multicentre study (NCT02953678) of ruxolitinib that enrolled patients with steroid-refractory acute GVHD grades 2 to 4.

To summarise, a better understanding of the pathogenesis of GVHD, particularly of the role of individual signalling pathways, cytokines, tissue regenerative approaches, protease inhibitor strategies and immunomodulatory cell types, will help foster novel therapeutic development. Future studies will need to investigate the effectiveness of novel therapeutic agents against anti-PD1 immunotherapy-induced GVHD or GVHD that occurs after post-transplant cyclophosphamide.

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