Animal models of graft-versus-host disease

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A B S T R A C T

Acute graft-*versus*-host disease (GvHD) is a severe complication that limits the success of allogeneic hematopoietic cell transplantation (alloHCT). Most knowledge about the biology of GvHD is derived from mouse models of this disease and, therefore, a critical analysis of potential advantages and disadvantages of the murine GvHD models is important to classify and understand the findings made in these models. The central events leading up to GvHD were characterized in three phases that include the tissue damage-phase, the T-cell priming-phase and the effector-phase, when the disease becomes clinically overt. The role of individual cytokines, chemokines, transcription factor or receptors was studied in these models by using gene deficient or transgenic mice in the donor or recipient compartments. Besides this, numerous studies have been performed in these models to prevent or treat GvHD. Several recent clinical trials were all based on previously reported findings made in the mouse model of GvHD, such as the trials on CCR5-blockade, donor statin treatment, vorinostat treatment or adoptive transfer of regulatory T cells for GvHD prevention. The different mouse models for GvHD and graft-*versus*-leukemia effects are critically reviewed and their impact on current clinical practice is discussed.

Learning goals

At the conclusion of this activity, participants should understand that:
- murine GvHD models contributed significantly to the understanding of the cytokine storm and the release of danger signals during GvHD;
- in humans the allogeneic T-cell response is more heterogenous compared to the inbred mouse strains where the expansion is more homogenous;
- there are different mouse models for chronic GvHD (cGvHD), such as cGvHD induction by defects in thymic function, sclerodermatous cGVHD models, and CD4 dependent stimulation of B cells in systemic lupus erythematosus cGVHD models;
- the different BLI-, MRI- or PET-based methods, as well as imaging at a cellular level, have contributed significantly to gaining a better understanding of the events that lead to the clinical picture of acute GvHD;
- several clinical trials for GvHD prevention, such as CCR5-blockade or vorinostat treatment, were motivated by findings from studies of mouse models of GvHD.

Mouse models of acute GvHD

The pathogenesis of acute GvHD is most frequently assessed in the mouse model, although in the early phase of allogeneic hematopoietic cell transplantation (alloHCT), dog models were crucial for understanding the role of MHC disparity in the 1960s.[1] Currently, canine models are still used to determine the effectiveness of cellular immunotherapy approaches.[2] Also non-human primate models[3] and rat models[4] of GvHD have been characterized and used to gain a better understanding of the pathophysiology of acute GvHD. The advantage of mouse models is based on multiple gene deficient and transgenic animals able to determine the role of individual genes or factors for GvHD. Furthermore, mice models represent lower costs on a per animal basis than those for higher vertebrates. Also the availability of laboratory reagents tailored for the use in mice, such as neutralizing monoclonal antibodies against multiple murine antigens, have led to the use of mice as a major animal model for GvHD. However, there are important differences between murine GvHD compared to the human disease that need to be considered critically when findings from one system are extrapolated into the other.

In contrast to the clinical situation, conditioning therapy in the mouse models most frequently involves total body irradiation (TBI) while few investigators have used chemotherapy in the mouse for myeloablative busulfan/cyclophosphamide-based conditioning[5] as well as fludarabine/cyclophosphamide-based reduced intensity conditioning.[6] Conversely, in the clinical situation, chemotherapy is most frequently applied as conditioning while TBI is applied mainly for the minority of patient who have acute lymphoblastic leukemia. Following conditioning, the murine recipients receive bone marrow to re-establish hematopoiesis and T cells derived

from the spleens of mice on the same genetic background as the bone marrow to induce GvHD.[7]

However, in humans, the T-cell response is more heterogenous compared to the inbred mouse strains where the expansion is more homogenous. This may be a reason why some of the findings in mouse models have not been successfully translated into clinical trials. This difference is likely to be responsible for the observation that many preclinical findings cannot be successfully applied to the clinic. One example are cytokine modifications that were tested in murine GvHD. IL-11 promoted T-cell polarization to a Th2 phenotype, reduced gut permeability, down-regulated IL-12, and reduced GvHD-related mortality in mice.[8,9] This approach was then investigated in a phase I/II double-blind, placebo-controlled study of recombinant human interleukin-11 for mucositis and acute GvHD prevention in patients.[10] Of 10 evaluable patients who received IL-11 in this trial, 4 died by Day 40 and one died on Day 85 because of transplant-related toxicity as patients receiving IL-11 experienced severe fluid retention.[10] This trial was not able to determine whether IL-11 given in this schedule can reduce the rate of GvHD, but the unexpected high mortality showed that a cytokine that was well tolerated by the mice induced severe side-effects in humans.

In the murine GvHD system, the number, phenotype and time point of transfer of donor T cells can be modified according to biological factors.[11] Also, based on differences in MHC class I and/or II molecules or multiple minor histocompatibility antigens, it can be determined whether the effectors of GvHD are either CD4 or CD8 T cells or both. Several murine minor mismatch GvHD models are illustrated in Table 1. The parameters analyzed in most experimental murine GvHD studies include animal survival, weight loss, and characteristic symptoms such as hunchback, diarrhea, and fur changes. Besides the clinical GvHD score, a histopathological scoring systems has been established to quantitatively assess the degree of GVHD severity.[15,16] When studying GvHD in the mouse, laboratories must consider differences in mouse vendor, age, sex, genetic drift, gut microbial flora and transplant protocols and each can have a marked impact on GVHD pathophysiology. Nonetheless, the mouse model has proven to be extremely useful for developing and testing new treatment approaches.

In order to induce organ specific GvHD, TCR-transgenic models have been developed. The specificity of the T cells in a TCR-transgenic mouse is restricted to a single peptide epitope that is either constitutively expressed by the transplanted allogeneic APCs or certain tissue such as the skin. In a skin-specific GvHD model, K14-mOVA transgenic mice that express membrane-associated ovalbumin (mOVA) under the control of a K14 promoter have been used.[17,18] These mice have also been extended to double transgenic mice by crossing them with OT-I mice that have a TCR recognizing the OVA peptide.[18] In this skin-specific acute GvHD model, the K14-mOVA transgenic mice develop the disease whereas double transgenic mice are protected despite injection of $CD8^+$ OT-I cells.[18] Multiple applications of TCR-transgenic mice have been described including TCRs directed against H-Y, TEa, TS1 and D011.10.[19-21] By using these TCR-transgenic mice as donors the role of antigen affinity during acute GvHD[22] was characterized as well as antigen-induced T-cell expansion.[23] These TCR-transgenic models aim to overcome the limitation of all MHC- and miHA-mismatched transplant GvHD models which are not suited to determine to which specific alloantigen the alloreactive T cells responded. Conversely, these models have the disadvantage of being highly artificial, because in patients, multiple T-cell clones with a variety of TCR affinities will expand and mutually influence each other; an effect that is lost with TCR-transgenic donor T cells.

Table 1. Mouse models for minor histocompatibility antigens.

Donor	Recipient	T cells mediating GvHD	Author (ref.)
B10.BR	CBA	CD8	Fanning[12]
DBA/2	B10.D2	CD8	de La Selle[13]
C3H.SW	B6	CD8	
B10.D2	DBA/2	CD4	Harper[14]
B6	BALB/b	CD4	

In order to mimic the heterogeneous TCR repertoire of human T cells, xenogeneic GvHD models have been developed. Early studies were conducted using non-obese diabetic severe combined immunodeficiency (NOD/SCID) mice and resulted in an engraftment of 1-20% of the human cells,[24] indicating that stable engraftment of the hematopoietic system of the disparate species was a major limitation of this approach. This was particularly true for the SCID model, as these mice still have active NK cells that reject upon missing self-recognition. A more successful engraftment was achieved in RAG2-deficient and IL-2-receptor-γ-deficient mice that lack functional T, B and NK cells.[25] Another model in which mice lack T, B and NK cells are NSG mice. Furthermore, these mice also have reduced function of macrophages and dendritic cells, and injection of human peripheral blood mononuclear cells causes acute GvHD in these mice.[26] Besides the limited engraftment, a major problem of xenogeneic transplants of human PBMCs into immunodeficient mice is the lack of human APCs to process mouse antigens and present them in the presence of class II MHC to the incoming human donor T cells which renders this model artificial.

Mouse models of chronic GvHD

The incidence of chronic GVvHD following alloHCT has been reported to range from 25% to 75% and is associated with significant morbidity and mortality.[27] Clinical cGVHD is an extremely complex and variable immunological disease, and, therefore, the establishment of murine models that recapitulate all disease features is very challenging. Several approaches to induce chronic GvHD have been performed, such as induction by defects in thymic function, sclerodermatous (Scl)-cGVHD models and CD4 dependent stimulation of B cells in systemic lupus erythematosus (SLE)-cGVHD models.[28] A SLE-cGVHD model that has been extensively applied to characterize cGVHD in mice uses adoptive transfer of immune cells from MHC antigen disparate donors. One model resulting in a cGvHD phenotype utilizes co-isogenic mice that differ only in the class II MHC molecule as a result of a mutant form of the class II I-A locus in MHC.[29,30] Comparable to clinical cGvHD, the phenotype that arises

from these models is connected to the generation of autoantibodies directed against dsDNA, ssDNA, and chromatin, and immune-complex glomerulonephritis.[31] Also, parent-into-F1 models have been used to induce cGvHD. The association of low precursor CTL numbers with cGVHD and high precursor CTL numbers with aGVHD has been demonstrated in other parent-into-F1 models, although the course and severity of the GVHD phenotype is variable.[32] The functional relevance of B-cell activity in SLE-cGVHD is supported by studies in which B-cell activation is blocked via inhibition of CD40 ligand[33] or blockade of T-cell co-stimulation by CTLA4Ig.[34] However, despite the similarities to human cGvHD, the relevance of the murine cGVHD models has been called into question for a number of reasons. One reason is that profiles of autoantibody expression in patients with cGVHD are highly heterogeneous, and include autoantibodies associated with other collagen vascular diseases,[35] which is different in the mouse. Also, the absence of bone marrow-derived stem cells in the donor inoculum and the absence of any host immunodepletion prior to cell transfer is very different from the setting of clinical alloHCT. Overall, murine models of cGVHD have been useful for a better understanding of important features in the pathophysiology, such as autoantibody production and fibrotic changes. Nevertheless, important clinical features connected to the development of cGVHD in alloHCT patients have so far not been reproducible in mice.

In vivo imaging modalities to monitor individual cell populations during GvHD

As the expansion of donor-derived T cells is common to all settings of acute GvHD, the quantification of this phenomenon is an important parameter to determine efficacy of therapeutic approaches against GvHD. *In vivo* T cells can be quantified by conventional methods including CFSE dilution[36] or BrdU uptake, or *in vivo* expansion of luciferase transgenic T cells quantified by bioluminescence imaging (BLI).[37-39] Following adoptive transfer, such labeled T cells can be detected in secondary lymph nodes, including Peyer's patches, mesenteric lymph nodes, and the spleen.[40] By using the methods and crossing luciferase transgenic mice[41] with gene deficient mice, the impact of a certain gene for migration or expansion can be evaluated as was done for IL-18.[42] A representative T-cell expansion in an allogeneic host is shown in Figure 1. While the luciferase-based BLI technique is well suited for fast dividing cells where the genomic integration of the reporter gene leads to increased signal intensity upon cell division, other cell populations such as dendritic cells

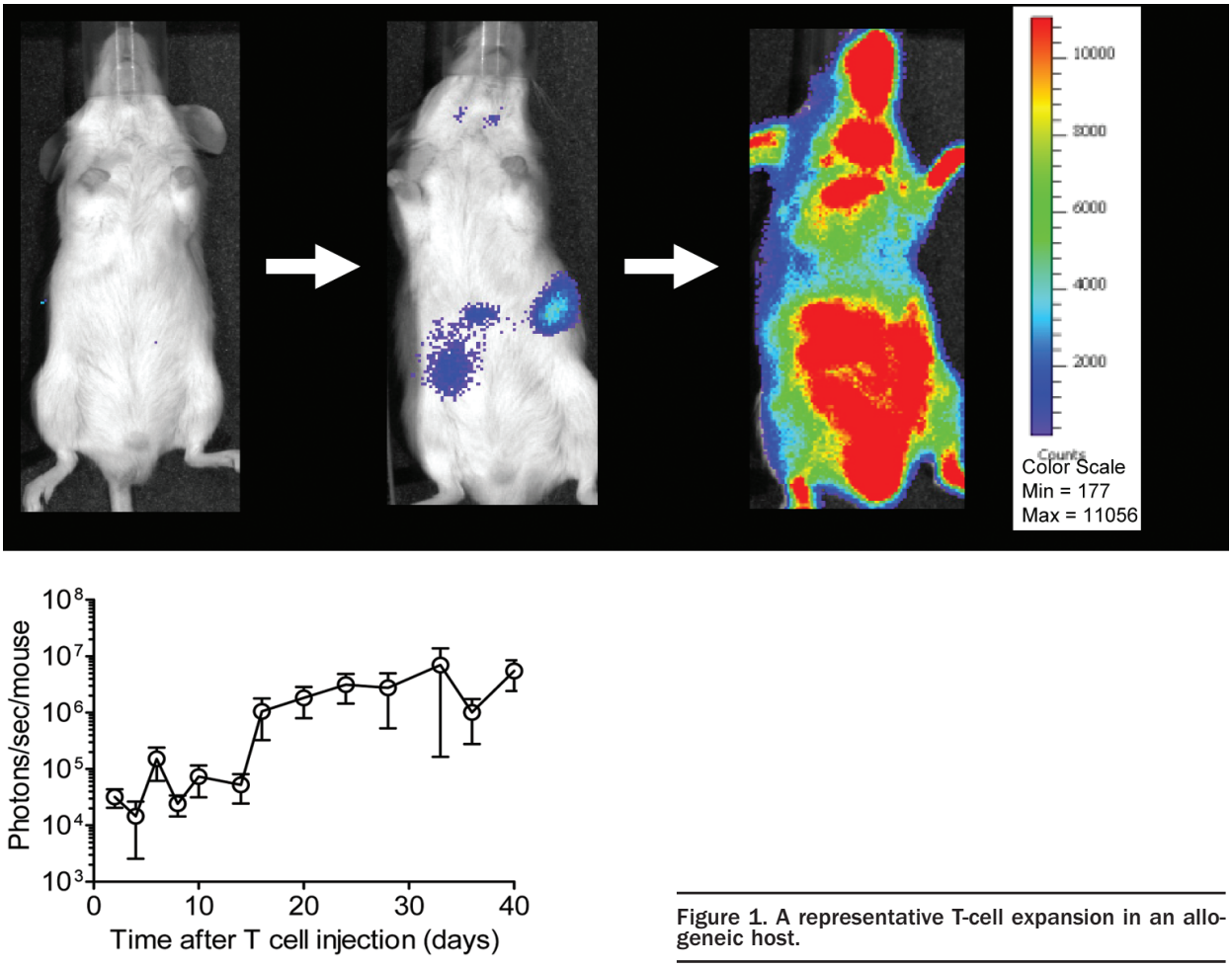

Figure 1. A representative T-cell expansion in an allogeneic host.

(DC) are well suited for magnetic resonance imaging (MRI)-based tracking. MRI offers high resolution imaging of specific anatomical regions *in vivo* following alloHCT.[43] As intracellular nanoparticles are diluted out when cells divide, MRI is particularly suitable for tracking DCs and other antigen-presenting cells because these cells have a low proliferative capacity and are efficient in Ag uptake.[44] By employing SPIO-IgG complexes, in our previous work,[43] we achieved a high uptake rate of the nanoparticles via Fc-γ R mediated endocytosis, a mechanism that has previously been used to study DC subsets.[45] By SPIO-based cell tracking, we characterized the migration of total CD11c$^+$ DC from a local injection site and found them to migrate towards lymph nodes of alloHCT recipients.[7,43] Besides the GvHD setting, DC were effectively tracked by means of MRI following *in vivo* administration in mice and in humans.[46,47] Not only cellular migration, but also the kinetics of ligand or receptor expression in different immune cell populations can provide additional valuable information regarding the time points at which a certain immunomodulatory treatment may be beneficial for GvHD. To monitor receptor expression non-invasively *in vivo*, we have previously used positron emission tomography (PET)-based imaging with radioactively labeled ligands in mice developing GvHD.[48] With this method, we could determine the level of alpha V integrin expression during inflammatory neoangiogenesis in the intestinal tract. Overall pre-clinical imaging of innate immune responses is a valuable tool to contribute to the understanding of GvHD biology in order to develop novel concepts to reduce the rate of GvHD in the clinic. The association of GvHD and the beneficial GVL effect was first described in mouse models[49] and, at the same time, multiple investigators have searched for avenues to separate both effects.[12,50,51] One of these very promising strategies involved the adoptive transfer of regulatory T cells[52,53] or mesenchymal stem cells (MSC).[54] The use of murine GvL models has also led to the identification of other immune effector cells besides conventional T cells, such as NK cells, cytokine-induced killer cells (CIK) or NKT cells.[55-57] Also the findings from the various mouse models of GvHD and GvL have shaped the understanding of the role of immunodominant epitopes, anti-tumor effects of donor lymphocyte infusions (DLI), the importance of alloantigens and cross-presentation on professional antigen presenting cells in GVL.[58,59]

To understand cellular migration and accumulation at a single-cell level, a multicolor light sheet fluorescence microscopy (LSFM) approach has been reported for GvHD.[60] With this method it was first possible to analyze intact mouse and human tissues by triple-color illumination. This allowed the investigators to determine changes in expression patterns of mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) and T-cell responses in Peyer's patches during GvHD.[60] The different BLI-, MRI- or PET-based methods, as well as imaging at a cellular level, have contributed significantly to a better understand of the events that lead to the clinical picture of acute GvHD.

Summary and outlook

Mouse models have been important for a better understanding of the biology of acute GvHD. Findings from GvHD mouse models have been translated into a clinical context and these have formed the basis of a number of clinically applied preventive and therapeutic strategies, all of which justify the use of such models in this field of research.

In particular, murine GvHD models contributed significantly to the understanding of the cytokine storm,[61] the release of danger signals,[62] the role of individual cytokines,[63-67] the role of CD4 and CD8 T cells,[68] regulatory T cells (Tregs),[52,53,69] host DCs[59] and NK cells[70] in GvHD. Beside the global reduction of cytokine release via reduced tissue-damaging protocols, the blockade of individual cytokines was investigated and data from mouse models of acute GvHD demonstrated that administration of an IL-1 receptor antagonist, either immediately after marrow infusion or immediately prior to the projected time of onset of acute GVHD, reduced GVHD-related mortality.[71] Later, the role of IL-1 in GvHD was independently confirmed by another group also using an IL-1 receptor antagonist.[72] A subsequent clinical phase I/II trial using the recombinant human interleukin-1 receptor with a significantly shorter half-life as compared to canakinumab demonstrated reduced GvHD severity when the drug was given in patients who had developed acute GvHD.[73] This finding was later confirmed by another study using an IL-1 antagonist in the therapeutic setting against acute GvHD.[74] Unfortunately, the design of anti-IL-1 treatment was then changed into a prophylactic approach with preemptive treatment from Days -4 to Day 10 after alloHCT.[75] In this study, no effect of IL-1 antagonist treatment on the incidence of acute GvHD was observed.[75] However, this could be due to the early administration long before the patient developed acute GvHD, and by the use of an IL-1 antagonist with a short half-life that had to be infused continuously.

Another example for a GvHD preventive approach that was derived from the mouse model was the application of tolerogenic cell types. Tregs as defined by expression of CD4, CD25, and forkhead box P3 (FOXP3) were shown to down-regulate autoreactive lymphocytes, and control immune responses at multiple cellular layers. The first studies showing that Tregs can prevent GvHD were performed in mice[52,69,76] and later developed into the clinic,[77] also motivated by the observation that, in humans, Tregs defects are associated with autoimmunity.[78] Isolation and quality assessment studies for adoptive transfer of Tregs to suppress GvHD had been initiated by Hoffmann *et al*.[79] which showed its feasibility. Another study used bead-purified Tregs given to patients three days prior to HLA-haploidentical CD34$^+$ cells supplemented with frozen/thawed mature donor T cells in the absence of any post-transplant immunosuppression.[80] These studies showed the safety of *ex vivo* purified natural Tregs, and found they promoted lymphoid reconstitution and did not overtly weaken the graft-*versus*-leukemia effect of the co-transferred mature T cells.[80] A clinical trial performed in the US included 23 patients who received a double umbilical cord blood (UCB) transplant and Tregs expanded from a third UCB unit.[81] The authors reported no infusional toxicity or increased risk for infection relapse or early mortality, and the incidence of grade II-IV acute GVHD was reduced compared to 108 historical controls treated (43% *vs*. 61%; P=0.05).[81] Another important observation

made in the mouse model was that Interleukin-(IL)2 is critical for Tregs development, expansion, activity, and survival.[38,82] To avoid IL-2 depletion, cyclosporine-free regimens have been studied in the clinic and are still under investigation.[83] Furthermore, strategies to enhance Tregs expansion after alloHCT by substitution of IL-2 have been very successfully applied in patients with chronic GvHD.[84]

However, despite these encouraging results, immunomodulatory cell populations like Tregs and MSCs that did not lead to the loss of GvL or graft-*versus*-infection effects in mouse models with intravenous injection of tumor cells could reduce these effects in humans. The location of the tumor may be important to determine how immunosuppressive Tregs are.[11,85] Therefore, carefully designed clinical studies are needed, and it will be important to monitor parameters such as minimal residual disease burden and CMV copy numbers in patients treated with Tregs or MSCs.

In the murine GvHD model, the migration of $CCR5^{+}CD8^{+}$ cells into the liver and gut was shown to be reduced when an anti-CCR5 antibody was applied, which translated into protection against GvHD-related mortality.[86,67] These findings were partly confirmed by murine studies in which mice that are genetically deficient for Ccr5 were used.[88] Consistent with a role of CCR5 in GvHD in humans, certain CCR5 polymorphisms are protective against GVHD.[89] Motivated by the data from the mouse models, a single-group phase I and II study on the preventive effect of effect of the CCR5 antagonist maraviroc on acute GvHD incidence was performed. This trial included 38 high-risk patients, and showed that acute liver and gut GVHD were not observed before Day 100, which was superior to historical controls.[90]

Another important trial that was based on findings in the mouse model assessed the efficacy of vorinostat in combination with standard GVHD prophylaxis after related-donor reduced-intensity conditioning.[91] In this phase I/II clinical trial, 50 patients were evaluated, and the authors reported that vorinostat in combination with standard GVHD prophylaxis was associated with a lower than expected incidence of severe acute GVHD.[91] Previous studies by the same group had shown in the mouse model that vorinostat reduced GvHD severity.[92]

Overall there are several lines of evidence that findings in the mouse model of GvHD can have a significant impact on treatment and prevention strategies used in the clinic for patients undergoing alloHCT.

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