REVIEW ARTICLE

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Translational and clinical advances in acute graft-*versus*-host disease

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ABSTRACT

Acute graft-*versus*-host disease (aGvHD) is induced by immunocompetent alloreactive T lymphocytes in the donor graft responding to polymorphic and non-polymorphic host antigens and causing inflammation in primarily the skin, gastrointestinal tract and liver. aGvHD remains an important toxicity of allogeneic transplantation, and the search for better prophylactic and therapeutic strategies is critical to improve transplant outcomes. In this review, we discuss the significant translational and clinical advances in the field which have evolved based on a better understanding of transplant immunology. Prophylactic advances have been primarily focused on the depletion of T lymphocytes and modulation of T-cell activation, proliferation, effector and regulatory functions. Therapeutic strategies beyond corticosteroids have focused on inhibiting key cytokine pathways, lymphocyte trafficking, and immunologic tolerance. We also briefly discuss important future trends in the field, the role of the intestinal microbiome and dysbiosis, as well as prognostic biomarkers for aGvHD which may improve stratification-based application of preventive and therapeutic strategies.

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) remains one of the most important curative modalities for marrow failure and various advanced/aggressive hematologic malignancies. Acute graft-*versus*-host disease (aGvHD) remains an important HSCT toxicity with significant associated morbidity and mortality. aGvHD clinical manifestations typically involve skin (rash), upper (nausea, anorexia) or lower (diarrhea, abdominal pain) gastrointestinal (GI) tract, or liver dysfunction (elevated bilirubin, transaminases).[1] Its pathology is typically induced by immunocompetent effector T lymphocytes responding to donor/recipient polymorphic and non-polymorphic antigens on host tissues, with activation, inflammation and eventual cytolytic activity.

Despite advances in HSCT, such as high resolution HLA genotyping and the routine use of calcineurin-inhibitor (CNI)-based prophylaxis, aGvHD incidence remains in the 30-35% range with HLA-matched donors. While aGvHD outcomes have improved (largely due to advances in supportive care, e.g., infectious disease interventions), patients with severe and steroid-refractory (SR) aGvHD still have impaired survival, estimated to be in the 5-30% range.

aGvHD control is a cornerstone of successful transplantation. Effective interventions should not cause excessive toxicity, impair the curative graft-*versus*-leukemia (GvL) effect of allotransplantation, or contribute to graft failure. In this review, we summarize aGvHD pathogenesis and discuss novel advances in the prevention and treatment of aGvHD that have evolved as our understanding of pathogenesis has grown. In addition, we highlight areas of burgeoning interest in the field: microbiota dysbiosis, and the development of aGvHD biomarkers.

Biology of acute graft-*versus*-host disease

In an early model of aGvHD, Antin and Ferrara described a three-step process comprising: (i) host tissue injury due to the conditioning regimen, with the production of inflammatory cytokines; (ii) stimulation and proliferation of effector T lymphocytes (Teff); and, finally, (iii) recruitment and activation of additional mononuclear effectors and amplification of a 'cytokine storm'.[2] This basic model has stood the test of time, although it has been refined thanks to a deeper and more sophisticated understand-

ing of transplant biology, briefly outlined below.

In the initial host tissue injury phase, exogenous and endogenous antigens classified as sterile damage-associated molecular patterns (DAMP; e.g., uric acid, ATP, heparan sulfate, HMGB-1 or IL-33) and pathogen-associated molecular patterns (PAMP; e.g., bacterial lipopolysaccharides) interact with antigen-presenting cells (APC) in the innate and adaptive immune systems, with activation of cytokine cascades (IL-1, IL-6, TNF-α, etc.) that set the stage for T-cell priming and expansion.[1]

The second phase involves Teff trafficking (mediated by L-selectin, CCR7, etc.) to lymphoid organs and host tissues. CD8+ and CD4+ Teff cells homing to the gut express high levels of integrin β7 (α4β7) which bind corresponding host tissue ligands. At their destination, Teff activation *via* APC-mediated host tissue:TCR interaction is initiated. This step, modulated by anti- *versus* co-stimulatory pathways and cytokine cascades, finally leads to the third phase, self-potentiating Teff cell proliferation and activation causing tissue damage *via* direct cellular cytotoxicity and indirectly *via* release of soluble mediators (TNF-α, IFN-γ, IL-1 and nitric oxide).[3] The canonical NOTCH pathway is involved in regulating GvHD pathogenesis[4] and using humanized monoclonal antibodies, it was shown that Notch-deprived T cells (with predominant roles for NOTCH1 and Dll-4) produce less inflammatory cytokines but proliferate normally, with a preferential increase in regulatory T cells (Tregs), without compromising GvL.[5] Selective NOTCH blockade offers potential for clinical translation.

Such immune activation is opposed by anti-inflammatory Tregs, a T-cell subset important in immunologic tolerance, in part *via* release of anti-inflammatory cytokines such as IL-10 and TGF-β.[6] Additionally, the balance of effector T-helper (Th) type 1 (IL-2, INF-γ) and type 2 (IL-4, IL-10) cytokine responses may govern the ultimate outcomes of inflammation since type 2 cytokines can inhibit potent proinflammatory type 1cytokines, and a Th1 to Th2 shift could be beneficial in aGvHD.[7] A distinct subset of CD4+ cells (characterized by production of *IL-17A* and *F*, IL-21 and IL-22) called Th17 cells has also been identified, which in murine models migrate to GvHD target organs causing severe pulmonary and GI lesions and GvHD lethality,[8] and may play a critical role in GvHD pathogenesis[9] antagonistic to Tregs. Invariant natural killer T (iNKT) cells (discussed below) are another cellular subset with putative immunoregulatory functions, in part *via* an increase in Treg numbers and IL-4 secretion, that may be important in GvHD pathophysiology.

Here we discuss novel advances in the prevention and therapy of aGvHD built on the understanding of these concepts.

Acute graft-*versus*-host disease prevention

Established determinants of acute graft-*versus*-host disease

The well-established impact of conditioning regimen intensity, donor-recipient HLA-mismatch and graft source (bone marrow [BM], peripheral blood stem cell [PBSC]) on aGvHD outcomes is briefly discussed below.

Conditioning regimen intensity - the impact of conditioning intensity on aGvHD is primarily due to tissue damage-induced DAMP/PAMP release (see above). In general, myeloablative conditioning (MAC) (particularly total body irra-

diation [TBI])-containing regimens are associated with higher aGvHD rates, an effect more pronounced with PBSC grafts.[10] Non-myeloablative (NMA) and reduced-intensity conditioning (RIC) transplants have been associated with lower aGvHD rates[11,12] than MAC, and even the newer reduced-toxicity regimens (e.g., ablative busulfan/fludarabine) as per a large randomized controlled trial (RCT).[13] There has, therefore, been a shift towards minimizing TBI except when absolutely necessary (e.g., acute lymphoblastic leukemia).

Novel therapeutic targeting of DAMP/PAMP:immune cell interactions are being investigated. For example, ATP (a DAMP) interacts with APC to activate inflammatory STAT1 signaling. Interruption of this pathway reduced GvHD in murine models[14] although translation into clinical practice is still awaited.

Donor-recipient human leukocyte antigen (HLA)-mismatch - HLA-mismatch is an aGvHD risk factor. Large registry studies document increased aGvHD rates (including severe aGvHD grades III-IV) and impaired survival for 1-2 locus HLA-mismatch *versus* 8 of 8 HLA-matched MAC and RIC HSCT.[15,16] With the advent of post-transplant cyclophosphamide (PTCy)-based regimens, the effect of HLA-mismatch may be less deleterious. PTCy was initially introduced in haploidentical (haplo) HSCT, but in a trial of matched and single-antigen mismatched unrelated donors (MUD, MMUD) it was found superior to standard CNI-based prophylaxis (discussed below).[17] The role of PTCy in single-antigen MMUD HSCT is being further explored, with one study showing better rates of acute and chronic GvHD, non-relapse mortality (NRM), and relapse with PTCy compared to anti-thymocyte globulin (ATG).[18] Many centers are adopting a PTCy-based platform for MUD/MMUD HSCT.

Graft source - while unmanipulated donor BM grafts were initially used in transplantation, there is now a secular trend towards use of PBSC grafts, due to logistical reasons and donor preference. In a large meta-analysis comparing the two graft sources, there was no difference in overall aGvHD rates, although severe grade III-IV aGvHD and chronic severe GvHD was lower with BM. However, relapse in that analysis appeared higher with BM grafts leading to impaired disease-free survival (DFS) and overall survival (OS) in late stage disease.[19] In a phase III RCT of MUD PBSC *versus* BM HSCT, OS was similar (albeit with relatively short follow-up) with no differences in aGvHD or relapse, but chronic GvHD rates were lower with BM.[20] Hence BM is arguably the better graft source, although the effect on relapse needs longer term follow-up. Cord blood transplants have resulted in similar rates of aGvHD as conventional sources although with lower rates of cGvHD.[21] It should be mentioned that many GvHD prophylaxis regimens have been tested in association with specific stem cell sources making the interpretation of these data difficult.

Innovations in acute graft-*versus*-host disease prophylaxis

Since the cardinal events in aGvHD etiopathogenesis involve T-cell trafficking, interaction with host antigens and activation to cause tissue injury, the cornerstone of aGvHD prevention remains depletion or modulation of donor T

lymphocytes.

Since the 1990s, standard of care (SOC) aGvHD prophylaxis has incorporated a CNI (e.g., tacrolimus [Tac], cyclosporine [CyA]) plus another agent (e.g., methotrexate [MTX]), mycophenolate mofetil [MMF], sirolimus [Siro]).[22,23] CNI inhibit alloreactive T-cell proliferation and activation. However, even with CNI-based platforms, rates of grade II-IV aGvHD are 30-40%, with 10-15% severe grade III-IV aGvHD. Furthermore, CNI are associated with various toxicities (e.g., renal dysfunction, thrombotic microangiopathy [TMA]) which can add to transplant-related mortality (TRM). Hence novel prophylactic therapies with improved efficacy and less toxicity are of great interest in transplantation. Recent advances in aGvHD prevention, some of which are challenging the established CNI-based platform, are discussed below.

In vivo T-cell depletion/modulation

Anti-thymocyte globulin - ATG is the polyclonal purified IgG fraction of sera from horses or rabbits immunized with human thymocytes or T-cell lines. *In vivo* T-cell depletion (TCD) with ATG has been extensively evaluated to reduce the incidence of acute and chronic GvHD with HLA-matched as well as cord blood and haploHSCT.

In the CNI-era, four RCT evaluated CNI/MTX prophylaxis ± ATG.[24] In the first, using horse ATG, a reduction in aGvHD was offset by higher rates of infection with no difference in NRM or OS; however, there was a reduction in severe chronic GvHD.[25,26] In the second, using rabbit ATG,[27,28] and the third, mainly using PBSC grafts, there was no effect on aGvHD, with a reduction in cGvHD.[29] These studies concluded that reduction in severe cGvHD with no deleterious effect on OS is a true ATG effect; however, aGvHD was not reduced. More recently, an RCT evaluated Tac/MTX ± anti T-lymphocyte globulin (ATLG) in MAC MUD HSCT, with a significant reduction in grade II-IV aGvHD and moderate/severe cGvHD. However, NRM and OS was impaired in the ATLG arm.[30] A higher dose of ATLG in the trial may have contributed to increased infections and mortality.

In pioneering studies by Storek *et al.*, persistence of therapeutic ATG levels on days +7 and +28 were found to reduce acute and chronic GvHD.[31] There is also evidence that excessive persistence or dosing of ATG may have immunosuppressive toxicity with increased NRM and relapse. Individualized ATG dosing, based on absolute lymphocyte count beyond recipient weight, could be a way forward to control GvHD without impairing NRM and relapse.[32]

Post-transplant cyclophosphamide - the use of PTCy-based GvHD prophylaxis has been a major advance allowing the widespread use of haploHSCT with increasing importance also in HLA-matched and mismatched HSCT.

Haplo-hematopoietic stem cell transplantation was initially associated with increased graft rejection and GvHD due to strong bidirectional donor *versus* recipient alloreactive responses. HaploHSCT regimens utilized highly immunosuppressive conditioning with high transplant-associated toxicity. The innovative use of PTCy dosed at 50 mg/kg on days +3 and +4 following NMA haploHSCT resulted in a grade II-IV aGvHD rate of 34%, with a low grade III-IV aGvHD rate of 6% and a trend towards reduction in severe cGvHD. Relapse rates were around 50%.[33] Numerous subsequent studies replicated these results, and PTCy is now the most widely used haploHSCT regimen.

It is worth noting that overall aGvHD rates with PTCy, at 30-80%, are not necessarily lower than SOC, but severe aGvHD and cGvHD rates are lower.

PTCy was initially thought to act *via* depletion of alloreactive T cells by elimination of proliferating cells and intrathymic clonal deletion of alloreactive T-cell precursors.[34] More recent data suggest important roles for Treg preservation and Teff exhaustion as additional mechanisms of effect.[34]

PTCy has also been evaluated in alternative donor HSCT. In a phase II RCT of MUD/MMUD PBSC HSCT, three GvHD prophylaxis regimens were compared with SOC Tac/MTX: PTCy/Tac/MMF, Tac/MTX/bortezomib, and Tac/MTX/maraviroc. The primary 1-year GvHD free, relapse-free survival (GRFS) endpoint was improved in the PTCy-based arm.[17] Interestingly, grade II-IV aGvHD was similar; however, impressive gains were seen for severe grade III-IV aGvHD. Chronic GvHD requiring immunosuppression also fared much better with PTCy.

Recently, a small European RCT compared PTCy/Tac/MMF to CyA/MMF in HLA-matched RIC PBSC HSCT. Grade II-IV aGvHD was lower with PTCy (*P*=0.014) while severe grade III-IV aGvHD was 6% *versus* 12%, respectively.[35] Importantly, CyA/MMF is considered inferior to Tac/MTX, and hence PTCy-based prophylaxis is being definitively evaluated in a large multi-center phase III RCT (BMT CTN 1703) of MUD PBSC RIC HSCT comparing PTCy/Tac/MMF with Tac/MTX.

Sirolimus - Siro is an mTOR inhibitor that synergizes with CNI in reducing Teff proliferation and activity. Siro inhibits CD8+ cells[36] while promoting Treg proliferation *in vitro*,[37] an attractive immunologic profile for GvHD prevention. Importantly, unlike CNI, it does not cause nephrotoxicity. Siro/MTX prophylaxis has been investigated in a large RCT of MAC HSCT, documenting similar grade II-IV but lower grade III-IV aGvHD compared to Tac/MTX.[38] In RIC transplants, a phase II RCT showed that combined Siro/Tac/MTX had less grade II-IV aGvHD but no survival benefit.[39] A recent phase III RCT of NMA HSCT concluded that adding Siro to CyA/MMF was superior to CyA/MMF.[40] Given that the combination of Tac and MMF is inferior to Tac/MTX in a phase II RCT in preventing grade II-IV aGvHD,[41] and CyA has also been shown to be inferior to Tac in the past for GvHD prophylaxis, it is unclear how these data impact centers that primarily use Tac/MTX-based regimens. Although less nephrotoxic, Siro has also been associated with higher rates of veno-occlusive disease (VOD), particularly with ablative busulfan and cyclophosphamide,[42] and is avoided in patients at a higher risk for VOD. It has also been associated with increased rates of TMA, particularly in combination with CNI.[43] Discontinuation of CNI typically resolves TMA in this setting.

Finally, the combination of Siro/PTCy as a CNI-free, less nephrotoxic regimen with acceptable rates of engraftment and aGvHD has been evaluated.[44] This is currently reserved for scenarios precluding CNI use (e.g., sickle cell HSCT, with renal dysfunction). Given the Treg-sparing effect of Siro,[45] novel combinations (e.g., with OX40L blockade) are being explored as GvHD prophylaxis platforms.[46]

Ex vivo T-cell depletion

A deeper understanding of transplant biology and the availability of sophisticated clinical-grade cell separation technology underpins advances in graft manipulation

involving both pan-T-cell and selective T-cell subset depletion for the clinic, reviewed below.

Pan-T-cell depletion - ex vivo TCD of the donor graft has been utilized as a method to prevent GvHD, considering competing risks of relapse and NRM. Methods have included monoclonal antibodies with or without complement,[47-50] immunotoxins,[51] and counter flow elutriation.[52]

Ex vivo TCD was evaluated in a multi-center RCT of TCD grafts *versus* CNI-based prophylaxis.[53] TCD was associated with lower rates of grade III-IV but not grade II-IV aGvHD, with no change in DFS. Graft failure and increased disease relapse (20% *vs*. 7%) was a concern, with increased relapse also noted in a seminal registry analysis.[54] Other studies also suggested increased rates of graft failure with TCD grafts, ameliorated by ATG or thiotepa conditioning to prevent host immune-mediated graft rejection.

T-cell depletion based on immunomagnetic CD34^{+} graft selection (to eliminate contaminating immune cells) was evaluated in a single-arm phase II multicenter trial and showed low rates of cGvHD and relapse.[55] This was compared to CNI-based prophylaxis in a retrospective analysis, where outcomes were similar, with lower rates of cGvHD in the CD34 arm.[56] *Ex vivo* TCD remains the primary mode of transplantation in certain centers, although infectious complications, particularly viral infections, can be problematic. To better define optimal GvHD prophylaxis, results from an ongoing RCT of *ex vivo* TCD *versus* PTCy with MMF only *versus* standard CNI-based regimen are eagerly awaited (*clinicaltrials.gov identifier: NCT02345850*).

Beyond pan-T-cell depletion, subset-selective T-cell depletion and modulation strategies to ameliorate GvHD without compromising GvL effect by using antibodies with narrow specificities has become an area of great interest.[50] Depletion of CD5^{+} T cells[51] and CD8^{+} T cells were tried in the 1990s, but abandoned primarily due to higher rates of relapse. Other novel strategies are discussed below.

α/β T-cell depletion - the majority of T lymphocytes express α/β T-cell receptors (TCR), while γ/δ TCR are expressed by 2-10% of circulating T cells. γ/δ T cells have important innate immune functions including rapid release of cytokines, and killing of tumor and virally infected cells without inducing GvHD.[57] They may have an important role in GvL effect and the preservation of NRM. Selective depletion of α/β T cells would preserve NK cells as well as γ/δ T cells. In a prospective study of 80 pediatric patients with acute leukemia, α/β TCD was studied with encouraging GRFS of 70%.[58] Ongoing studies are further evaluating this approach in adult and pediatric populations, including a CNI-free GvHD prophylaxis strategy for acute leukemia patients undergoing 1-2 locus MMUD MAC HSCT (*clinicaltrials.gov identifier: NCT03717480*).

CD45RA (naïve) T-cell depletion - conceptually, it is naïve T cells in the donor allograft that are primarily alloreactive. In a study in healthy individuals, the bulk of allo-HLA reactivity was derived from subsets enriched for naïve T cells.[59] Hence, removal of CD45RA^{+} naïve T cells from the donor graft could help prevent aGvHD alloreactivity. The CD45RA^{-} target fraction contains effector and central memory T cells that show preserved reactivity to common viral and fungal pathogens.[60] In a two-step immunomagnetic bead procedure for naïve TCD, Bleakley *et al.*[61] reported on a first-in-human single-arm trial (n=35) for patients with acute leukemia transplanted with HLA-matched related donors. Although 34 of 35 patients engrafted with lower rates of cGvHD, rates of aGvHD remained relatively high

(66%), suggesting a lack of efficacy with this approach alone.[62] A combinatorial approach using α/β TCD combined with CD45RA naïve cell depletion was not much better, with aGvHD rates in the 58% range.[63] Hence, for the moment, this approach remains only investigational.

CD6 depletion - CD6 is a co-stimulatory receptor, predominantly expressed on T cells that bind to activated leukocyte cell adhesion molecule (ALCAM), a ligand expressed on APC and various host tissues and plays an integral role in modulating T-cell activation, proliferation, differentiation and trafficking. CD6 depletion using a monoclonal antibody (mAb) (anti-T12, CD6) that recognized mature T cells but not other cellular elements (e.g., B, natural-killer [NK] cells, and myeloid precursors) was clinically evaluated in a single arm trial with 112 patients with a grade II-IV aGvHD rate of 18%.[48] More recently, itolizumab a humanized anti-CD6 mAb, was evaluated in human xenograft models, suggesting that itolizumab can modulate pathogenic Teff activity.[64] Itolizumab has been provided fast track status by the US Food and Drug Administration (FDA) for this indication and is undergoing evaluation in a phase I/II study for first-line treatment (with steroids) of severe aGvHD (*clinicaltrials.gov identifier: NCT03763318*).

Graft engineering: Treg/Tcon add back strategies - in haploHSCT, in the early 1990s, CD34^{+} cell selection was used by the Perugia group to generate T-cell depleted peripheral blood progenitor cell grafts. Although GvHD rates were low, there was poor immune reconstitution (IR) and high rates of infection.[65] The Perugia group then pioneered the use of a 'megadose' of CD34^{+} cells to facilitate engraftment and improve IR based on the increased tolerability effect of such a dose of CD34^{+} cells.[66] Subsequently, further TCD by negative selection of CD3/CD19^{+} cells was used. Most recently, CD34^{+} selection followed by graduated add back of Tregs and conventional T cells (Tcons) (in a 2:1 ratio) have been adopted, with promising early results for enhanced IR and GvL, but aGvHD remains a concern.[67] Further iterations of this approach may yield enhanced clinical benefit, despite their complexity and cost.

Regulatory T-cell enhancement

Tregs - Tregs are CD4^{+}CD25^{+}Foxp3^{+} cells which play an important role in immunologic homeostasis and the control of aberrant or overactive immune effectors. Tregs can be derived 'naturally' from the thymus (nTregs) or converted from CD4^{+}CD25^{-} cells (inducible or iTregs).[6] iTregs require IL-2 and TGF-β to fully develop their suppressive function. Blazar *et al.* showed that *ex vivo* activation and expansion of Treg is feasible, with efficacy in murine GvHD models.[68] Other approaches have utilized fucosylation[69] and TL1A/TNFRSF25 stimulation[70] for *ex vivo* Tregs. In clinical transplantation, they have confirmed the feasibility and safety of *ex vivo* Treg expansion and adoptive transfer, with preliminary clinical efficacy for aGvHD prevention in both cord[71] and haploHSCT.[67] However, concerns about stability of expanded Tregs has been a barrier to translation into the clinic.

Invariant natural killer T cells - invariant NK T (iNKT) cells are a rare T-lymphocyte subset which co-express both T- and NK-cell markers and are considered a bridge between innate and adaptive immunity. Their semi-invariant TCR recognizes glycolipid antigens presented by the major histocompatibility complex (MHC) class I-like molecule Cd1d. Despite their rarity, they have strong immunomodulatory functions through the secretion of IL-4 and IL-10,

as well as providing active immunologic surveillance against cancer.[72] Murine models suggested that iNKT cells have a protective effect against GvHD without impairing the GvL effect. This occurs in part *via* a switch of donor T cells to a Th2 cytokine profile and/or IL-4 dependent Treg expansion.[73,74] Observational studies suggest lower acute and chronic GvHD with improved iNKT cell reconstitution. Clinical translation has involved RIC HSCT utilizing total lymphoid irradiation plus ATG (TLI-ATG) conditioning (offering iNKT expansion in murine models) with promising outcomes,[75] as well as more direct *ex vivo* expansion and adoptive iNKT transfer peri-transplant, where clinical data are eagerly anticipated. KRN7000 (synthetic derivative of α-galactosylceramide and a CD1d ligand) when embedded in a lipid bilayer constitutes RGI-2001or REGiMMUNE which can expand FoxP3^{+} Tregs *via* iNKT cells in mice to reduce aGvHD lethality.[76] Recently a phase IIa trial of a combination of Siro and RGI-2001 showed lower incidence of overall and severe aGvHD in responders compared to non-responders.[77] Although promising, iNKT targeted approaches have not been widely adopted for the moment and more mature data are awaited.

Cytokine targeting

Tocilizumab - interleukin-6 (IL-6) is a key inflammatory cytokine in the early pathogenesis of aGvHD in murine models.[78] A logical next step was to investigate the role of IL-6 blocking agents in preventing aGvHD. Tocilizumab is a humanized mAb against the IL-6 receptor (IL-6R). Based on promising phase II data,[79] a placebo-controlled phase III study from Australia was reported, which, however, showed no significant difference in grades II-IV or III-IV aGvHD.[80] This is a salient reminder that, given the complex pathophysiology of aGvHD, with crosstalk between myriad cytokines and immune effector cells, it is possible that targeting multiple cytokine pathways will be required for efficacy.

Targeting T-cell co-stimulatory pathways

As mentioned previously, following initial engagement of an APC with the TCR, a number of secondary co-stimulatory signals come into play which are necessary to complete alloreactive T-cell activation, proliferation and eventual development of aGvHD. CD28 is a co-stimulatory receptor while CTLA-4 is a co-inhibitory receptor on the T cell, both of which bind to B7-1/CD80 and B7-2/CD86 ligands on APC. CTLA-4-Ig (abatacept) is the soluble extracellular portion of CTLA-4 complexed with immunoglobulin heavy chain which blocks CD28/CTLA-4 (CD28>CTLA-4) co-stimulation with an eventual T-cell inhibitory signal. Blazar *et al.* showed in murine models that blockade of the CD28/CTLA-4 and CD80/CD86 interaction reduced aGvHD lethality.[81] Following a promising feasibility study, Kean *et al.* then tested abatacept added to SOC *versus* SOC in a phase II RCT with 8/8 and 7/8 HLA-matched donors. There was significant reduction in grades III-IV aGvHD in the abatacept arm with improved OS[82] leading to FDA breakthrough designation for this drug. To avoid the undesirable effect of concomitantly blocking inhibitory pathways, more selective approaches to CD28 blockade are being investigated. FR104, an antagonistic CD28-specific pegylated-Fab' has shown promise with and without Siro in non-human primate models, with the caveat that a worrying inhibitory effect was seen on

the INF-γ axis with deaths secondary to sepsis.[83] The modulation of co-stimulatory/inhibitory pathways is one of the important new frontiers in aGvHD prevention.

These prophylactic strategies, along with the level of evidence supporting them, are summarized in Table 1.

Advances in acute graft-*versus*-host disease therapy

Systemic steroids, while not FDA-approved for this indication, remain a cornerstone of the initial treatment of moderate-severe aGvHD. In a seminal study, Blazar *et al.* showed that first-line therapy of aGvHD with corticosteroids (60 mg daily followed by an 8-week taper) resulted in response rates of 50% and 1-year survival of 53%.[84] Higher doses of steroids did not result in better outcomes. In a study comparing 10 mg/kg to 2 mg/kg of methylprednisone, both resulted in transplant mortality of 30% at one year with no improvement in aGvHD responses at higher doses.[85] SR-aGvHD treatment remains a difficult problem, with 6-month survival in the 50% range, and long-term survival of only 5-30%.[86] Stratification systems such as the Minnesota risk score that take into account patterns of aGvHD by target organ involvement can further refine the prediction of transplant-related mortality, and are being considered in clinical trial risk stratification.[84] Finally, even when aGvHD is controlled, patients often succumb to infections exacerbated by additional immunosuppressive therapies. Novel therapies are, therefore, a critical unmet need. Here we outline some of the more promising approaches currently available or in early translation to the clinic.

Cytokine pathways

JAK-STAT pathway - the Janus Kinases (JAK) are intracellular tyrosine kinases investigated as GvHD therapeutic targets given their important role in cytokine signaling and effects on immune effector cells. In murine models, the role of IFNγ on T-lymphocyte trafficking to GvHD target organs (particularly the GI tract) *via* CXCR upregulation was studied. Inhibition of interferon (IFN)γR signaling *via* JAK1/JAK2 inhibitors resulted in decreased CXCR3 expression and altered Teff trafficking to target organs, reducing GvHD.[87] Ruxolitinib (Rux) is a potent oral JAK-1/JAK-2 inhibitor. In a proof of concept study, Rux reduced Teff proliferation and activity, increased Tregs and decreased cytokine production, with excellent responses in six SR-aGvHD patients.[88]

A retrospective survey of off-label Rux in SR aGvHD documented overall response rate (ORR) of 81.5% (complete responses [CR] 46%). Cytopenias and cytomegalovirus (CMV) reactivation were seen.[89] A phase II single-arm multicenter study of Rux (REACH-1) in 71 patients documented ORR at 28 days of 54.9% (complete remission/CR, 26.8%), irrespective of aGvHD grade and steroid refractoriness.[90] In addition to cytopenias and CMV reactivation, serious bacterial infections were reported. The phase III RCT of Rux *versus* investigator's choice for SR aGvHD has now been reported (REACH-2). Rux was superior in terms of ORR; however, there was no difference in cumulative incidence of 18-month NRM.[91] Infections and cytopenias remain limiting toxicities. The FDA has approved Rux for SR aGvHD.

In contrast, failure of the selective JAK1 inhibitor itaci-

Table 1. Prophylactic strategies for acute graft-*versus*-host-disease.

Prophylaxis strategy	Intervention	Level of evidence	Comments
In vivo T-cell depletion/modulation	Calcineurin inhibitors (tacrolimus, cyclosporine)	Phase III RCT	Tac *vs.* CyA, less aGvHD with Tac but no survival advantage.
	ATG	Phase III RCT	Either no reduction in aGvHD or reduction in aGvHD with significant increase in NRM.
	PTCy	Phase II/III RCT	Lower rates of severe aGvHD but not grades II-IV aGvHD compared to CNI.
	Sirolimus (mTOR inhibitor)	Phase II/III RCT	Lower rates of grades III-IV aGvHD in MAC and grades II-IV aGvHD in RIC HSCT but no survival advantage.
Ex vivo T-cell depletion	Pan T-cell depletion	Phase II/III RCT	Lower rates of grades III-IV aGvHD but no survival advantage. Graft failure sometimes an issue.
	α/β T-cell depletion	Phase II single arm	Promising GRFS of 70% in pediatric acute leukemia. Adult studies ongoing.
	CD45RA (naïve) T-cell depletion	First-in-human phase I/II	High aGvHD rates of 66% and hence investigational only for the moment.
	CD6 depletion Itolizumab	Phase II single arm Ongoing phase I/II	aGvHD rates of 18%; monoclonal antibody (itolizumab) with FDA fast-track status now being tested.
	Treg:Tcon add back strategies	First-in-human phase I/II	Operationally complex and for the moment difficult to generalize.
Regulatory T-cell enhancement	Tregs	Phase I	Preliminary safety results encouraging; concerns about stability of *ex vivo* Treg expansion.
	iNKT cells	Phase I/II	TLI-ATG regimen *via* iNKT cells with reported GvHD in only 2 of 37 recipients. REGIMMUNE/sirolimus combination promising.
Cytokine targeting	Tociluzumab	Phase III RCT	Tocilizumab *vs.* placebo; no improvement in aGvHD of any grade.
Targeting T-cell co-stimulatory pathways	CD28/CTLA-4 targeting (abatacept)	Phase II RCT	CTLA-4 Ig (abatacept)+SOC compared to SOC in 8/8 and 7/8 HLA-matched HSCT with lower rates of grades III-IV aGvHD outcomes and OS leading to FDA breakthrough designation.

aGvD: acute graft-*versus*-host disease; HSCT: hematopoietic stem cell transplantation; PTCy: post-transplant cyclophosphamide; ATG: anti-thymocyte globulin; RCT: randomized controlled trial; iNKT: invariant natural killer T cells; OS: overall survival; SOC: standard of care; FDA: US Food and Drug Administration; NRM: non-relapse mortality; aGvHD: acute graft-*versus*-host disease; GRFS: GvHD free, relapse-free survival; CNI: calcineurin inhibitor.

tinib when added to steroids (*vs.* steroids alone) for upfront therapy of aGvHD in the closed GRAVITAS 301 trial (*clinicaltrials.gov NCT03139604*) is notable. JAK-1 inhibition is capable of selectively suppressing Th1 and Th17 Teff cell subsets, with preserved activation of anti-inflammatory Treg cells dependent on the JAK2/JAK3 pathway; however, the drug failed clinical efficacy, highlighting limitations in clinical trial design, optimal therapeutic target identification, or both. Data on the efficacy of selective JAK2 inhibitors in aGvHD are eagerly awaited, but it is possible that combination JAK1/2 blockade may be required for appropriate suppression of activation in Teff cells.

Although a number of cytokine-directed therapies previously failed in the therapy of aGvHD (denileukin diftitox, tocilizumab, anti TNF-α), the efficacy of Rux is a milestone in the field, and a testament to the critical role of a 'cytokine storm' in aGvHD.

Alpha-1-antitrypsin - alpha-1-antitrypsin (AAT) is a serine protease inhibitor produced by the liver which has myriad functions including inhibition of proinflammatory plasma cytokines and induction of anti-inflammatory IL10, and *in vivo* induction of Treg. In preclinical aGvHD models, AAT reduced inflammatory cytokines, altered the ratio of Teff and Tregs and reduced levels of DAMP.[92] In a phase I/II open label single center study in SR aGvHD patients (n=12), responses were seen in 8 of 12 patients with no significant toxicity.[93] In a larger phase II multicenter study (n=40), ORR at D28 was 65% (CR 35%).[94] Upfront AAT is being evaluated in a Blood and Marrow Transplant Clinical

Trials Network (BMT-CTN) phase III RCT evaluating corticosteroids ± AAT (*clinicaltrials.gov NCT04167514*) as a promising non-toxic agent for high-risk aGvHD.

Targeting lymphocyte trafficking

Vedolizumab - lymphocyte trafficking to GvHD target organs is a key event leading to aGvHD. In the lower GI tract, Peyer's patches (PP) and gut-associated lymphoid tissue (GALT) are the targets for alloreactive CD8⁺ T cells. Gut-tropic CD8⁺ cells express high levels of integrin β7 (α4β7) that binds its ligand mucosal addressin cell adhesion molecule 1 (MAdCAM 1) in the PP and GALT. Vedolizumab, a humanized mAb, targets α4β7 integrins and prevents Teff trafficking to the gut. A small proof of concept study (n=6) demonstrated responses in all patients with SR lower GI GvHD. In an international, retrospective review to evaluate the off-label use of vedolizumab (n=29), ORR was 64% and OS at 6 months was 54%.[95] CMV reactivation and *Clostridium difficile* colitis were noted. Natalizumab, a selective α4 subunit adhesion molecule inhibitor was studied in a phase II study with a response rate of approximately 30%.[96] Vedolizumab is being studied in larger prophylactic (*clinicaltrials.gov NCT03657160*) and therapeutic (*clinicaltrials.gov NCT02993783*) trials for aGvHD.

Targeting immunologic tolerance

Extracorporeal photochemotherapy - extracorporeal photochemotherapy (ECP) has been used for cGvHD for decades, and more recently for aGvHD with some suc-

cess. Although the mechanism by which ECP improves GvHD is a matter of debate, its immunomodulatory effects include Treg upregulation, a change from Th1 to Th2 cytokine profile, as well as modulation of APC.[97] Importantly ECP may not result in additional immunosuppression in GvHD patients. In a RCT evaluating ECP in cGvHD therapy, there was no increased risk of infection in the ECP arm,[98] which, if also true in the aGvHD setting, would be a major benefit. Initially evaluated in pediatric cohorts, ECP resulted in a response rate of 67% in a small study of adult aGvHD.[99] In another small ECP study (n=23), CR was achieved in 70%, 42% and 0% of patients with grades II, III and IV aGvHD respectively. With regards to end-organ based efficacy, complete responses were seen in 66%, 27% and 40% of patients with skin, liver and gut involvement, respectively.[100] However, the data are limited to small non-randomized studies and efficacy needs to be confirmed.

Finally, another novel therapeutic intervention for aGvHD, fecal microbiota transplantation (FMT), is further discussed in the section on microbiome and the role of dysbiosis.

These therapeutic strategies, along with the level of evidence supporting them, are summarized in Table 2.

Future trends

Finally, we highlight the emerging role of early prognostic biomarkers as well as the potentially critical role of the intestinal microbiome in influencing aGvHD and transplant outcomes.

Novel biomarkers in acute graft-*versus*-host disease

Identifying predictive biomarkers for aGvHD development and/or prognosis has been an important question in the field. Hypothesis-driven markers based on the pathophysiology of aGvHD include acute phase reactants (e.g., IL-6, C-reactive protein [CRP]), Th1 cytokines (e.g., IL-12, IL-18), anti-inflammatory cytokines (e.g., IL-10, TGF-β), other circulating markers (e.g., IL-8, HGF, cytokeratin-18, CD30), and lymphocyte trafficking molecules (e.g., CXCL10, CCL8) have been evaluated with limited success. In contrast, unbiased marker discovery typically involved proteomic screening of GvHD and non-GvHD samples. In a discovery study from Ann Arbor, IL-2Rα, TNFR1, HGF and IL-8 identified early after aGvHD onset demonstrated impressive accuracy confirmed in a larger validation set.[101] Another panel comprising IL-2Rα, TNFR1 and elafin has also been validated.[102]

The Mount Sinai Acute GvHD International Consortium (MAGIC) was established to identify potential biomarkers to risk stratify GvHD. Investigators tested previously identified biomarkers, namely suppressor of tumorigenicity-2 (ST2) and regenerating islet-derived protein 3-α (REG3α), in SR aGvHD and found that marker elevation 7 days after aGvHD was a better predictor of NRM than the Minnesota clinical risk score.[103] Another approach has evaluated markers of endothelial toxicity documenting follistatin and endoglin as being associated with higher rates of grade III-IV aGvHD and NRM.[104]

The appropriate clinical application of these biomarker panels is a complex issue, with the underlying principle that test results should change therapy and, ideally, outcome. Risk-adapted approaches have proposed using these panels

in two different ways: (i) early post-transplant prior to diagnosis of aGvHD, with allocation of high-risk patients to novel GvHD trials; and (ii) after the diagnosis of aGvHD, to stratify patients at high NRM risk and risk-adapt therapy accordingly.

Future clinical trials that use biomarkers to risk stratify aGvHD patients for eligibility or therapy will be important to prospectively evaluate their utility as a first step to their broader use in clinical practice.

The microbiome in acute graft-*versus*-host disease

The many micro-organisms which constitute the human gut are collectively called the intestinal microbiota while their genetic make-up has often been referred to as the 'microbiome'.[105] Diversity is a hallmark of the healthy gut microbiome. There is a growing appreciation of the role of the microbiome in various health and disease states. In HSCT, the loss of microbiota diversity (dysbiosis) has been associated with the risk of aGvHD.[105]

This association between aGvHD and gut dysbiosis relates to immunologic and metabolic imbalances in the gut wrought by HSCT, with loss of diversity of the microbiome. Under normal circumstances, diverse gut commensals result in healthy tissue immune cells, including recruitment of Treg cells, secretion of TGF-β and IL-10, as well as TH17 cells secreting IL-17 and IL-22.[106] Another protective immune response modulated by gut bacteria relates to their production of short chain fatty acids (SCFA), a nutritional source for intestinal epithelial cells. Disruption of the intestinal microbiome triggered by conditioning chemoradiotherapy and antibiotic use during transplantation results in overgrowth of bacteria (e.g., enterococci, *Proteus spp.*), and reduction in firmicutes (e.g., *Blautia spp.*), which generally are producers of SCFA, is considered an inciting stimulus for GvHD.[107]

Further studies are needed to develop actionable targets in this arena. It is a complex endeavor given the variations in gut microbiome over different geographical areas, across transplant strategies, and inpatient and outpatient settings. It is heartening that a recent study from four international centers showed that the patterns of loss of microbiome diversity during HSCT was similar across countries, and that lower diversity at time of neutrophil engraftment was associated with higher mortality.[108] A large biorepository of stool samples along with blood and other samples is being built as the correlative arm of the large BMT CTN RCT 1703 (Mi-immune) study in which the biology of the microbiome and correlations with transplant outcomes will be interrogated.

Fecal microbiota transplant as an effort to repopulate the gut with normal gut flora has been proposed as a means to control aGvHD, based on data limited to pilot studies[109] and limited case series.[110] Infection with extended spectrum β-lactamase (ESBL) producing *Escherichia coli* bacteria has been reported in at least two transplant patients post allogeneic transplantation who underwent FMT, one of whom died.[111] Hence the safety and efficacy of FMT in aGvHD remains an open question.

Conclusion

To summarize, aGvHD remains an important problem in HSCT. However, where effective treatment options had previously been very limited, there are now multiple exciting translational advances.

Table 2. Therapeutic strategies for acute graft-*versus*-host disease.

Prophylaxis strategy	Intervention	Level of evidence	Comments
Non-specific immunosuppression	Corticosteroids	Phase II/III RCT	RCT comparing different steroid doses, no RCT comparing steroids *vs.* placebo.
Cytokine targeting	JAK-2 inhibition: ruxolitinib	Phase III RCT	Ruxolitinib *vs.* investigator's choice in SR-aGvHD: superior ORR but no difference in 18-month NRM.
	JAK-1 inhibition: itacitinib	Phase III RCT	Itacitinib *vs.* itacitinib+steroids in upfront therapy: negative trial.
	Alpha-1 anti-trypsin (AAT)	Phase II, ongoing Phase III RCT	ORR of 65% in SR-aGvHD in Phase II, ongoing phase III RCT of AAT+steroids *vs.* steroids alone for high-risk aGvHD.
Targeting lymphocyte trafficking	Vedolizumab	Proof-of-concept, retrospective review, ongoing Phase II randomized study	ORR of 64% in retrospective studies, CMV reactivation, *C Diff*. colitis seen
	Natalizumab	Phase II single-arm	ORR of approx. 30%
Targeting immunologic tolerance	Extra-corporeal photochemotherapy	Phase II single-arm	ORR varies from 40-70% in small non-randomized studies.
	NOTCH inhibition	Investigational	No reported clinical studies at this time
Targeting dysbiosis	Fecal microbiota transplant	Pilot studies, case series	Concern for fatal bloodstream infection hence still investigational

aGvD: acute graft-*versus*-host disease; AAT: alpha-1-antitrypsin; aGvHD: acute graft-*versus*-host disease; ATG: anti-thymocyte globulin; *C Diff.: Clostridium difficile* colitis; CMV: cytomegalovirus; HSCT: hematopoietic stem cell transplantation; NRM: non-relapse mortality; ORR: overall response rate; RCT: randomized controlled trial; SR: steroid-refractory.

In the arena of prevention, PTCy-based GvHD prophylaxis has been a significant advance and some selective methods of T-cell depletion and modulation of co-stimulatory pathways appear promising. In the therapeutic arena, cytokine targeting with Rux is an exciting novel therapy for SR-aGvHD, while immunomodulatory strategies (e.g., ECP, AAT) offer therapeutic potential without immunosuppressive toxicity, and strategies targeting lymphocyte trafficking and inhibition of key canonical pathways (e.g., Notch) offer future potential. For the more long-term future, the importance of the gut microbiome in aGvHD is becoming increasingly apparent, and offers an opportunity for future therapeutic targeting (e.g., probiotics, metabolic modifications).

A long-term rational approach to aGvHD care would involve precision prognostics pre- and peri-transplantation (e.g., plasma biomarkers, microbiota dysbiosis, etc.) to select patients for innovative GvHD preventive strategies, as well as the early identification of high-risk patients at aGvHD onset, for novel treatment trials, ideally avoiding additional immunologic dysfunction or impairing GvL.

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