New insights into the basic biology of acute graft-versus-host-disease

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New insights into the basic biology of acute graft-versus-host-disease

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ABSTRACT

Although allogeneic hematopoietic stem cell transplantation is an important therapy for many hematologic and non-hematologic diseases, acute graft-versus-host disease (aGvHD) is a major obstacle to its success. The pathogenesis of aGvHD is divided into three distinct phases which occur largely as the result of interactions between infused donor T cells and numerous cell types of both hematopoietic and non-hematopoietic origin. In light of the disease’s immensely complex biology, epigenetics has emerged as a framework with which to examine aGvHD. This review focuses on new findings that clarify the roles that specific epigenetic regulators play in T-cell-mediated aGvHD development and discusses how their modulation could disrupt that process with beneficial effects. DNA methyltransferases, histone methyltransferases and histone deacetylases are the most closely studied regulators across aGvHD priming, induction and effector phases and have been manipulated using drugs and other methods in both murine models and clinical trials, with varying degrees of success.

Antigen-presenting cells, effector T cells and memory T cells, among others, are targeted and affected by these regulators in different ways. Finally, our review highlights new directions for study and potential novel targets for modulation to abrogate aGvHD.

Introduction

The success of allogeneic hematopoietic stem cell transplantation (allo-HSCT) is significantly hampered by acute graft-versus-host disease (aGvHD), which is caused by donor T cells that recognize and react to histocompatibility differences between the donor and host. It occurs in sequential priming, induction and effector phases (Figure 1). During priming, preparative irradiation and chemotherapeutic regimens for allo-HSCT can damage the patient’s tissues, leading to release of damage-associated molecular patterns (DAMP) and pathogen-associated molecular patterns (PAMP), as well as activation of host antigen-presenting cells (APC) such as dendritic cells. Activated APC, including hematopoietic and non-hematopoietic cells, upregulate antigen-presenting molecules and costimulatory molecules to prime transplanted donor T cells. During induction, T-cell receptors on donor T cells react to alloantigens presented by host APC and undergo robust proliferation and differentiation into effector T cells that produce pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-17. Upon persistent exposure to host alloantigens, most of these effector cells (~90%) undergo apoptotic contraction, but a proportion survive and become memory T cells. The final effector phase is characterized by infiltration of alloreactive effector cells into aGvHD target organs. Tissues already damaged by preparative treatments produce chemokines, recruiting T cells to their vicinity. The effector T cells recognize and react to host alloantigens, mediating host tissue injury. The damaged host tissues recruit more alloreactive T cells and other types of inflammatory cells (e.g., monocytes/macrophages and granulocytes), leading to feed-forward amplification and continuation of aGvHD (Figure 1). Chronic GvHD may arise following or independently of aGvHD, but due to the conditions’ differing pathogeneses and clinical manifestations, chronic GvHD will not be discussed in this review.

Because aGvHD is T-cell-mediated, significant progress has been made in understanding how alloreactive T cells are induced and sustained. APC may be primed...
to produce special cytokines (e.g., IL-12, IL-23) and Notch ligands (e.g., Delta-like 1 and 4; DLL1 and DLL4) which instruct antigen-activated T cells to differentiate into distinct lineages of GvHD-mediating effector T cells.5,12-15 Other groups have reviewed these topics elegantly, so we focus on a related area of investigation: understanding how extracellular stimuli are converted to gene programs that promote or abrogate alloreactive T-cell development and responses, and leveraging them to reduce aGvHD.

Epigenetic modifications are one such mechanism. Epigenetics refers to heritable molecular determinants of phenotype that are independent of DNA sequence. Major contributors include DNA methylation on cytosine nucleotides, histone modification and chromatin structure. Proteins governing these modifications have loosely been termed epigenetic regulators.16 This review will discuss advances in our understanding of epigenetic regulation, either by direct effects or via interactions with other molecules, of alloreactive T-cell responses and these responses’ roles in aGvHD; we identify the roles that specific regulators play and interventions targeting these regulators for aGvHD prevention and treatment (Table 1). We also acknowledge the contributions of non-hematopoietic cells to the development of aGvHD, whether via their own function or their impact on T cells.

**Epigenetic effects on and sensitization of antigen-presenting cells**

To allow for proper engraftment, allo-HSCT patients may undergo conditioning regimens before donor T cells are infused. Consequently, DAMP from injured cells, PAMP from gut bacteria and pro-inflammatory cytokines are released, priming APC.1 In the setting of murine allo-HSCT, non-hematopoietic APC, alongside professional hematopoietic APC, are also known to prime alloreactive T cells.2,5,7 Upon activation following tissue damage, APC upregulate major histocompatibility complex class II and
costimulatory molecules (e.g., CD40, CD80, CD86) and secrete cytokines (e.g., IL-4, IL-12, IL-23, DLL1, DLL4), shaping T-cell responses.\textsuperscript{12,13,20,23} Immunosuppressive molecules such as IL-10, indoleamine-2,3-dioxygenase (IDO) and programmed death ligand 1 (PD-L1) may be upregulated to repress alloreactive T-cell responses, shifting them to become tolerogenic.\textsuperscript{10,21} Epigenetic regulators convert these signals into the aforementioned markers and molecules.

**Histone deacetylases’ multiple functions in the sensitization of hematopoietic antigen-presenting cells**

Two classes of enzyme regulate histone acetylation status: histone acetyltransferases (HAT) and histone deacetylases (HDAC). HAT acetylate histone lysine substrates and open compacted chromatin, allowing transcription factors to access DNA.\textsuperscript{22} HDAC decrease histone lysine tail acetylation, repressing gene transcription.\textsuperscript{16} Epigenetic studies of hematopoietic APC sensitization have primarily focused on the impact of HDAC (Figure 1).

One of the first studies anchoring epigenetics to acute GvHD, helmed by Reddy and colleagues, brought to light the role of histone acetylation in aGvHD.\textsuperscript{21} HDAC are important for APC production of pro-inflammatory cytokines and immunosuppressive molecules.\textsuperscript{22,23} Preclinical studies have shown that in vivo administration of the pan-HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) reduced aGvHD.\textsuperscript{24} SAHA treatment did not impair T-cell responses to host antigens, but significantly decreased the production of inflammatory cytokines, TNF-α, IL-1 and IFN-γ, by APC. Subsequent studies confirmed that treatment with SAHA resulted in a marked decrease in pro-inflammatory cytokines (e.g., TNF-α, IL-12, IL-6) in APC, which are important in promoting alloreactive T-cell responses.\textsuperscript{20} SAHA inhibited IL-6 production in dendritic cells stimulated by variable toll-like receptor (TLR) agonists (e.g., TLR2, TLR3, TLR4 and TLR9). In lipopolysaccharide-stimulated dendritic cells, SAHA treatment induced high-level expression of IDO to suppress alloreactive T-cell responses.\textsuperscript{20}

Villagran et al. highlighted the importance of HDAC11 in repressing the negative regulation that murine APC exerted on T-cell responses.\textsuperscript{23} Using chromatin immunoprecipitation, researchers determined that upon overexpression of HDAC11 in APC, there was decreased acetylation of histone 4 (H4) at the distal Il10 promoter. This was associated with decreased IL-10 transcription upon lipopolysaccharide stimulation. In contrast, HDAC11 knockdown using shRNA had the opposite effect, resulting in the induction of IL-10 expression. Accordingly, silencing HDAC11 expression in APC impaired antigen-specific T-cell responses, whereas overexpression of HDAC11 in APC caused tolerant CD4+ T cells to transition to an immunogenic phenotype.\textsuperscript{24}

HDAC6 is a positive regulator of tolerogenic APC. Normally, HDAC6 forms a complex with signal transducer and activator of transcription (STAT) 3 that is recruited to the Il10 promoter. Silencing HDAC6 resulted in decreased STAT3 phosphorylation and reduced IL-10 production.\textsuperscript{25} The opposing effects of HDAC11 and HDAC6

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**Table 1. Preclinical studies of acute graft-versus-host disease investigating epigenetic mechanisms.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cells</th>
<th>Key findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>EZH2</td>
<td>CD8+/CD4+ T cells</td>
<td>Enh2 KO impairs proliferation, differentiation and expansion; it reduces aGvHD but preserves GvL.\textsuperscript{20} Enh2 inhibition with D2Nep inhibits ongoing GvHD but preserves the Gv effect.\textsuperscript{26}</td>
</tr>
<tr>
<td></td>
<td>CD8+/CD4+ T cells</td>
<td>Administration of the EZH2 inhibitor GSK126, which specifically reduces H3K27me3 without affecting the protein, failed to prevent aGvHD in mice. In contrast, targeting T-cell EZH2 protein by inhibiting HSP90 reduced aGvHD in mice undergoing allo-HSCT.\textsuperscript{27}</td>
</tr>
<tr>
<td></td>
<td>CD8+ T cells</td>
<td>EZH2 controls CD8+ T memory precursor formation and antitumor activity.\textsuperscript{28}</td>
</tr>
<tr>
<td>DNMT</td>
<td>CD8+/CD4+ T cells</td>
<td>Inhibition impairs activation, expansion, and secretion of cytokines.\textsuperscript{29}</td>
</tr>
<tr>
<td></td>
<td>Treg</td>
<td>Inhibition by Aza increased Treg frequency through hypomethylation of Foxp3.\textsuperscript{30,31,32}</td>
</tr>
<tr>
<td>HDAC (Pan)</td>
<td>CD8+/CD4+ T cells</td>
<td>Pan-inhibition using SAHA results in reduced proliferative and cytotoxic activity of anti-CD3 activated T cells.\textsuperscript{33}</td>
</tr>
<tr>
<td>HDAC6</td>
<td>CD8+ T cells</td>
<td>Inhibition of HDAC6 impairs CD8+ T-cell proliferation and function in a GvHD-like model.\textsuperscript{34}</td>
</tr>
<tr>
<td>HDAC11</td>
<td>CD8+/CD4+ T cells</td>
<td>KO of Hdac11 increased T-cell proliferation rates and effector function resulting in more rapid and potent aGvHD.\textsuperscript{35}</td>
</tr>
<tr>
<td>SIRT3</td>
<td>CD8+/CD4+ T cells</td>
<td>Loss of Sirt3 results in decreased aGvHD severity due to decreased activation and production of ROS while maintaining GvT.\textsuperscript{36}</td>
</tr>
<tr>
<td>HDAC (Pan)</td>
<td>APC</td>
<td>Pan-inhibition of HDAC with SAHA reduced aGvHD, resulting in a drastic decrease in pro-inflammatory cytokine expression and induced high level expression of IDO to suppress alloreactive T cells.\textsuperscript{37,38}</td>
</tr>
<tr>
<td>HDAC (Pan)</td>
<td>IEC</td>
<td>Butyrate treatment reduced GvHD severity, improved IEC junction integrity and reduced IEC apoptosis. In addition, the decrease in H4 acetylation, butyrate transporter and receptor levels due to allo-HSCT inflammation were reversed.\textsuperscript{39}</td>
</tr>
</tbody>
</table>

EZH2: enhancer of zeste homolog 2; KO: knockout; aGvHD: acute graft-versus-host disease; GvL: graft-versus-leukemia; H3K27me3: histone 3 lysine 27 trimethylation; HSP90: heat shock protein 90; allo-HSCT: allogeneic hematopoietic stem cell transplantation; DNMT: DNA methyltransferases; Treg: regulatory T cell; Aza: 5-azacytidine; HDAC: histone deacetylase; SAHA: suberoylanilide hydroxamic acid; ROS: reactive oxygen species; GvT: graft-versus-tumor; IDO: indoleamine-2,3-dioxygenase; APC: antigen-presenting cell; IEC: intestinal epithelial cell; H4: histone 4
in regulating IL-10, a cytokine that can tip the balance between reactivity and tolerance in dendritic cells, underlining the importance of understanding how individual HDAC regulate APC function. More specific HDAC inhibitors, rather than pan-HDAC inhibitors, may be appropriate targets for further study.

**Sensitization of non-hematopoietic cells**

Emerging evidence indicates the importance of non-hematopoietic cells in aGvHD. Koyama et al. demonstrated that antigen presentation from non-hematopoietic cells could induce lethal aGvHD independently of T-cell interactions with hematopoietic APC. Microbiota in the gastrointestinal tract can secrete IL-12 to induce major histocompatibility complex II upregulation on intestinal epithelial cells (IEC), initiating lethal aGvHD. In addition, fibroblastic stromal cells in the lymph nodes have been shown to drive aGvHD through the presentation of Delta-like Notch ligands, DLL1 and DLL4 specifically. Inhibition of the Notch ligands and receptors conferred protection against GvHD in murine models. However, we have a limited understanding of the epigenetic effects these non-hematopoietic cells have on aGvHD. In the light of these striking findings, this represents an important avenue for future investigation.

**Epigenetic control of alloreactive T cells**

Upon encountering allogeneic host APC, infused donor T cells are activated and undergo robust proliferation and differentiation into effector T cells (Figure 1), which include IFN-γ-producing CD4+ Th1 cells, IL-4-producing CD4+ Th2 cells, IL-17-producing CD4+ Th17 cells and cytotoxic CD8+ T cells. Effector T cells mediate tissue injury during aGvHD. Alloantigen-sensitized donor T cells can also become memory T cells that mediate persistence of host tissue injury. Over the past two decades, much research has been undertaken to understand the molecular mechanisms that control the generation and maintenance of alloreactive effector and memory T cells during the induction phase of aGvHD.

**Epigenetic programming of effector T-cell responses EZH2**

Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase that catalyzes histone 3 lysine 27 trimethylation (H3K27me3), which primarily silences genes, and is a core component of the polycomb repressive complex-2 (PRC2). Evidence suggests that EZH2 is involved in Th1 and Th2 polarization as well as the proliferation and differentiation of hematopoietic stem cells. EZH2 is also involved in cancer development and progression, which has stimulated efforts to develop methods of inhibiting the enzyme. EZH2 plays an essential role in T-cell immune responses. Studies by our group and others have demonstrated the functional relevance of EZH2 in regulating antigen-driven T-cell responses. Using experimental murine models, we discovered EZH2’s role in regulating allogeneic T-cell proliferation, differentiation and function. Conditional loss of Ezh2 in donor T cells inhibited aGvHD in mice. Although EZH2-deficient T cells could be activated and underwent initial proliferation, their ability to undergo continual proliferation and expansion became defective during the later stage of aGvHD induction. Unexpectedly, as a gene silencer, EZH2 was required to promote the expression of transcription factors T-bet and STAT4, which are critical for effector differentiation. Subsequent studies revealed that EZH2 regulation of transcription factor expression and function depends on the differentiation stage of antigen-driven T cells. Ezh2 knockout in T cells impaired their differentiation into IFN-γ-producing effector cells. However, Ezh2 ablation, EZH2 protein inhibition and EZH2 protein destabilization all did not affect graft-versus-leukemia activity, leading to improved overall survival in recipients. Thus, targeting EZH2 may represent an effective therapeutic strategy for aGvHD prevention and treatment.

**HDAC1, HDAC6 and HDAC11**

HDAC are important for regulating the proliferative and cytotoxic capabilities of activated T cells. Pharmacological inhibition of HDAC by SAHA has been shown to suppress T-cell-receptor-mediated T-cell proliferation through the induction of apoptosis. Hdac1 deletion in a murine allergic asthma model showed a significant increase in airway inflammation and Th2 cytokine production. Upon Hdac1 deletion, in vitro studies noted an enhanced induction of Th1 and Th2 cells. Thus, HDAC1 plays a negative regulatory role for the functions of Th1 and Th2 subsets. In T cells, HDAC11 may suppress the graft-versus-host reaction. Hdac11 knockout resulted in increased T-cell proliferation and release of pro-inflammatory cytokines associated with upregulation of eomesodermin (EOMES) and T-bet which are important in effector differentiation. Indeed, decreased expression of HDAC11 exacerbated aGvHD in mice.

HDAC6 can deacetylate non-histone proteins such as heat shock protein 90 (HSP90). Acetylation disrupts HSP90’s chaperone function and inhibits LCK phosphorylation. In a GvHD-like model involving OT-I T-cell transplants to K14-mnOVA mice, control mice developed mucosal and skin lesions, while inhibition of HDAC6 using a specific inhibitor, ACY-1215, prevented similar lesions from forming for 14 days after transplantation. This protective effect was accompanied by dramatically decreased production of CD8+ effector T cells that secreted high levels of IL-2 and IFN-γ. Further studies of these HDAC should be conducted in GvHD models to definitively validate their roles in driving or mitigating aGvHD.

**SIRT3**

SIRT3 is a mitochondrial HDAC that regulates metabolic enzyme acetylation. SIRT3 is expressed in metabolically stressed cells such as alloreactive T cells. Loss of SIRT3 in donor T cells led to decreased GvHD severity in mice. The protective effect associated with Sirt3 deletion was associated with a reduction in reactive oxygen species and decreased activation and expression of chemokine receptor CXCR3.

**DNMT**

Because DNA methyltransferases (DNMT) – DNMT1, DNMT3A, DNMT3B and DNMT3L – can enact global transcription suppression, they have been widely studied in the context of immunity. DNMT1 is the principal enzyme that maintains methylation across DNA replication. DNMT3A and DNMT3B contribute to methylation maintenance and are also responsible for de novo DNA
methylation.41 DNMT inhibitors such as 5-azacytidine (Aza) have been shown to impair T-cell activation, expansion and cytokine release early in culture via downregulation of cell cycle- and cytokine-related genes.42 Sánchez-Abarca et al. treated mice undergoing allo-HSCT with Aza and found that early treatment prevented aGvHD development without increasing regulatory T cells (Treg); researchers speculated that this was likely due to Aza inhibition of T-cell expansion, which had been expanded in vitro.43 In a humanized murine allo-HSCT, xenogenic GVHD model, Aza treatment was also noted to decrease the frequency of IFN-γ-secreting CD4+ human T cells and granzyme B- and perforin 1-secreting CD8+ human T cells in vivo.44 Using Dnmt3a conditional knockout mice, a recent study by Youngblood et al. revealed the importance of DNMT3A in the regulation of T-cell exhaustion.45 Moving forward, similar genetic approaches will be useful in understanding the mechanisms of the precise effects of DNMT inhibition on aGvHD.

Epigenetic programming of alloreactive memory T cells

A hallmark of aGvHD is cytopathic injury mediated by persistent alloreactive effector T cells, which can occur within weeks and persist for years after transplantation.17,92 Data from our17,92 studies suggest that memory T cells that develop during aGvHD sustain alloreactive effector cells.17,92 These allograft-sensitized memory T cells differ from naturally-occurring T cells because the ability of memory T cells to mediate aGvHD is limited by their T-cell receptor repertoires.45 Memory T cells are generated during the primary immune response from proliferating T cells upon APC activation.46 After re-encountering antigens, they undergo rapid and robust proliferation and elaboration of effector function. They have stem cell-like self-renewal properties, are distinguishable from both naïve and effector T cells and are resistant to existing immunosuppressive agents.47-48 In fact, whether or not their resilience contributes to the low response rates to current aGvHD therapies (~40%) is the subject of ongoing debate.17,92

EZH2

EZH2 is required for the development of memory precursors early after antigenic priming, for the maturation of memory T cells and for the recall response of mature memory T cells.49 EZH2 deficiency in activated CD8+ T cells caused significant skewing toward central memory precursors and drastically increased the relative proportion of terminally differentiated effector cells that were unable to contribute to further expansion.49 EZH2 repressed the expression of Blimp-1, ID2 and EOMES, which promote effector differentiation, and promoted and sustained expression of ID8, a gatekeeper critical for memory formation and survival.49 Given EZH2’s crucial roles in the regulation of alloreactive T cells, EZH2-mediated memory formation may be responsible for the generation and maintenance of alloreactive memory T cells during aGvHD.

Other regulators

Histone methyltransferase SUV39H1 may play a role in repressing memory genes. Upon infection with Listeria monocytogenes, SUV39H1-defective CD8+ T cells demonstrated enhanced “long-term memory reprogramming,” allowing them to persist in mice.45 The Mixed-Lineage Leukemia gene encodes histone lysine methyltransferase 2A and may be a regulator of memory Th2 cells.50 Protein arginine methyltransferase 5 (PRMT5) has also been implicated as a supporter of memory T-cell reactivation; its inhibition suppressed memory Th1 responses in experimental autoimmune encephalitis.51 We anticipate continued studies of these epigenetic regulators that consider their relevance to aGvHD.

Epigenetic regulation of regulatory T cells

Treg with CD4+CD25+FOXP3+ phenotype can suppress immune responses via cytokine- and contact-dependent mechanisms.15,46 Stable Forkhead box P3 (FOXP3) expression has been considered a critical determinant of Treg identity and activity, but some suggest this characterization may be incomplete, pointing to the importance of independent epigenetic alterations.53 Natural Treg (nTreg) and induced Treg (iTreg) are the most closely-studied Treg subsets in aGvHD; nTreg develop within the thymus while iTreg arise from activated CD4+ T cells in the periphery.15 Because they can repress T-cell proliferation and survival, Treg have been established as an important cell population in reducing aGvHD.53,54 Indeed, infusion of nTreg into allo-HSCT mice abrogated aGvHD lethality,57 and iTreg have been shown to suppress aGvHD in allogeneic models.52,53 While nTreg use has achieved preliminarily promising results in clinical trials,15 the potential of iTreg is less clear. Each has disadvantages; there are few nTreg in the peripheral blood and their use requires ex vivo expansion.46 On the other hand, iTreg, with unstable FOXP3 expression, are often unable to maintain a suppressive phenotype.58,59

DNMT

Researchers have attempted to stabilize FOXP3 expression by maintaining demethylation of the Foxp3 locus.55 As DNMT are independent epigenetic regulators, they are thought to contribute to FOXP3 suppression. Indeed, without demethylation of a CpG island in the Foxp3 locus, cells’ FOXP3 expression and suppressive ability are limited.60 The use of DNMT inhibitors to sustain Treg stability and abrogate aGvHD has achieved some success.

Choi et al. showed that treatment with the DNMT inhibitors decitabine and Aza induced Treg from CD4+CD25+ cells.60 Transplantation of decitabine- and Aza-treated cells into mice undergoing allo-HSCT reduced clinical aGvHD and improved survival.61 Directly treating mice with Aza after allo-HSCT resulted in similar effects. Interestingly, these suppressive effects of iTreg were found to be maintained even in Foxp3 knockout cells, suggesting that their anti-GVHD activity may be downstream or independent of FOXP3 expression.62 In a humanized murine allo-HSCT, xenogenic GVHD model, in vivo Aza treatment was associated with longer survival and lower xenogeneic GVHD scores.63 Researchers suggested that Aza treatment induced Il2 promoter hypomethylation, leading to increased IL-2 expression and augmented Treg proliferation.63

EZH2

EZH2 is known to co-localize with FOXP368 and has also been implicated in the maintenance of Treg identity after activation.69 Tumes et al. noted that iTreg differenti-
ation is impaired without EZH2. Ablation of Ezh2 led to autoimmunity associated with a faulty FOXP3-dependent gene expression program in activated Treg. Ezh2 deletion in vivo murine Treg reprogrammed them to express an effector phenotype that could potentially be unfavorable for aGvHD. In vitro pharmacological EZH2 inhibition also impaired iTreg differentiation, resulting in a significantly decreased frequency of iTreg. Human iTreg treated with the same inhibitor were unable to maintain suppressive activity. However, since inhibition of EZH2 potently sustained persistence and expansion of effector T cells, the impact of pharmacological inhibition of EZH2 on Treg is likely context-dependent.

HDAC

Akimova et al. established that human Treg express a unique combination of HDAC compared to effector T cells, and that treatment with several different HDAC inhibitors augmented the suppressive function of Treg in vitro. Specific HDAC have been implicated in modulating Treg function, including HDAC3 (for both nTreg and iTreg), HDAC9 and HDAC6. Inhibition of HDAC9 had a positive effect on FOXP3 expression and nTreg generation. Deletion of Hdac6 or Sirt1 resulted in similar increases in FOXP3 expression and augmented nTreg function. Interestingly, combined pharmacological inhibition of HDAC6 and SIRT1 had a synergistic effect on increasing nTreg function in vivo in mice. It is likely that the two enzymes share mechanisms in their effect via the deacetylation of FOXP3. However, because these studies were not conducted in the context of GvHD, their results should be taken as an indication for GvHD study in the future.

In the gut, butyrate and other short-chain fatty acids (SCFA) produced by commensal bacteria may also assist with the induction and maintenance of Treg in the periphery. Possessing more Lachnospiraceae- and Ruminococcaceae-family bacteria, which belong to the class Clostridia, was correlated with greater H5 acetylation, a greater Treg/Th17 cell ratio and greater protection against aGvHD. Members of the Clostridium class produce SCFA that comprise colonocytes’ primary energy source. The SCFA butyrate has notable HDAC inhibitory activity. Butyrate delivery via drinking water increased peripheral Treg in mice treated with broad-spectrum antibiotics. Notably, this increase did not occur in mice deficient in the conserved non-coding sequence (CNS) 1 enhancer, which is part of the Foxp3 locus. Treg isolated from these mice exhibited improved suppressor function in vitro compared to antibiotic-treated mice that did not receive butyrate. Treating CD4+ T cells with butyrate during non-specific activation in vitro was also able to induce Treg, so researchers examined the effect of the treatment on Foxp3 locus deacetylation. Butyrate-treated naïve CD4+FoxP3 T cells that were non-specifically activated for 3 days showed significant increases in Foxp3 promoter and CNS 1, 2, and 3 acetylation at H3K27.

Furusawa et al. noted that feeding mice with butyrylated high-amylase maize starches significantly increased differentiation of colonic Treg; these Treg were able to suppress chronic intestinal inflammation brought on by adoptive transfer of CD4+CD45RB+ cells into Rag1-/- mice. Through chromatin immunoprecipitation analysis, researchers verified that butyrate treatment increased global acetylation levels, but also acetylation at histone H3 at (i) the Foxp3 promoter region and CNS3 prior to FOXP3 induction and (ii) CNS1 over the course of Treg differentiation. Though not directly related to aGvHD, these are important findings pertaining to gut inflammation that should provide direction for further study.

Other regulators

Endothelial cell dysfunction, specifically the loss of endothelial cell-derived thrombomodulin, has been associated with steroid-refractory aGvHD. Ranjan et al. showed that thrombomodulin is essential for the generation of protease-activated protein C; incubation of human T cells with activated protein C prior to their transplantation into humanized mice increased Treg frequency and improved xenogeneic GvHD compared to non-incubated human T cells. This activity was speculated to take place via an epigenetic pathway that has yet to be investigated.

Epigenetic programs that influence tissue injury and regeneration during acute graft-versus-host disease

The effector phase is characterized by migration and infiltration of alloreactive effector cells into aGvHD target organs and cytotoxic attack (Figure 1). Areas surrounding tissue commonly affected by aGvHD produce chemokines (e.g., CXCL9 and CXCL10) that recruit effector T cells. The cells recognize major histocompatibility complex and/or minor histocompatibility antigen mismatches and attack tissue via a cytotoxic response mediated by cell-surface factors and cytokines. Alloreactive T cells attack tissue through mechanisms that include Fas-Fas ligand interactions, perforin- and granzyme-mediated killing and TNF-α induction of cell death.

Concurrently, tissue regeneration from both cytotoxic damage and potentially pre-allo-HSCT conditioning comprimes. Intestinal stem cells (ISC) are crucial for the regeneration of the intestinal epithelium after injury. However, ISC are also a target of effector T cells during aGvHD, causing the intestinal epithelium to be trapped in a cycle of repeated damage. Interestingly, IL-22 plays a central role in protecting the intestinal epithelium and ISC. During aGvHD, IL-23-responsive intestinal lymphoid cells produce and secrete IL-22. However, intestinal lymphoid cells are also targeted and eliminated during disease progression, leading to IL-22 deficiency and further ISC damage. Regeneration can be boosted through Wnt pathway stimulation using the Wnt agonist R-Spondin1. Treatment with R-Spondin1 before allo-HSCT expanded ISC and treatment after transplant enhanced surviving ISC proliferation, allowing for fortification of the intestinal lumen and aGvHD inhibition. Little is known about epigenetic regulation of the Wnt pathway-dependent and IL-22-mediated regeneration processes in the context of GvHD. Recent studies have suggested that BMI1, a polycomb repressive complex-1 (PRC1) component important for hematopoietic stem cell renewal, is expressed in the ISC and progenitor compartments. PRC1 is known to enact its function in gene
silencing via recognition of H3K27me3. Bmi1 knockout resulted in reduced ISC proliferation and significant increases in cell cycle regulators p16INK4a and p19ARF84. BMI1 contributes to ISC self-renewal, which is co-regulated by the Wnt pathway and Notch. The interaction between BMI1 and the Wnt pathway in regulating stem cell self-renewal has been validated in a separate study. Mathewson et al. showed that the levels of the HDAC inhibitor butyrate in IEC were significantly decreased after exposure to allo-HSCT inflammation; this led to decreased histone H4 acetylation and decreased expression of the butyrate transporter and receptor, SLC5A8 and GPR43, respectively, in IEC. Increasing intragastric butyrate levels restored H4 acetylation, decreased GvHD and increased ISC proliferation and significant results in cell cycle regulators p16INK4a and p19ARF84. BMI1 contributes to ISC self-renewal, which is co-regulated by the Wnt pathway and Notch. The interaction between BMI1 and the Wnt pathway in regulating stem cell self-renewal has been validated in a separate study. Yeste et al. noted that STAT3 regulates Il22 promoter accessibility. When STAT3-deficient CD4+ T cells were activated in the presence of IL-21, which normally induces Il22 transcription, IL-22 production was significantly decreased. STAT3-deficient cells also showed decreases in H3 and H4 acetylation, decreases in H3K4me3 and increases in H3K9me3 and H3K27me3 at the Il22 promoter. This could involve a number of epigenetic regulators, including histone methyltransferases (e.g., G9A, Suv39H1, EZH2) and enzymes that modify histone acetylation. Recent studies have also implicated the microbiome in aGvHD development and exacerbation. The inflammatory conditions associated with aGvHD, as well as pre-transplant preparatory regimens, can harm commensal bacteria populations and compromise the normal functioning of gastrointestinal cells, which in turn result in more severe aGvHD. For instance, aGvHD inflammation was associated with a loss of SCFA-producing bacteria in the Clostridiales order in both humans and mice. Mathewson et al. showed that the levels of the HDAC inhibitor butyrate in IEC were significantly decreased after exposure to allo-HSCT inflammation; this led to decreased histone H4 acetylation and decreased expression of the butyrate transporter and receptor, SLC5A8 and GPR43, respectively, in IEC. Increasing intragastric butyrate levels restored H4 acetylation, decreased GvHD severity and improved IEC junction integrity. Butyrate treatment was also associated with significantly less IEC apoptosis; among other effects, treatment led to lower expression of pro-apoptotic proteins, higher expression of the anti-apoptotic protein BCL-2 and higher expression of junctional proteins occludin and JAM. The promoter regions of Bcl220 (encoding BCL-2) and Flr (encoding JAM) were noted to be directly associated with H4 acetylation. Though the study did not discuss these changes’ impact on recipient physiology, it is plausible that increased IEC junction integrity and decreased apoptosis prevented immune cell infiltration and PAMP/DAMP from escaping the gut, reducing the severity of the aGvHD.

Deeper examination of this aspect of aGvHD biology and its associated pathways is crucial because it represents a less commonly pursued paradigm in aGvHD treatment: fostering recovery of damaged tissues (Figure 1).

Pharmacological modulation of acute graft-versus-host disease by targeting epigenetic pathways

Development of epigenetic therapy is a particularly active area of cancer research because of such therapies’ potential to selectively target chromatin-modifying enzyme-mediated disease mechanisms. Epigenetic therapy may produce fewer adverse effects than conventional cytotoxic chemotherapies and may influence response to immunotherapy in various cancers. This logic applies to the search for drugs which modify epigenetic mechanisms controlling alloreactive T-cell responses to reduce aGvHD while preserving graft-versus-leukemia activity (Table 2).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Main Conclusion</th>
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<tbody>
<tr>
<td>Vorinostat (SAHA)</td>
<td>HDAC inhibition with vorinostat in combination with standard prophylaxis resulted in reduced incidence of severe aGvHD. Phase I/II trial.</td>
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<tr>
<td></td>
<td>HDAC inhibition with vorinostat was safe and efficacious in unrelated donor allo-HSCT patients receiving myeloablative conditioning and methotrexate. Results showed a low cumulative incidence of severe aGvHD. Phase II trial.</td>
</tr>
<tr>
<td>Panobinostat</td>
<td>HDAC inhibition with panobinostat in addition to glucocorticoids as primary therapy for aGvHD was deemed safe. However, the study did not have sufficient power to address efficacy. Phase I/II trial.</td>
</tr>
<tr>
<td>5-Azacytidine</td>
<td>DMNT inhibition by Aza after donor lymphocyte infusion as salvage therapy was well tolerated and no patients developed grade III-IV aGvHD. Phase I trial.</td>
</tr>
<tr>
<td></td>
<td>DMNT inhibition by Aza after allogeneic stem cell transplantation increased circulating Treg in patients. Phase I/II trials.</td>
</tr>
</tbody>
</table>

SAHA: suberoylanilide hydroxamic acid; HDAC: histone deacetylase; aGvHD: acute graft-versus-host disease; allo-HSCT: allogeneic hematopoietic stem cell transplantation; Aza: 5-Azacytidine; DMNT: DNA methyltransferase; Treg: regulatory T cell
The results are inconclusive because the trial lacked sufficient power, but are nevertheless promising.34

DNMT inhibitors

Clinically, Aza and decitabine have been used in the context of allo-HSCT for the express purpose of reducing the disease burden before transplantation and as maintenance and salvage therapy.35 In studies using Aza or decitabine treatment in the setting of blood cancers or myelodysplastic syndromes to reduce disease burden before transplantation, there were no significant findings with regard to aGvHD.46-48 DNMT inhibitor treatment after allo-HSCT has typically been a component of salvage or maintenance therapy and has had some success in mitigating aGvHD. Ghobadi et al. treated patients with Aza after donor lymphocyte infusion; no patients developed severe aGvHD (III-IV) and there was no aGvHD-caused mortality.49 Similarly, Schroeder et al. provided Aza treatment alongside donor lymphocyte infusion upon patients’ relapse and saw a 3.2-fold increase in Treg and a 1.9-fold increase in Treg frequency after four cycles of Aza treatment in patients who relapsed early after allo-HSCT.46 Goodyear et al. found that although the incidence of aGvHD was lower in treatment groups than in control groups, Treg increases were only observed within the first 3 months of treatment.50 These results suggest that early treatment may be required for a beneficial effect on aGvHD.

Because of its comparative success, it may be fruitful for future clinical trials to expand on the post-transplant, early HDAC inhibitor treatment paradigm. Of note, DNMT inhibitors were not typically used for aGvHD prevention, so patients often received other treatments (e.g., methotrexate) which were not standardized across studies.

EZH2 inhibitors

In vivo administration of GSK126 failed to reduce aGvHD and did not affect the development of alloreactive effector T cells in preclinical studies.51 This is in contrast to observations that EZH2 deficiency led to aGvHD blockade in various murine allo-HSCT models.52 The mechanism of action of EZH2 in mediating aGvHD induction is therefore likely independent of its canonical target H3K27me3.53 Notably, EZH2 protein depletion by DZNep led to arrest of ongoing GvHD in experimental mice,54 indicating that targeting EZH2 may lead to new strategies to treat ongoing GvHD.

An interaction between HSP90 and EZH2 has also been shown to be vital for the stability and function of EZH2.55 A lack of HSP90 marks EZH2 for ubiquitination via the proteasome. Treatment of activated T cells with the HSP90 inhibitor AUY922 significantly decreased EZH2 protein levels while leaving histone methylation intact. HSP90 inhibitor treatment significantly decreased alloactive T-cell responses and aGvHD in mice, affirming EZH2’s involvement in aGvHD pathogenesis and the non-canonical hypothesis.56

The Food and Drug Administration has approved the EZH2 inhibitor tazemetostat specifically for the treatment of epithelioid sarcoma. We anticipate that this inhibitor may be used to target alloreactive memory T cells to reduce aGvHD in the future.

Future directions

As the epigenetics of aGvHD biology is a young area of study, there is much room for further investigation, both in elucidating mechanisms surrounding the action of known enzymes and in exploring the roles of new regulators documented here and beyond. Nevertheless, enormous progress has been made through the identification of critical enzymes and mechanisms. Next steps will be to further map how their pathways intersect amid the multitude of cell types and interactions that comprise aGvHD. Some epigenetic regulators (e.g., EZH2 and HDAC6) have points of commonality in their mechanisms of action (via HSP90).57,58,59 Advances will illuminate these locations of confluence such that more effective, integrated therapies may be developed. Additionally, a single regulator (e.g., HDAC11) may have beneficial or detrimental effects at different stages of cell development; understanding these situations will be vital for treatment. Also bringing promise for epigenetic intervention are those aspects of aGvHD pathogenesis that are T-cell-independent, such as microbiome injury.

Different tissues, hematopoietic and non-hematopoietic, have distinct roles in mediating aGvHD immunopathology. Further investigation of the epigenetics surrounding the role of non-hematopoietic APC would likely be beneficial for the field. In addition, tissue-intrinsic mechanisms that contribute to inhibition of aGvHD have been somewhat overlooked. These include those controlling tissue regeneration,80-82 and those modulating tissue-resident APC, which are critical for local aGvHD induction.83 Furthermore, aGvHD blocks peripheral tolerance of host-reactive T cells by elimination of lymph node fibroblastic reticular cells that induce T-cell tolerance in the gut.84 Thus, future studies should also investigate the epigenetic mechanisms that regulate tissue regeneration and regulation of the graft-versus-host reaction, as suggested by Reddy and colleagues.85

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