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Recipient NK cell inactivation and intestinal barrier loss are required for MHC-matched graft-versus-host disease

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Abstract

Previous studies have shown a correlation between pre-transplant conditioning intensity, intestinal barrier loss, and graft-versus-host disease (GVHD) severity. However, since irradiation and other forms of pre-transplant conditioning have pleiotropic effects, the precise role of intestinal barrier loss in GVHD pathogenesis remains unclear. We developed GVHD models that allowed us to isolate the specific contributions of distinct pre-transplant variables. First, intestinal damage was required for the induction of minor mismatch (MHC-matched) GVHD, but was not necessary for major mismatch GVHD, demonstrating fundamental pathogenic distinctions between these forms of disease. Moreover, recipient NK cells prevented minor mismatch GVHD by limiting expansion and target organ infiltration of alloreactive T cells via a perforin-dependent mechanism, revealing a previously unrecognized immunoregulatory function of recipient NK cells in GVHD. Minor mismatch GVHD required MyD88-mediated TLR4 signaling on donor cells, and intestinal damage could be bypassed by parenteral LPS administration, indicating a critical role for the influx of bacterial components triggered by intestinal barrier loss. In all, the data demonstrate that pre-transplant conditioning plays a dual role in promoting minor mismatch GVHD by both depleting recipient NK cells and inducing intestinal barrier loss.

INTRODUCTION

Intestinal barrier loss is associated with many autoimmune and inflammatory disorders(1), including graft-versus-host disease (GVHD), a life-threatening complication of allogeneic bone marrow transplantation (BMT) and hematopoietic stem cell transplantation (HSCT). The luminal microbiome contributes to the pathogenesis of GVHD, as germ-free mice are at least partially protected(2) and antibiotics can provide benefit in human subjects(3). In addition, altered signaling by Pattern Recognition Receptors (PRRs), including NOD2 and many of the toll-like receptors (TLRs), has been implicated in GVHD(4). In particular,

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CONFLICT OF INTEREST DISCLOSURE

The authors have no conflict of interests to disclose.

AUTHORSHIP CONTRIBUTIONS

Study concept and design (SCN, PAS, JRT); data acquisition (SCN, HAK, KLE, GS, NEJ, GFK, EDM, JRT); analysis and interpretation of data (SCN, PAS, JRT); drafting of the manuscript (SCN, PAS, JRT); critical revision of the manuscript (SCN, PAS, JRT); obtained funding (JRT).

experimental models have shown that LPS-induced TLR4 signaling contributes to GVHD following F1 (major mismatch) BMT(5, 6) and that the severity of this GVHD correlates with the magnitude of LPS-induced TNF production by donor cells(6). These data support a model in which intestinal damage, including barrier loss induced by pre-transplant conditioning allows translocation of gut microbiota and microbial products that stimulate GVHD development and progression. While attractive, this model has not been fully tested, as most experimental studies have used severe MHC-mismatch GVHD models in which non-immune contributors to disease (i.e. intestinal damage) are likely underestimated. Moreover, both disease models and human trials rely on pre-transplant conditioning, which causes intestinal damage, to permit donor cell engraftment. Thus, it has not been possible to ask if intestinal damage is strictly required for GVHD initiation.

Pre-transplant conditioning has many effects beyond the gastrointestinal tract, including damage to skin, lungs and multiple other organ systems(7), creation of space that allows expansion of alloreactive donor cells, depletion of regulatory cell populations, and activation of recipient dendritic cells(8). Thus, despite a strong correlation between conditioning damage-induced organ injury and GVHD severity(9–12), it remains unclear whether the intestinal damage merely enhances disease severity or is required for GVHD development. The goal of our study was to dissect the individual contributions of these distinct effects of pre-transplant conditioning on GVHD pathogenesis.

We developed mouse models of minor antigen mismatch (MHC-matched) allogeneic transplantation and compared these with established MHC-mismatch models. *Rag1*^{-/-} immunodeficient recipients were used to permit donor cell engraftment without pre-transplant conditioning. This allowed direct experimental manipulation of distinct parameters and their contributions to GVHD pathogenesis. The data demonstrate that intestinal barrier loss is required to initiate minor but not major mismatch GVHD. Consistent with exposure of luminal bacterial products as a critical contribution of intestinal barrier loss to minor mismatch GVHD, MyD88-mediated TLR4 signaling was required on donor, but not recipient, cells, and parenteral LPS administration overcame the requirement for intestinal damage. Finally, our data demonstrate a previously unrecognized immunoregulatory function whereby recipient NK cells prevent development of minor mismatch GVHD by limiting alloreactive T cell expansion and target organ infiltration.

RESULTS

Minor mismatch GVHD is biphasic

To elucidate the mechanisms driving systemic immune activation in GVHD, we established mouse models of minor and major antigen mismatch GVHD. For minor mismatch (MHC-matched) GVHD, cells from 129 (H-2^b) mice were transferred into B6 (H-2^b) recipients, while cells from Balb/c (H-2^d) mice were transferred into B6 recipients to induce major mismatch (MHC-mismatched) GVHD. After lethal irradiation, recipients were transplanted with bone marrow cells and splenocytes (hereafter, this combination is referred to as BMT). Major mismatch BMT led to severe weight loss, clinical signs of disease, and characteristic histopathology (Fig. 1, Supp. Fig. 1); no mice survived past 24 days (Fig. 1C). Weight loss and clinical signs of disease developed more slowly after minor mismatch BMT (Fig. 1A,

B), which was associated with histopathological features typical of GVHD in human patients (Fig. 1E, Supp. Fig. 1). Despite disease, more than 50% of minor mismatch BMT recipients survived past 35 days, and a few survived >100 days (Fig. 1C). Recipients of syngeneic BMT recovered fully and survived long term.

All mice displayed weight loss, increased clinical scores, and increased intestinal permeability in the first week after irradiation and BMT (Fig. 1F). In minor mismatch and syngeneic recipients, weight stabilized, clinical scores decreased, and intestinal barrier function was restored within 2 weeks (Fig. 1F), indicating that these early effects were due to irradiation. In contrast, major mismatch recipients did not demonstrate clinical improvement or restoration of intestinal barrier function. This suggests that onset of major mismatch GVHD overlapped with and masked resolution of irradiation injury, making it difficult to resolve the two processes. In minor mismatch recipients, a second phase of barrier loss developed within 28 days (Fig. 1F) and coincided with increasing clinical scores (Fig. 1B). Thus, an initial phase of irradiation-induced barrier loss followed by transient recovery precedes clinical presentation of minor mismatch GVHD.

Pre-transplant irradiation and allogeneic splenocyte transfer induce GVHD in *Rag1*^{-/-} recipients

To assess the contribution of pre-transplant irradiation to GVHD pathogenesis, we performed experiments involving the adoptive transfer (AT) of donor splenocytes into *Rag1*^{-/-} hosts. The use of *Rag1*^{-/-} recipients allows engraftment of MHC-matched donor splenocytes without irradiation. Bone marrow was omitted from the donor inoculum as it has no effect on GVHD outcome and is not required for recipient survival at the sublethal irradiation dose (7 Gy) used. Experiments comparing 129 AT (splenocytes only) to 129 BMT (splenocytes and bone marrow) into irradiated B6 recipients confirmed that the omission of bone marrow had no effect on *Rag1*^{-/-} recipient bone marrow histopathology and peripheral blood counts (Supp. Fig. 2).

Using the splenocyte AT experimental system, we found that syngeneic (B6), minor mismatch (129), or major mismatch (Balb/c) AT into non-irradiated B6 *Rag1*^{-/-} recipients failed to induce GVHD (Fig. 2A, B). In contrast, recipients that received sublethal irradiation prior to minor or major mismatch AT developed GVHD, including weight loss, clinical signs of disease, and characteristic histopathology (Fig. 2C, D). Irradiated recipients that received a syngeneic AT recovered fully in a manner similar to mice that received syngeneic BMT (compare Fig. 1A, B to Fig. 2A, B).

When recipients of minor mismatch 129 splenocytes were assessed 7 days after AT, a time at which transient irradiation-induced intestinal damage had resolved, increased intestinal permeability was observed in mice that received pre-transplant irradiation. In contrast, intestinal permeability of mice that received AT without irradiation was similar to controls (Fig. 2E). Immunofluorescent analysis of intestinal epithelial tight junction structure showed ZO-1 displacement and increased claudin-2 expression in mice that received minor mismatch AT and irradiation, but not in those that received only minor mismatch AT (Supp. Fig. 3). These results are consistent with reduced tight junction barrier function and explain, in part, the increased permeability observed in irradiated recipients of a minor mismatch AT.

To further investigate the mechanisms by which irradiation contributes to GVHD pathogenesis, we considered the possibility that in the absence of irradiation, donor T cells were rejected by CD3⁺/NK1.1⁺ recipient NK cells, which are present in abundance in B6 *Rag1*^{-/-} mice (Fig. 2F). Irradiation dramatically reduced NK cell numbers (Fig. 2G). Consistent with the above hypothesis, irradiated recipients of major mismatch (Balb/c) splenocytes had a greater number of CD3⁺/NK1.1⁻ donor T cells than non-irradiated B6 *Rag1*^{-/-} recipients. In contrast, the number of minor mismatch (129) donor T cells were comparable in irradiated and non-irradiated B6 *Rag1*^{-/-} recipients (Fig. 2F). These data suggest that major, but not minor, mismatch donor cells were rejected by NK cells in non-irradiated recipients and that irradiation prevented this rejection by clearing recipient NK cells.

Acute NK cell depletion or perforin-deficiency allows major but not minor mismatch GVHD in *Rag1*^{-/-} recipients without pre-transplant irradiation

To test the hypothesis that NK cells prevented GVHD in non-irradiated recipients, B6 *Rag1*^{-/-} mice were treated with depleting anti-NK1.1 antibody (PK136) or control antibody prior to AT. This treatment allowed development of GVHD after major, but not minor, mismatch AT (Fig. 3A–C). Mice that received major mismatch AT also exhibited elevated intestinal permeability (Fig. 3D). These results suggest that, for major mismatch AT, the requirement for pre-transplant irradiation partly reflects the need for NK cell depletion to prevent rejection. These data also demonstrate that conditioning-induced intestinal damage is not required for major mismatch GVHD pathogenesis, and that major mismatch allogeneic donor cells can induce any necessary intestinal damage. In contrast, the contributions of pre-transplant irradiation to minor mismatch GVHD cannot be explained solely by NK depletion.

While antibody-mediated NK cell depletion may simply remove NK cells and thereby allow major mismatch donor cells to survive, acute NK1.1⁺ cell clearance might also create a niche for donor cell engraftment. Alternatively, NK cell death induced by anti-NK cell antibodies could generate a “danger signal” that stimulates GVHD (13, 14). To discriminate between these possibilities, GVHD was assessed in B6 *Rag2*^{-/-}/*Pfp*^{-/-} recipients. In addition to an absence of mature T and B cells, *Rag2*^{-/-}/*Pfp*^{-/-} mice lack perforin, which is required for NK cell cytolytic activity (15, 16). Both minor and major mismatch donor cells were readily accepted following transfer into *Rag2*^{-/-}/*Pfp*^{-/-} mice. Major mismatch AT into *Rag2*^{-/-}/*Pfp*^{-/-} B6 recipients resulted in GVHD, including weight loss, elevated clinical scores, characteristic histopathology, and increased serum IFN γ , TNF, and IL-6 (Fig. 3E–G, Supp. Fig. 4, 5). In contrast, GVHD did not develop following minor mismatch AT into *Rag2*^{-/-}/*Pfp*^{-/-} B6 recipients despite increases in cytokine production. Thus, while immune activation, as indicated by cytokine production, occurred following both major and minor mismatch AT into *Rag2*^{-/-}/*Pfp*^{-/-} recipients, GVHD only developed after major mismatch AT. This suggests that effects of irradiation other than NK cell depletion are required for development of minor mismatch GVHD.

To better define the underlying mechanisms responsible for the difference in GVHD development following major or minor mismatch AT into non-irradiated *Rag2*^{-/-}/*Pfp*^{-/-}

recipients, we performed *in vivo* analyses of donor cell killing efficiency. Major mismatch Balb/c donor cells were extremely efficient at killing allogeneic (B6) targets, while minor mismatch 129 donor cells were relatively inefficient at killing B6 targets under these conditions (Fig. 3H). Thus, the elimination of perforin-dependent donor cell rejection is sufficient to allow major mismatch donor cells to survive, kill recipient cells, and drive GVHD. Further, neither cell and tissue damage, e.g. irradiation or NK cell death, nor additional clearance of a proliferative niche, are required for major mismatch GVHD. In contrast, despite donor cell survival, neither recipient NK cell depletion nor perforin deficiency are sufficient to allow GVHD development following minor mismatch AT without prior irradiation.

Colonic damage and NK cell inactivation synergize to promote AT-induced minor mismatch GVHD

Donor cell rejection could not explain the failure of minor mismatch AT (without irradiation) to induce GVHD (Fig. 2F). We therefore asked if irradiation-induced intestinal damage was critical for minor mismatch GVHD. In this regard irradiation has many effects, which makes it difficult to assess the contribution of intestinal damage to GVHD pathogenesis. To overcome this, B6 *Rag1*^{-/-} recipients of minor mismatch AT were treated with dextran sulfate sodium (DSS), which induces colitis and colonic barrier loss that resolves within one week of DSS discontinuation (17, 18). Despite significant colonic damage, GVHD did not develop in DSS-treated recipients of minor mismatch AT (Fig. 4A–C, Supp. Fig. 6). Further, unlike irradiation, DSS did not increase donor cell cytotoxicity against allogeneic targets (Fig. 4D). Irradiation effects other than intestinal damage are, therefore, required for minor mismatch GVHD development. One of these effects could be creation of a niche, or space, for donor cell proliferation. However, *in vivo* analysis of CFSE-labeled donor cells 6 days after AT showed significant proliferation regardless of prior irradiation or DSS treatment (Supp. Fig. 7), thereby excluding a role for a “proliferative niche” created by NK cell clearance in minor mismatch GVHD. Taken together, these studies demonstrate that effects of irradiation beyond intestinal damage are required for development of minor mismatch GVHD.

Although not required for donor cell survival or proliferation following minor mismatch AT, irradiation does clear recipient NK cells (Fig. 2G). Recent studies have reported incompletely-defined roles for NK cells in regulating T cell proliferation and differentiation into central memory CD8⁺ T cells in non-GVHD models (19, 20). We therefore asked whether combined colonic damage and NK cell depletion was sufficient to allow minor mismatch GVHD development following minor mismatch AT. DSS coupled with anti-NK1.1 (PK136), but not control antibody, treatment was sufficient to drive minor mismatch GVHD (Fig. 4). Notably, disease was evident in the small intestine, which is not damaged by DSS, as well as liver, skin, and lung (Fig. 4C, Supp. Fig. 6). Thus, the minor mismatch GVHD that developed under these conditions was systemic and not simply a reflection of direct DSS-induced intestinal damage. Further, development of minor mismatch GVHD was accompanied by increased *in vivo* donor cell cytotoxicity against allogeneic targets (Fig. 4D). Thus, NK cell depletion and colonic damage synergize to drive T cell-mediated cell and tissue damage and minor mismatch GVHD.

In contrast to major mismatch, minor mismatch AT into *Rag2*^{-/-}/*Pfp*^{-/-} B6 recipients did not cause GVHD (Fig. 4E–H). However, DSS treatment of *Rag2*^{-/-}/*Pfp*^{-/-} B6 recipients prior to minor mismatch AT resulted in GVHD, including characteristic features of systemic GVHD, including involvement of small intestine, liver, lungs, and skin (Fig. 4E–H, Supp. Fig. 8) and increased intestinal permeability (Fig. 4I). DSS-treated *Rag2*^{-/-}/*Pfp*^{-/-} B6 recipients of syngeneic AT recovered completely (Fig. 4E–H), indicating that the systemic disease observed was not an artifact of DSS treatment. These data also demonstrate that minor mismatch GVHD does not require creation of space for donor cell expansion. Notably, survival of *Rag2*^{-/-}/*Pfp*^{-/-} B6 recipients subjected to minor mismatch AT and DSS treatment was similar to that following minor mismatch BMT in lethally irradiated recipients (compare Fig. 4G and Fig. 1C), thereby confirming the development of robust systemic GVHD. Thus, recipient NK cells prevent GVHD by limiting functional maturation of donor-derived cytolytic T cells via a perforin-dependent mechanism.

Recipient NK cell depletion results in increased effector T cell expansion in secondary lymphoid tissues and GVHD target organs

Based on the unexpected observation that recipient NK cells were able to prevent minor mismatch GVHD, we sought to investigate the mechanism of NK cell regulation of alloreactive T cells in more detail. To do this, *Rag1*^{-/-} recipients were treated with DSS and an NK cell-depleting antibody or control IgG prior to minor mismatch AT. By 3 weeks after AT, NK cell-depleting antibody-treated recipients had a significant increase in effector memory CD8⁺ T cells within the spleen, mesenteric lymph nodes (MLN), and axillary/brachial lymph nodes (A/B), as judged by both CD44^{hi}CD62L^{lo} and granzyme B expression (Fig. 5A, B). In addition, effector CD4⁺IFN γ ⁺ T cells were increased in MLN (Fig. 5C). These results are consistent with previous studies in non-GVHD models demonstrating a role for NK cells in restricting the expansion of virus-specific CD8⁺ T cells and the homeostatic proliferation of T cells in lymphopenic hosts (19, 20). We also investigated donor CD3⁺ T cell GVHD target organ tissue infiltration immunohistochemically. Mice that received NK cell-depleting antibody had substantially greater T cell infiltration in small intestine, liver, and skin (Fig. 5D, E). Taken together, these data provide evidence that recipient NK cells restrict minor mismatch GVHD by limiting the effector differentiation and target organ infiltration of alloreactive T cells.

Donor TLR4 and MyD88 signaling are required for minor mismatch GVHD

The data above show that, in addition to NK cell inactivation, intestinal damage and barrier loss are required for minor mismatch GVHD. To investigate the effect of barrier loss in more detail, we evaluated the role of several well-defined innate pathogen sensors in GVHD development. LPS, a cell wall component of Gram-negative bacteria, has been implicated in major mismatch GVHD (5, 6). Further, donor and recipient TLR4 (a receptor for LPS) mutations have been associated with reduced GVHD severity in human (minor mismatch) HSCT(21, 22). Previous studies in mouse models of major mismatch GVHD have also shown that donor TLR4 signaling enhances the development of disease (6). However, no studies have investigated the role of TLR4 signaling in mouse models of minor mismatch GVHD.

To assess the role of TLR4 signaling in minor mismatch GVHD, we performed experiments in which donor cells or recipient mice were deficient in TLR4. Our data demonstrate that following BMT from 129 donor mice into lethally irradiated B6 recipients, disease severity was comparable in *TLR4*^{+/+} and *TLR4*^{-/-} recipients, (Fig. 6A, B), suggesting that TLR4 signaling on recipient cells, including antigen-presenting cells, is not required for minor mismatch GVHD. In contrast, when *TLR4*^{+/+} or *TLR4*^{-/-} B6 splenocytes were adoptively transferred to 129 *Rag2*^{-/-} mice treated with 3% DSS and NK cell-depleting antibody, GVHD developed only in mice that received *TLR4*^{+/+} B6 splenocytes (Fig. 6C, D). Thus, TLR4 signaling on donor-derived cells is required for the development of minor mismatch GVHD.

MyD88 and TRIF are intracellular proteins that initiate separate inflammatory signaling cascades following TLR4 ligation(23). To determine which of these pathways was involved in the TLR4 signaling that drives minor mismatch GVHD, the ability of *MyD88*^{-/-} or *TRIF*^{-/-} B6 splenocytes to induce disease in DSS-treated, NK cell-depleted 129 *Rag2*^{-/-} recipients was assessed. As shown in Figure 6, the severity of minor mismatch GVHD was diminished following transfer of *MyD88*^{-/-} donor cells, but was not impacted by the transfer of *TRIF*^{-/-} donor cells. This was apparent in terms of weight loss (Fig. 6E) and clinical scores (Fig. 6F). These results demonstrate that MyD88-dependent signaling on donor cells contributes to minor mismatch GVHD, and that TLR4-TRIF signaling is not required.

The intestinal microbiota and microbial products promote minor mismatch GVHD following dysregulation of the intestinal barrier

Having established a role for donor MyD88-dependent TLR4 signaling in minor mismatch GVHD, we next sought to determine the contribution of the intestinal microbiota and microbial products to GVHD pathogenesis. To determine whether the effect of intestinal damage on GVHD is dependent on the microbiota, we treated B6 *Rag1*^{-/-} mice with a vancomycin/imipenem antibiotic cocktail in their drinking water for one week prior to and one week after minor mismatch AT with DSS and NK cell depletion. Despite discontinuation at day 7 after AT, antibiotic treatment markedly reduced GVHD severity that persisted to at least day 35. The milder disease was accompanied by weight gain (Fig. 7A), absence of clinical features of GVHD (Fig. 7B), and markedly reduced histopathology scores (from 19±3 in mice that did not receive antibiotics to 12±0.5 in antibiotic-treated mice, P=0.036 by two-sided Mann-Whitney U test). These data suggest that intestinal damage-dependent translocation of microbes or microbial products is critical for early stages of GVHD pathogenesis.

Given the above results, including data showing that MyD88-mediated TLR4 signaling on donor cells is required for GVHD pathogenesis, we hypothesized that intestinal damage and barrier loss promotes minor mismatch GVHD by allowing LPS, other bacterial products, or live microorganisms to access the systemic circulation. To assess this, B6 *Rag2*^{-/-}/*Pfp*^{-/-} mice were treated with a single intraperitoneal dose of purified LPS prior to syngeneic or minor mismatch AT. Mice that received LPS and syngeneic AT experienced transient, mild weight loss (Fig. 7C), and clinical score increases (Fig. 7D) in the first week after AT, but

recovered thereafter. In contrast, mice that received LPS and minor mismatch AT continued to lose weight (Fig. 7C) and developed clinical features of GVHD (Fig. 7D). Further, mice that received LPS developed histopathology typical of GVHD (Fig. 7E, F). Thus, microbial components, i.e. LPS, are sufficient to drive GVHD in the absence of intestinal barrier loss when paired with NK cell inactivation and minor mismatch AT.

DISCUSSION

In this study we developed GVHD models to dissect the roles of intestinal damage, barrier loss, and NK cell function in disease pathogenesis. The data show that strong antigenic stimuli, such as the major histocompatibility antigens associated with major mismatch transplantation, are sufficient to drive disease in the absence of pre-transplant conditioning-induced tissue damage. In contrast, weaker antigenic stimuli, i.e. minor mismatch antigens, that are common in HLA-matched BMT and HSCT performed clinically, are unable to drive disease without intestinal barrier loss. Moreover, we have uncovered a previously unrecognized regulatory role for MHC-matched recipient NK cells in preventing minor mismatch GVHD. Together, our data show that in recipients lacking functional NK cells, intestinal barrier loss is both necessary and sufficient to drive minor mismatch GVHD (Supp. Fig. 9).

Although NK cells have long been of interest in GVHD (24, 25), NK cells of donor origin, which reduce GVHD while enhancing graft anti-tumor activity (26–33), are the most well-studied. With regard to recipient NK cells, a recent study suggested that recipient NK cells control proliferation of antigen-stimulated donor T cells by a perforin-independent, Fas-dependent mechanism (16). That work used a male antigen-specific CD4+ T cell-mediated GVHD model, where clinical disease presentation is extremely mild (16), but another study has described a perforin-independent NK cell regulatory function in major mismatch skin transplantation (19). In contrast to these reports, our data using a robust minor antigen mismatch GVHD model show that recipient NK cells regulate minor mismatch GVHD pathogenesis by a perforin-dependent mechanism. Importantly, recipient NK cells did not impact the engraftment of minor mismatch donor T cells, but did prevent the development of GVHD. These observations are consistent with reports that NK cells may regulate activated, MHC-matched T cells in colitis and viral infection by a perforin-dependent mechanism (34–36). If this immunoregulatory activity can be defined and therapeutically modulated, recipient NK cells may become useful as a means to treat or prevent GVHD and, possibly, other T cell-mediated diseases. Conversely, we found that elimination of recipient NK cells was not sufficient to allow initiation of minor mismatch GVHD. We show here that intestinal barrier dysfunction provides the required second signal. Further, we show that the critical role of barrier loss in this context is to allow systemic exposure to LPS, or other bacterial products, particularly during disease initiation.

The absence of a requisite role for intestinal damage in major mismatch GVHD is consistent with reports that F1 (major mismatch) GVHD can develop without pre-transplant conditioning (37–39). However, pre-transplant irradiation has been suggested to promote antigen-presenting cell activation (13, 40) and recruitment of donor-derived reactive T cells to peripheral tissues (41). Our data support this hypothesis, as lethal irradiation with major

mismatch BMT caused more severe GVHD than major mismatch AT without irradiation. However, significant major mismatch GVHD did develop in the absence of pre-transplant irradiation. Thus, our data show that intestinal damage is not required for development of major mismatch GVHD.

The conclusion that intestinal damage is not required for major mismatch GVHD conflicts with a widely-accepted model of GVHD pathogenesis (9, 42). This model, developed primarily on the basis of MHC-mismatch (major mismatch) experimental systems, is supported by clinical studies showing a correlation between conditioning intensity and GVHD severity in patients receiving HLA-matched (minor mismatch) HSCT/BMT (11, 43). However, neither experimental nor clinical studies have been able to demonstrate a causal relationship between intestinal damage and GVHD severity (11, 44). This is primarily because previous work has not completely eliminated pre-transplant intestinal damage. Further, the preconditioning regimens used caused both intestinal and extra-intestinal damage, making it impossible to distinguish intestinal damage from other effects of pre-transplant conditioning. Thus, the data presented here, which are the first to specifically evaluate the role of intestinal barrier loss in the pathogenesis of clinically relevant minor mismatch GVHD, emphasize the potential utility of conditioning regimens that reduce or eliminate intestinal damage or barrier loss.

Overall, our data demonstrate that pre-transplant conditioning fulfills dual roles in GVHD by simultaneously inhibiting recipient NK cell-dependent donor cell immunoregulation and inducing intestinal barrier loss. The latter creates a proinflammatory host environment that allows systemic access of luminal microbial components, such as LPS, which activate donor cell TLR4 and MyD88 to promote to systemic GVHD (Supp. Fig. 9). These results provide further support for the therapeutic use of antibiotics in HSCT/BMT in order to reduce GVHD severity in patients (45). Given the increased understanding of the role of the microbiota in GVHD (46–48), it is likely that patients would also benefit from adjunct probiotic therapy or, possibly, TLR4 antagonists (49, 50).

The most intriguing therapeutic implications that arise from our studies are related to the unexpected immunoregulatory role of recipient NK cells in minor mismatch GVHD. Although not yet demonstrated experimentally, these data suggest that improved transplantation protocols could involve harvesting recipient NK cells prior to pre-transplant conditioning and then infusing these cells back into the recipient after HSCT/BMT to limit GVHD pathogenesis. Further studies of the role of recipient NK cells and the control of MHC-matched alloreactive T cells will help determine the feasibility of this approach.

In sum, our results provide the first direct evidence that pre-transplant intestinal damage not only enhances GVHD severity but is required for clinically relevant minor mismatch GVHD. These data therefore support the hypothesis that reduction of pre-transplant conditioning-induced intestinal damage or modulation of the intestinal microbiota may be an effective preventative approach in GVHD. The results also suggest that a previously unrecognized regulatory role of MHC-matched recipient NK cells may serve as a novel approach for preventing GVHD.

MATERIALS AND METHODS

Mice

Female 129S6 (H-2^b, Taconic), Balb/c (H-2^d, Harlan), or C57BL/6J (Jackson) mice were used as donors. 129 *Rag2*^{-/-} recipients were purchased from Taconic Farms. C57BL/6 (H-2^b) *TLR4*^{-/-}, *MyD88*^{-/-}, and *TRIF*^{-/-} mice were purchased from Jackson Laboratory. All mice were bred and maintained at the University of Chicago. All experiments were in compliance with the Institutional Animal Care and Use Committee regulations at the University of Chicago, and were performed under an approved protocol. 200 µg of anti-NK1.1 (clone PK136), control IgG, or 20 µl anti-asialo GM1 (Wako Chemicals) was injected i.p. 2 days prior to AT. B6 *Rag1*^{-/-} and *Rag2*^{-/-}/*Pfp*^{-/-} recipients received 3% DSS in their drinking water for 4 days prior to AT. 129 *Rag2*^{-/-} recipients received 3% DSS in their drinking water for 5 days prior to AT. LPS (0.8 mg/kg) from *E. coli* (Sigma) was injected i.p. 2 hours prior to AT. Antibiotic-treated mice received vancomycin and imipenem in their drinking water (50 mg/kg/day) from days -7 to +7 relative to AT.

Bone marrow transplants and adoptive transfers

Female B6, 129, or Balb/c mice between the ages of 6–12 weeks were used as donors. Male B6 recipients, between the ages of 7–12 weeks, were lethally irradiated with 11 Gy 24 hours prior to BMT with 5 million bone marrow cells and 30 million splenocytes by retroorbital injection. For AT, sex-matched B6 *Rag1*^{-/-} recipients or B6 *Rag2*^{-/-}/*Pfp*^{-/-} recipients received only splenocytes, with or without irradiation (7 Gy) 24 hours prior to AT. Clinical signs were scored from 0–2 for posture, activity, fur texture, and hair loss, giving a maximum score of 8. Mice were euthanized when weight loss exceeded 20% for two consecutive measurements, as required by the University of Chicago IACUC.

Histopathological scoring

Histopathological analysis of GVHD target organs was performed by a subspecialty-trained anatomic pathologist blinded to the experimental conditions. Jejunum, liver, and skin were scored at 0.5 point intervals from 0–3 based on 4 features, for a maximum organ score of 12 and a maximum total histopathological score of 36. For small intestine (jejunum), crypt cell apoptosis, inflammatory infiltrate, architectural integrity, and reactive epithelial cytology were assessed. For liver, features scored were portal infiltrate, bile duct damage, lobular activity, and vascular destruction. Skin damage was assessed in perioccipital skin sections, based on follicle and sebaceous gland loss, epithelial damage, dermal fibrosis, and reticular dermal inflammation.

Intestinal permeability assay

Mice were denied access to food for 3 hours prior to gavage with 150 µl of 80 mg/ml FITC-4kD dextran. Serum was harvested 3 hours later and analyzed in a Synergy HT plate reader (Bio-Tek). Permeability was normalized to an age-matched control mouse.

Immunofluorescence and immunohistochemistry

For immunofluorescence, 1 cm jejunal sections were snap frozen in OCT. 5 μ m frozen sections were fixed in 1% PFA in PBS and immunostained using mouse anti-ZO-1 (Invitrogen), rabbit anti-claudin-2 (Abcam), rabbit-anti claudin-15 (Invitrogen), or rabbit anti-E-cadherin (Cell Signaling), followed by Alexa Fluor 488- or 594-conjugated secondary antibodies (Invitrogen), along with Hoechst 33342 (Invitrogen). Stained sections were mounted in Prolong Gold (Invitrogen) and imaged using an epifluorescence microscope with a Plan-Neofluar 40X/1.3 oil objective (Axioplan 2, Zeiss). Images were deconvolved for 10 iterations using Autoquant X3 (Media Cybernetics) and analyzed using Metamorph (Universal Imaging Corp.).

For immunohistochemistry, tissue sections were de-paraffinized, rehydrated, antigen was unmasked by boiling in 0.01 M sodium citrate, pH 6.0, and endogenous peroxidases were quenched by incubation with 3% hydrogen peroxide. Slides were incubated with 10% goat serum for 30 min and stained with rabbit anti-CD3 (Abcam) at 4°C overnight. Anti-rabbit horseradish peroxidase was applied to slides for 30 min and developed using an Envision+ System-HRP (DAB) kit (Dako). Methyl green was used as a counterstain.

Flow cytometry and ELISA

Single cell suspensions were prepared from isolated spleens or lymph nodes and stained with fluorophore-conjugated antibodies against NK1.1, CD3, CD4, CD8, CD44, CD62L, IFN γ (all from eBioscience), or granzyme B (BD Biosciences). T cells were stimulated with PMA/ionomycin (Sigma) for 4 hours prior to staining for intracellular IFN γ . Flow cytometry was performed with a FACSCanto (BD) and analyzed with FlowJo version 9.4.11 (Tree Star). Serum IFN γ , TNF, and IL-6 were quantified using Ready-SET-Go! ELISA kits (eBioscience).

In vivo allogeneic cytotoxicity

All in vivo allogeneic cytotoxicity assays were performed 1 week after the primary AT. Donor splenocytes were depleted of T cells by negative selection using CD3 MACS beads (Miltenyi). The target (B6) and control (either 129 or Balb/c) populations were labeled with high- or low-dose CFSE (1 μ M or 0.1 μ M), respectively. 20 million total cells (1:1 ratio) were injected retroorbitally. 24 hours later, splenocytes were harvested and analyzed by FACS. Specific killing was calculated as fractional reduction of the target population relative to the control population.

In vivo analysis of donor T cell proliferation

Donor splenocytes were labeled with 1 μ M CFSE prior to AT. Splenocytes were harvested 6 days later, and the extent of CFSE dilution was analyzed by flow cytometry to assess donor CD4⁺ and CD8⁺ T cell proliferation.

Data analysis

All data presented are mean $\pm$ SEM. P values and the type of statistical analysis performed are described in the figure legends. In the figures, a single asterisk (*) is used to denote $P < 0.05$, and two asterisks (**) for $P < 0.01$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Minor mismatch GVHD occurs in two phases and is less severe than major mismatch GVHD

Wild-type B6 recipients were lethally irradiated followed by a syngeneic (syn, B6, n=5), minor mismatch (minor, 129, n=14), or major mismatch (major, Balb/c, n=16) BMT (bone marrow and splenocytes). (A) Weight, (B) clinical signs of disease, and (C) survival are shown. Data are pooled from >5 independent experiments. $P < 0.001$, comparing minor mismatch BMT and major mismatch BMT to syngeneic BMT by two-sided Student's t-test for weight or two-sided Mann-Whitney U test for clinical score, except $P = 0.0096$ comparing the clinical score of major mismatch BMT to syngeneic BMT. $P = 0.001$ for survival by Kaplan-Meier log rank test comparing minor mismatch BMT to syngeneic BMT and $P < 0.001$ comparing major mismatch BMT to syngeneic BMT. (D) Pathology scores of GVHD target organs at 5 weeks post BMT for syngeneic and minor mismatch and at 3 weeks, or time of death if disease severity required early sacrifice, for major mismatch recipients. Each dot represents an individual mouse. Data are pooled from 2 independent experiments. All pathology scores were evaluated by two-sided Mann-Whitney U test. Intestine: $P = 0.013$ comparing minor mismatch BMT to syngeneic BMT, $P = 0.013$ comparing major mismatch BMT to syngeneic BMT. Liver: $P = 0.004$ comparing minor mismatch BMT to syngeneic BMT, $P = 0.012$ comparing major mismatch BMT to syngeneic BMT. Skin: $P = 0.008$ comparing minor mismatch BMT to syngeneic BMT, $P = 0.020$ comparing major mismatch BMT to syngeneic BMT. Total pathology score: $P = 0.016$ comparing minor mismatch BMT to syngeneic BMT, $P = 0.029$ comparing major mismatch BMT to syngeneic

BMT. (E) Histopathology of GVHD target organs. Scale bars: intestine, 50 μm , inset, 10 μm ; liver, 100 μm , inset, 10 μm ; skin, 50 μm . Yellow arrows in the intestine insets denote apoptotic cells. (F) Relative intestinal permeability to 4-kDa FITC-dextran at indicated times after BMT ($n=2-4/\text{group/time point}$). Values were normalized to a non-treated WT control (control permeability=1.0). Data are pooled from 2 independent experiments. $P=0.040$ at day 7 comparing major mismatch BMT to syngeneic BMT and $P=0.011$ comparing minor mismatch BMT to syngeneic BMT by two-sided Student's t-test. $P=0.005$ at day 28 comparing minor mismatch BMT to syngeneic BMT by two-sided Student's t-test. $P=0.041$ at day 35 comparing minor mismatch BMT to syngeneic BMT by two-sided Student's t-test.

Figure 2. Pre-transplant irradiation is required for major and minor mismatch GVHD
 B6 *Rag1*^{-/-} recipients did or did not receive 7 Gy irradiation prior to a syngeneic (B6), minor (129), or major mismatch (Balb/c) splenocyte adoptive transfer (AT) (n=4–10/group). (A) Weight and (B) clinical signs of disease. Data are pooled from >5 independent experiments. For weight, P=0.01 for minor mismatch and P<0.001 for major mismatch comparing matched ATs, unconditioned vs. conditioned, by two-sided Student's t-test. For clinical score, P=0.047 for minor mismatch and P=0.010 for major mismatch comparing matched ATs, unconditioned vs. conditioned, by two-sided Mann-Whitney U test. (C) Representative intestinal histopathology of mice that received 7 Gy irradiation + Balb/c AT at day 7, showing dense inflammatory infiltrate and crypt cell apoptosis (yellow arrows). Scale bars: 50 μ m, inset, 10 μ m. (D) Pathology scores of GVHD target organs 25 days after AT. Each dot represents an individual mouse. Data are pooled from 2 independent experiments. All pathology scores were evaluated by two-sided Mann-Whitney U test and compared matched ATs, unconditioned vs. conditioned. Liver: P=0.036. Skin: P=0.036. Total pathology score: P=0.036. (E) Relative intestinal permeability to 4-kDa FITC-dextran gavage 7 days after AT (n=4–6/group). Values were normalized to a non-treated *Rag1*^{-/-} control (control permeability=1). Data are pooled from 3 independent experiments. P=0.031 comparing minor mismatch AT with irradiation to minor mismatch AT without irradiation by two-sided Student's t-test. (F) Dot plots of CD3⁺/NK1.1⁺ cells in the spleen 35 days after AT or 7 days after AT (7 Gy + Balb/c). Lymphocytes were gated based on forward and side scatter. Data are representative of three independent experiments. (G) NK1.1⁺/CD3⁺ splenocytes in B6 *Rag1*^{-/-} that did or did not receive 7 Gy irradiation 3 days earlier (n=4/

group). Data are pooled from 2 independent experiments. $P < 0.001$ by two-sided Student's t-test.

Figure 3. NK cell inactivation is sufficient to drive major, but not minor, mismatch GVHD (A–D) B6 *Rag1*^{-/-} mice were treated with anti-NK1.1 (clone PK136) 2 days prior to minor (129) or major (Balb/c) mismatch AT. (A) Weight and (B) clinical signs of disease. Data are pooled from 3 independent experiments each with n=5/group. For weight, P=0.013 by two-sided Student's t-test. For clinical score, P=0.042 by two-sided Mann-Whitney U test. (C) Pathology scores of GVHD target organs 35 days after AT. Each dot represents an individual mouse. Data are pooled from 2 independent experiments. All pathology scores were evaluated by two-sided Mann-Whitney U test. Skin: P=0.035. Total pathology score: P=0.036. (D) Relative intestinal permeability to 4-kDa FITC-dextran 7 days after AT (n=8/group). Values were normalized to a non-treated *Rag1*^{-/-} control (control permeability=1). Data are pooled from 4 independent experiments. P=0.033 by two-sided Student's t-test. (E–

H) B6 *Rag2*^{-/-}/*Pfp*^{-/-} mice received a minor (129) or major (Balb/c) mismatch AT. (E) Weight and (F) clinical signs of disease (n=4–5/group). Data are pooled from 3 independent experiments. For weight, P=0.046 by two-sided Student's t-test. For clinical score, P=0.044 by two-sided Mann-Whitney U test. (G) Pathology scores of GVHD target organs 35 days after AT. Each dot represents an individual mouse. Data are pooled from 2 independent experiments. All pathology scores were evaluated by two-sided Mann-Whitney U test. Skin: P=0.036. Total pathology score: P=0.029. (H) In vivo cytotoxicity against allogeneic targets 7 days after AT (n=3/group). Specific killing was calculated based on the percent reduction of the target population relative to the control population. Data are pooled from 2 independent experiments. P=0.011 by two-sided Student's t-test.

Figure 4. Combined intestinal damage and NK cell inactivation can drive minor mismatch GVHD

(A–C) B6 *Rag1*^{−/−} were treated with 3% DSS + anti-NK1.1 (clone PK136) or 3% DSS + control antibody prior to minor mismatch (129) AT, or DSS only. (A) Weight and (B) clinical signs of disease. Data are pooled from 3 independent experiments (n=4–5/group). All mice survived until sacrifice 35 days after AT. For weight, P=0.018 by two-sided Student's t-test, and for clinical score, P=0.044 by two-sided Mann-Whitney U test, comparing mice that received DSS and a 129 AT with NK cell depletion vs. mice that received DSS and a 129 AT with control antibody. (C) Pathology scores of GVHD target organs 25 days after AT. Each dot represents an individual mouse. Data are pooled from 2

independent experiments. $P=0.029$ by two-sided Mann-Whitney U test, comparing total pathology scores of mice that received DSS and a 129 AT with NK cell depletion vs. mice that received DSS and a 129 AT with control antibody. (D) B6 *Rag1*^{-/-} mice received 7 Gy irradiation, 3% DSS, 3% DSS + anti-NK1.1, 3% DSS + control antibody, or no treatment prior to minor mismatch (129) AT. In vivo cytotoxicity against allogeneic targets was evaluated 7 days after AT ($n=4-5$ /group). $P=0.007$ comparing mice that received 7 Gy and a 129 AT vs. 129 AT only by two-sided Student's t-test. $P=0.028$ comparing DSS and a 129 AT with NK cell depletion vs. mice that received DSS and a 129 AT with control antibody by two-sided Student's t-test. (E-I) B6 *Rag2*^{-/-}*Pfp*^{-/-} mice were treated with 3% DSS prior to minor mismatch (129) or syngeneic (B6) AT. (E) Weight, (F) clinical signs of disease, and (G) survival ($n=5-7$ /group). For weight, $P=0.008$ by two-sided Student's t-test. For clinical score, $P=0.010$ by two-sided Mann-Whitney U test. For survival, $P=0.034$ by Kaplan-Meier log rank test. (H) Histopathology of GVHD target organs 21 days after AT. In mice that received DSS + 129 AT, there is abundant crypt cell apoptosis in the jejunum (yellow arrows), portal inflammation and bile duct damage in the liver, and hyperkeratosis, inflammatory infiltrate, and epithelial hyperplasia in the skin. Scale bars: intestine, 50 μ m, inset, 10 μ m; liver, 100 μ m, inset, 20 μ m; skin, 50 μ m. (I) Relative intestinal permeability to 4-kDa FITC-dextran gavage 7 days after AT ($n=3-4$ /group). Values were normalized to a non-treated *Rag2*^{-/-}*Pfp*^{-/-} control (control permeability=1.0). $P=0.036$ comparing mice that received DSS and a 129 AT vs. mice that received DSS and a B6 AT and $P=0.024$ vs. mice that received a 129 AT only by two-sided Student's t-test.

Figure 5. NK cell depletion increases alloreactive T cell expansion and target organ infiltration
 B6 *Rag1*^{-/-} received 3% DSS, anti-NK1.1 (clone PK136) or control antibody (IgG), and a 129 AT (n=5–6 mice/group). Mice were sacrificed 21 days after AT. (A–C) Flow cytometry analysis of secondary lymph nodes. (C) was evaluated after 4 hour PMA/ionomycin stimulation. Each dot represents an individual mouse. Data are pooled from 2 independent experiments. Lymph node results were evaluated by two-sided Student's t-test. (A) Spleen: P=0.006. MLN: P=0.002. Axillary/brachial lymph node (A/B): P=0.023. (B) Spleen: P=0.001. MLN: P=0.007. A/B: P=0.011. (C) MLN: P=0.033 by two-sided Student's t-test. (D–E) Immunohistochemical staining of CD3⁺ cells in GVHD target tissue. (D) Representative staining in liver, magnified to illustrate CD3⁺ cells (black arrows). Scale bar: 10 μ m. (E) Quantification of CD3⁺ cells. Each dot represents an individual area. 3–6 representative areas/organ for each mouse were scored (5–6 mice/group). Organ infiltration was evaluated by two-sided Mann-Whitney U test. Intestine: P=0.001. Liver: P<0.001. Skin: P=0.0099.

Figure 6. Donor TLR4 and MyD88, but not recipient TLR4 or donor TRIF, are necessary for minor mismatch GVHD

(A–B) B6 TLR4^{-/-} or WT recipients were lethally irradiated followed by a 129 BMT (n=4–6/group). (A) Weight and (B) clinical signs of disease. Half of TLR4^{-/-} recipients died by 35 days after BMT. Data are pooled from 2 independent experiments. (C–F) 129 *Rag2*^{-/-} mice received 3% DSS, anti-asialo GM1 (NK depl.), and a minor mismatch (B6) AT from donors that lack TLR4, MyD88, or TRIF. Data are pooled from 3 independent experiments. (C) Weight and (D) clinical signs of disease, (n=4–5/group). For weight, P=0.045 by two-sided Student's t-test. For clinical score, P=0.030 by two-sided Mann-Whitney U test. (E) Weight and (F) clinical signs of disease (n=7–8/group). For weight, P=0.016 by two-sided

Student's t-test compared to WT AT. For clinical score, $P=0.039$ by two-sided Mann-Whitney U test compared to WT AT.

Figure 7. The enteric microbiome contributes to the initiation of minor mismatch GVHD (A–B) B6 *Rag1*^{−/−} mice received 3% DSS, anti-NK1.1 (clone PK136), and a minor mismatch (129) AT. One group additionally received 50 mg/kg/day vanocymyin/imipenem in their drinking water from day −7 to +7 relative to AT. (n=4/group). (A) Weight and (B) clinical signs of disease. Data are pooled from 2 independent experiments. For weight, $P=0.015$ by two-sided Student's t-test. For clinical score, $P=0.042$ by two-sided Mann-Whitney U test. (C–F) B6 *Rag2*^{−/−} *Pfp*^{−/−} mice received an i.p. injection of 0.8 mg/kg purified LPS 2 hours prior to AT (n=6–7/group). (C) Weight and (D) clinical signs of disease. Data are pooled from 3 independent experiments. For weight, $P=0.040$ by two-sided

Student's t-test. For clinical score, $P=0.042$ by two-sided Mann-Whitney U test. (E) Pathology scores of GVHD target organs 19–21 days after AT. Each dot represents an individual mouse. Data are pooled from 2 independent experiments. All pathology scores were evaluated by two-sided Mann-Whitney U test. Intestine: $P=0.003$. Liver: $P=0.003$. Skin: $P=0.003$. Total pathology score: $P=0.003$. (F) Histopathology of GVHD target organs 21 days after AT. Scale bars: intestine, 50 μm , inset, 10 μm ; liver, 100 μm , inset, 20 μm ; skin, 50 μm . Yellow arrows in the intestine insets denote apoptotic cells.