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Sam C. Nalle *et al.*

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Editor's Summary

Double Trouble Before Transplant

Graft-versus-host disease, a condition where transplanted immune cells attack the body of the transplant recipient, is a common complication of bone marrow or stem cell transplant. Although there are treatments available for treating graft-versus-host disease, they don't always work well. Nalle *et al.* have now developed several different mouse models of this condition to provide insight into its causes. The authors used these mouse models to pinpoint two different ways that pretransplant conditioning contributes to graft-versus-host disease. This knowledge should help scientists search for new treatments against this disease, as well as identify safer ways to prepare patients for transplant.

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GRAFT-VERSUS-HOST DISEASE

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

Sam C. Nalle, H. Aimee Kwak, Karen L. Edelblum, Nora E. Joseph, Gurminder Singh, Galina F. Khramtsova, Eric D. Mortenson, Peter A. Savage,* Jerrold R. Turner*

Previous studies have shown a correlation between pretransplant conditioning intensity, intestinal barrier loss, and graft-versus-host disease (GVHD) severity. However, because irradiation and other forms of pretransplant 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 pretransplant variables. Intestinal damage was required for the induction of minor mismatch [major histocompatibility complex (MHC)–matched] GVHD, but was not necessary for major mismatch GVHD, demonstrating fundamental pathogenic distinctions between these forms of disease. Moreover, recipient natural killer (NK) cells prevented minor mismatch GVHD by limiting expansion and target organ infiltration of alloreactive T cells via a perforin-dependent mechanism, revealing an immunoregulatory function of MHC-matched recipient NK cells in GVHD. Minor mismatch GVHD required MyD88-mediated Toll-like receptor 4 (TLR4) signaling on donor cells, and intestinal damage could be bypassed by parenteral lipopolysaccharide (LPS) administration, indicating a critical role for the influx of bacterial components triggered by intestinal barrier loss. In all, the data demonstrate that pretransplant 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, in that 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, including NOD2 and many of the Toll-like receptors (TLRs), has been implicated in GVHD (*4*). In particular, experimental models have shown that lipopolysaccharide (LPS)–induced TLR4 signaling contributes to GVHD after F1 (major mismatch) BMT (*5*, *6*) and that the severity of this GVHD correlates with the magnitude of LPS-induced tumor necrosis factor (TNF) production by donor cells (*6*). These data support a model in which intestinal damage, including barrier loss induced by pretransplant conditioning, allows translocation of gut microbiota and microbial products that stimulate GVHD development and progression. Although attractive, this model has not been fully tested, because most experimental studies have used severe major histocompatibility complex (MHC)–mismatch GVHD models in which nonimmune contributors to disease (such as intestinal damage) are difficult to assess. Moreover, both disease models and human trials rely on pretransplant 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.

Pretransplant conditioning has many effects beyond the gastrointestinal tract, including damage to the 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 pre-transplant 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 pretransplant 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 pretransplant 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 to 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 an immunoregulatory function whereby recipient natural killer (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 mice of the 129 strain (H-2^b) were transferred into B6 (H-2^b) recipients, whereas cells from Balb/c (H-2^d) mice were transferred into B6

Department of Pathology, The University of Chicago, Chicago, IL 60637, USA.
*Corresponding author. E-mail: jturner@bsd.uchicago.edu (J.R.T.); psavage@bsd.uchicago.edu (P.A.S.)

recipients to induce major mismatch (MHC-mismatched) GVHD. After lethal irradiation, recipients were transplanted with bone marrow cells and splenocytes (hereinafter, this combination is referred to as BMT). Major mismatch BMT led to severe weight loss, clinical signs of disease, and characteristic histopathology (Fig. 1, fig. S1, and table S1); no mice survived past 24 days (Fig. 1C). Weight loss and clinical signs of disease developed more slowly after minor mismatch BMT (Fig. 1, A and B, and table S1), which was associated with histopathological features typical of GVHD in human patients (Fig. 1E and fig. S1). 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 and table S1). In minor mismatch and syngeneic recipients, weight stabilized, clinical scores decreased, and intestinal barrier function was restored within 2 weeks (Fig. 1F and table S1), 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 table S1) and coincided with increasing clinical scores (Fig. 1B and table S1). Thus, an initial phase of irradiation-induced barrier loss followed by transient recovery precedes clinical presentation of minor mismatch GVHD.

Irradiation and allogeneic splenocyte transfer induce GVHD in *Rag1*$^{-/-}$ recipients

To assess the contribution of pretransplant 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 because it has no effect on GVHD outcome and is not required for recipient

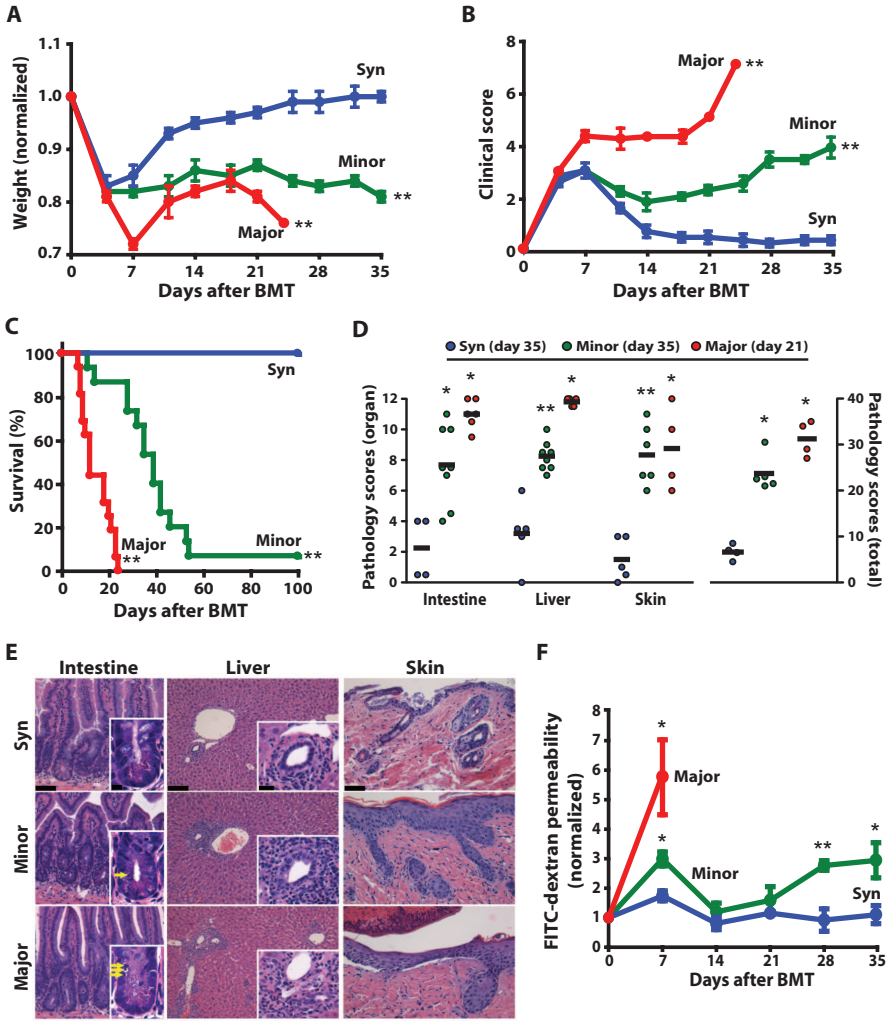

Fig. 1. Minor mismatch GVHD occurs in two phases. Wild-type B6 recipients were lethally irradiated and then received a syngeneic (syn, B6, $n = 9$), minor mismatch (minor, 129, $n = 14$), or major mismatch (major, Balb/c, $n = 16$) BMT (bone marrow and splenocytes). (**A**) Weight. (**B**) Clinical signs of disease. (**C**) Survival. Data are pooled from more than five 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 after 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 two 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, $P = 0.011$ 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-kD fluorescein isothiocyanate (FITC)–dextran at the indicated times after BMT ($n = 2$ to 4 per group per time point). Values were normalized to a nontreated wild-type (WT) control (control permeability = 1.0). Data are pooled from two independent experiments. $P = 0.047$ 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.

survival at the sublethal irradiation dose (7 Gy) used. Experiments comparing AT (splenocytes only) to BMT (splenocytes and bone marrow) of cells isolated from mice of the 129 strain into irradiated B6 recipients confirmed that the omission of bone marrow had no effect on *Rag1*$^{-/-}$ recipient bone marrow histopathology or peripheral blood counts (fig. S2).

We used the splenocyte AT experimental system and found that syngeneic (B6), minor mismatch (129), or major mismatch (Balb/c) AT into nonirradiated B6 *Rag1*$^{-/-}$ recipients failed to induce GVHD (Fig. 2, A and B, and table S1). In contrast, recipients that received sublethal irradiation before minor or major mismatch AT developed GVHD, including weight loss, clinical signs of disease, and characteristic histopathology (Fig. 2, A to D, and table S1). Irradiated recipients that received a syngeneic AT recovered fully in a manner similar to mice that received syngeneic BMT (compare Fig. 1, A and B, and Fig. 2, A and B).

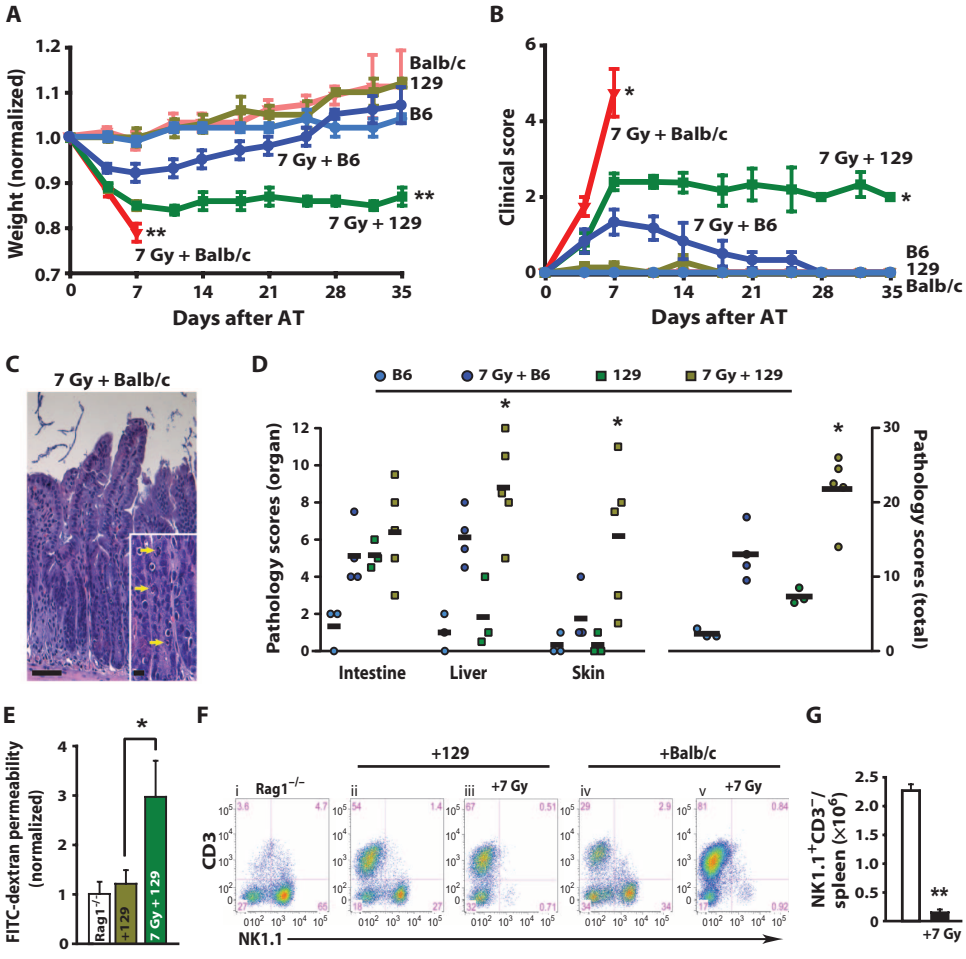

Fig. 2. Pretransplant irradiation is required for GVHD. B6 *Rag1*$^{-/-}$ recipients did or did not receive 7-Gy irradiation before a syngeneic (B6), minor (129), or major mismatch (Balb/c) splenocyte AT (n = 4 to 10 per group). (**A**) Weight. (**B**) Clinical signs of disease. Data are pooled from more than five independent experiments. For weight, $P < 0.001$ for minor mismatch and major mismatch comparing matched ATs, unconditioned versus 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 versus 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 μm (inset, 10 μm). (**D**) Pathology scores of GVHD target organs 25 days after AT. Each dot represents an individual mouse. Data are pooled from two independent experiments. All pathology scores were evaluated by two-sided Mann-Whitney U test and compared matched ATs, unconditioned versus conditioned. Liver: $P = 0.036$. Skin: $P = 0.036$. Total pathology score: $P = 0.036$. (**E**) Relative intestinal permeability to 4-kD FITC-dextran gavage 7 days after AT (n = 4 to 6 per group). Values were normalized to a nontreated *Rag1*$^{-/-}$ control (control permeability = 1). Data are pooled from three 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 7 days after AT (7 Gy + Balb/c) or 35 days after AT (all other ATs). Lymphocytes were gated on the basis of 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 per group). Data are pooled from two independent experiments. $P < 0.001$ by two-sided Student's t test.

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 pretransplant irradiation. In contrast, intestinal permeability of mice that received AT without irradiation was similar to controls (Fig. 2E and table S1). Immunofluorescence 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 (fig. S3). 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 NK1.1$^+$/CD3$^-$ recipient NK cells, which are present in abundance in B6 *Rag1*$^{-/-}$ mice (Fig. 2F). Irradiation markedly reduced NK cell numbers (Fig. 2G and table S1). Consistent with the above hypothesis, irradiated recipients of major mismatch (Balb/c) splenocytes had a greater number of NK1.1$^+$/CD3$^-$ donor T cells than did the nonirradiated B6 *Rag1*$^{-/-}$ recipients. In contrast, the numbers of minor mismatch (129) donor T cells were comparable in irradiated and nonirradiated B6 *Rag1*$^{-/-}$ recipients (Fig. 2F). These data suggested that major, but not minor, mismatch donor cells were rejected by NK cells in nonirradiated recipients and that irradiation prevented this rejection by clearing recipient NK cells.

NK cell inactivation allows major, but not minor, mismatch GVHD without pretransplant irradiation

To test the hypothesis that NK cells prevented GVHD in nonirradiated recipients, we treated B6 *Rag1*$^{-/-}$ mice with depleting

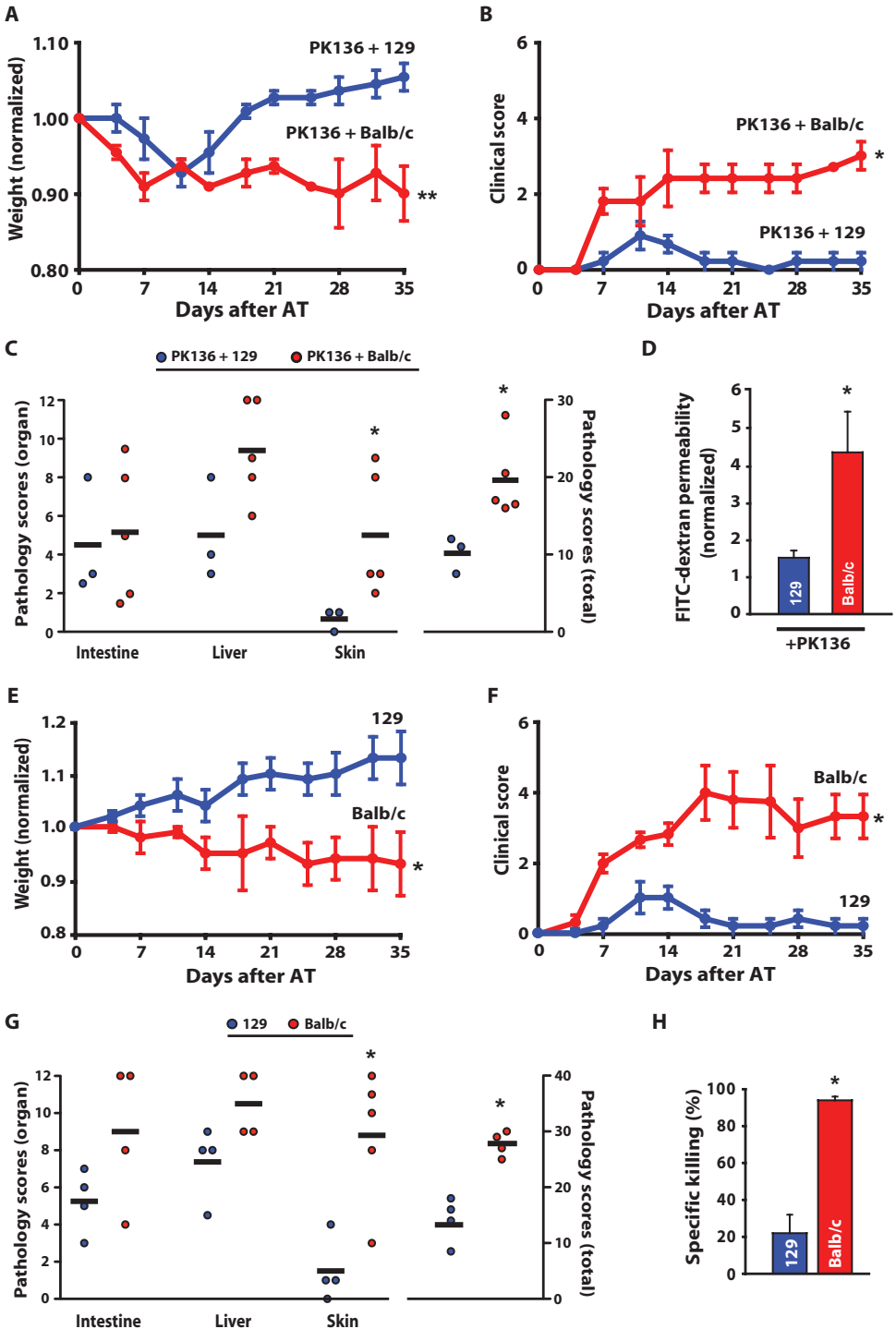

Fig. 3. NK cell inactivation is permissive for major, but not minor, mismatch GVHD. (**A** to **D**) B6 *Rag1*$^{-/-}$ mice were treated with anti-NK1.1 (clone PK136) 2 days before minor (129) or major (Balb/c) mismatch AT. (A) Weight. (B) Clinical signs of disease. Data are pooled from three independent experiments each with $n = 4$ per group. For weight, $P = 0.005$ 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 two 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-kD FITC-dextran 7 days after AT ($n = 6$ to 8 per group). Values were normalized to a nontreated *Rag1*$^{-/-}$ control (control permeability = 1). Data are pooled from four independent experiments. $P = 0.033$ by two-sided Student's t test. (**E** to **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 to 5 per group). Data are pooled from three 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 two 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 ($n = 2$ to 3 per group). Specific killing was calculated on the basis of the percent reduction of the target population relative to the control population. Data are pooled from two independent experiments. $P = 0.011$ by two-sided Student's t test.

anti-NK1.1 antibody (PK136) or control antibody before AT. This treatment allowed development of GVHD after major, but not minor, mismatch AT (Fig. 3, A to C, and table S1). Mice that received major mismatch AT also exhibited elevated intestinal permeability (Fig. 3D and table S1). These data 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 pretransplant irradiation to minor mismatch GVHD cannot be explained solely by NK depletion.

Although 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, we assessed GVHD 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 after 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 interferon-γ (IFNγ), TNF, and interleukin-6 (IL-6) (Fig. 3, E to G, figs. S4 and S5, and table S1). In contrast, GVHD did not develop after minor mismatch AT into *Rag2*$^{-/-}$/*Pfp*$^{-/-}$ B6 recipients despite increases in cytokine production. Thus, although immune activation, as indicated by cytokine production, occurred after 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 after major or minor mismatch AT into nonirradiated *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, whereas minor mismatch 129 donor cells were relatively inefficient at killing B6 targets under these conditions (Fig. 3H and table S1). 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, such as irradiation or NK cell death, nor additional clearance of a proliferative niche is required for major mismatch GVHD. In contrast, despite donor cell survival, neither recipient NK cell depletion nor perforin deficiency is sufficient to allow GVHD development after minor mismatch AT without previous irradiation.

Colonic damage and NK cell inactivation combine to promote 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, we treated B6 *Rag1*$^{-/-}$ recipients of minor mismatch AT with dextran sulfate sodium (DSS), which induces colitis and colonic barrier loss that resolves within 1 week of DSS discontinuation (*17*, *18*). Despite substantial colonic damage, GVHD did not develop in DSS-treated recipients of minor mismatch AT (Fig. 4, A to C, fig. S6, and table S1). Further, unlike irradiation, DSS did not increase donor cell cytotoxicity against allogeneic targets (Fig. 4D and table S1). Irradiation effects other than intestinal damage are therefore required for minor mismatch GVHD development. One of these effects could be the creation of a niche, or space, for donor cell proliferation. However, in vivo analysis of donor cells labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) 6 days after AT revealed that >80% of donor cells had undergone proliferation regardless of previous treatment (fig. S7), thereby excluding a role for a "proliferative niche" created by NK cell clearance in minor mismatch GVHD. 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 after minor mismatch AT, irradiation does clear recipient NK cells (Fig. 2G and table S1). 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 the combination of colonic damage and NK cell depletion was sufficient to allow minor mismatch GVHD development after minor mismatch AT. Treatment with DSS coupled with anti-NK1.1 (PK136), but not control antibody, 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 in the liver, skin, and lung (Fig. 4C and fig. S6). 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 and table S1). 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. 3, E to H, and table S1). However, DSS treatment of *Rag2*$^{-/-}$/*Pfp*$^{-/-}$ B6 recipients before minor mismatch AT resulted in GVHD, including characteristic features of systemic GVHD, such as involvement of the small intestine, liver, lung, and skin (Fig. 4, E to H, fig. S8, and table S1) and increased intestinal permeability (Fig. 4I and table S1). DSS-treated *Rag2*$^{-/-}$/*Pfp*$^{-/-}$ B6 recipients of syngeneic AT recovered completely (Fig. 4, E to H, and table S1), 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. The survival of *Rag2*$^{-/-}$/*Pfp*$^{-/-}$ B6 recipients subjected to minor mismatch AT and DSS treatment was similar to that seen after minor mismatch BMT in lethally irradiated recipients (compare Figs. 4G and 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 augments effector T cell expansion in target organs

On the basis of the 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, we treated *Rag1*$^{-/-}$ recipients with DSS and an NK cell–depleting antibody or control immunoglobulin G (IgG) before minor mismatch AT. By 3 weeks after AT, recipients treated with NK cell–depleting antibody had a marked increase in effector memory CD8$^+$ T cells within the spleen, mesenteric lymph nodes (MLNs), and axillary/brachial lymph nodes (A/B), as judged by both CD44^{hi}CD62L^{lo} and granzyme B expression (Fig. 5, A and B). In addition, effector CD4$^+$IFNγ$^+$ T cells were increased in MLNs (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 the infiltration of donor CD3$^+$ T cells in GVHD target organ tissue using immunohistochemistry. Mice that received NK cell–depleting antibody had substantially greater T cell infiltration in the small intestine, liver, and skin (Fig. 5, D and E). 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

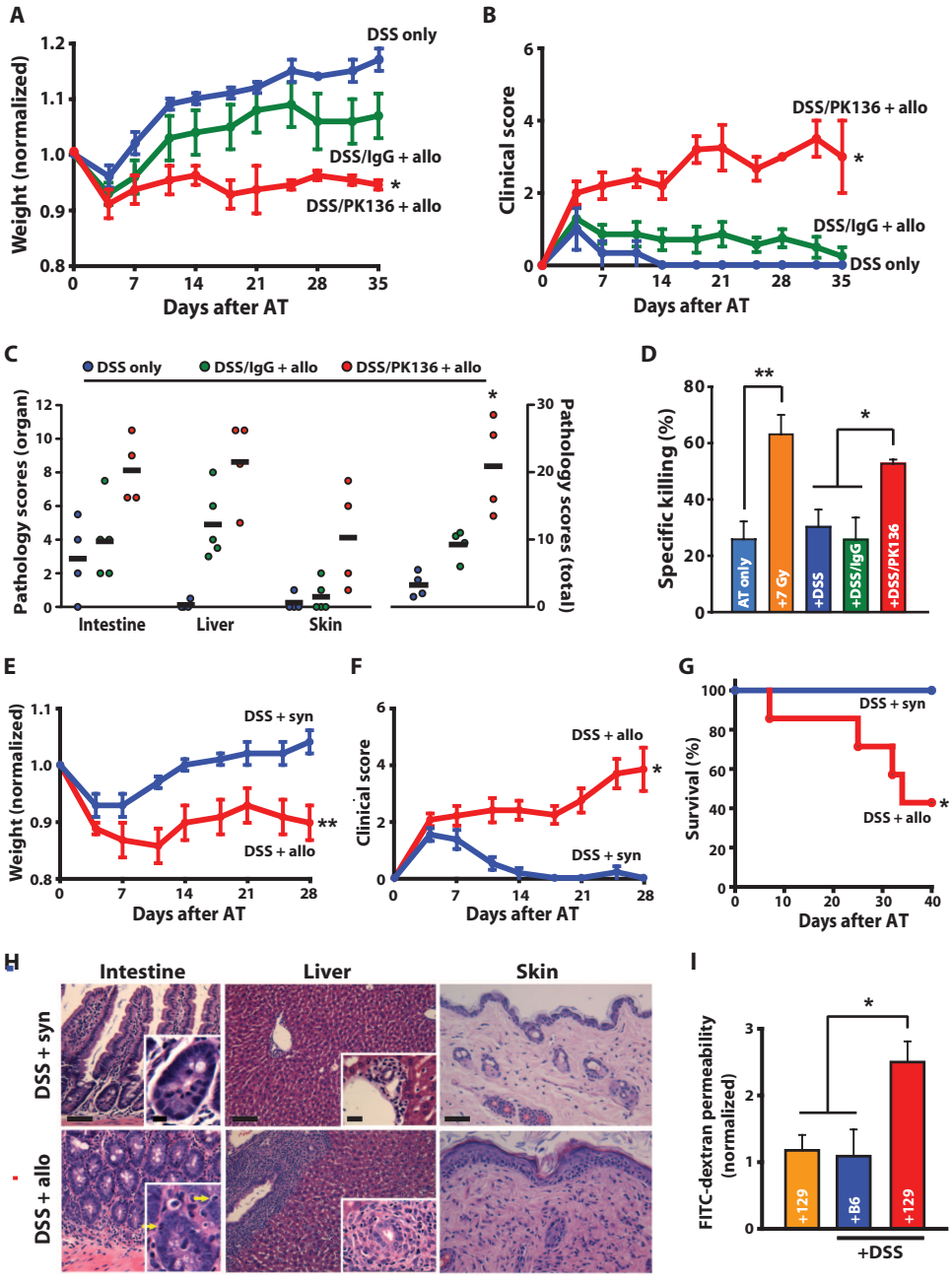

Fig. 4. Combined intestinal damage and NK cell inactivation can drive minor mismatch GVHD. (**A** to **C**) B6 *Rag1*$^{-/-}$ were treated with 3% DSS + anti-NK1.1 (clone PK136) or 3% DSS + control antibody before minor mismatch (129) AT, or DSS only. (A) Weight. (B) Clinical signs of disease. Data are pooled from three independent experiments (n = 3 to 7 per group). All mice survived until 35 days after AT unless sacrificed earlier. 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 versus 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 two 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 versus 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 before minor mismatch (129) AT. In vivo cytotoxicity against allogeneic targets was evaluated 7 days after AT (n = 3 to 4 per group). P = 0.007 comparing mice that received 7 Gy and a 129 AT versus 129 AT only by two-sided Student's t test. P = 0.028 comparing DSS and a 129 AT with NK cell depletion versus mice that received DSS and a 129 AT with control antibody by two-sided Student's t test. (**E** to **I**) B6 *Rag2*$^{-/-}$*Pfp*$^{-/-}$ mice were treated with 3% DSS before minor mismatch (129) or syngeneic (B6) AT. (E) Weight, (F) clinical signs of disease, and (G) survival (n = 6 to 7 per 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 μm (inset, 10 μm); liver, 100 μm (inset, 20 μm); skin, 50 μm. (I) Relative intestinal permeability to 4-kD FITC-dextran gavage 7 days after AT (n = 3 to 4 per group). Values were normalized to a nontreated *Rag2*$^{-/-}$*Pfp*$^{-/-}$ control (control permeability = 1.0). P = 0.036 comparing mice that received DSS and a 129 AT versus mice that received DSS and a B6 AT and P = 0.024 versus mice that received a 129 AT only by two-sided Student's t test.

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 demonstrated that after BMT from 129 donor mice into lethally irradiated B6 recipients, disease severity was

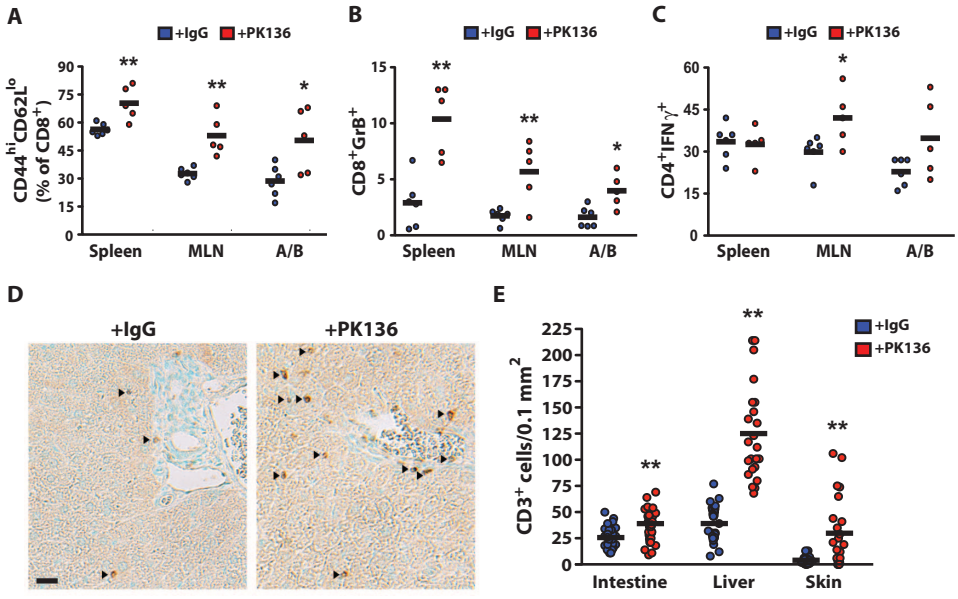

Fig. 5. NK cell depletion increases T cell expansion and tissue infiltration. B6 *Rag1*$^{-/-}$ received 3% DSS, anti-NK1.1 (clone PK136) or control antibody (IgG), and a 129 AT (n = 5 to 6 mice per group). Mice were sacrificed 21 days after AT. (**A** to **C**) Flow cytometric analysis of secondary lymph nodes. The analysis in (C) was performed after 4 hours of stimulation with phorbol 12-myristate 13-acetate (PMA)/ionomycin. Each dot represents an individual mouse. Data are pooled from two 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** and **E**) Immunohistochemical staining of CD3$^+$ cells in GVHD target tissue. (D) Representative staining in liver, magnified to illustrate CD3$^+$ cells (black arrowheads). Scale bar, 10 μm. (E) Quantification of CD3$^+$ cells. Each dot represents an individual area. Three to six representative areas per organ for each mouse were scored (five to six mice per group). Organ infiltration was evaluated by two-sided Mann-Whitney U test. Intestine: $P = 0.001$. Liver: $P < 0.001$. Skin: $P = 0.0099$.

comparable in *TLR4*$^{+/+}$ and *TLR4*$^{-/-}$ recipients (Fig. 6, A and B, and table S1), 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. 6, C and D, and table S1). 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 after TLR4 ligation (*23*). To determine which of these pathways was involved in the TLR4 signaling that drives minor mismatch GVHD, we assessed the ability of *MyD88*$^{-/-}$ or *TRIF*$^{-/-}$ B6 splenocytes to induce disease in DSS-treated, NK cell–depleted 129 *Rag2*$^{-/-}$ recipients. As shown in Fig. 6, the severity of minor mismatch GVHD was diminished after transfer of *MyD88*$^{-/-}$ donor cells, but was not affected by the transfer of *TRIF*$^{-/-}$ donor cells. This was apparent in terms of weight loss (Fig. 6E and table S1) and clinical scores (Fig. 6F and table S1). These results demonstrate that MyD88-dependent signaling on donor cells contributes to minor mismatch GVHD and that TLR4-TRIF signaling is not required.

Intestinal microbiota and microbial products promote minor mismatch GVHD

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 1 week before and 1 week after minor mismatch AT with DSS and NK cell depletion. Despite discontinuation at day 7 after AT, antibiotic treatment markedly reduced GVHD severity, and this effect persisted to at least day 35. The milder disease was accompanied by weight gain (Fig. 7A and table S1), absence of clinical features of GVHD (Fig. 7B and table S1), 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 promote minor mismatch GVHD by allowing LPS, other bacterial products, or live microorganisms to access the systemic circulation. To assess this, we treated B6 *Rag2*$^{-/-}$*Pfp*$^{-/-}$ mice with a single intraperitoneal dose of purified LPS before syngeneic or minor mismatch AT. Mice that received LPS and syngeneic AT experienced transient, mild weight loss (Fig. 7C and table S1), and increased clinical scores (Fig. 7D and table S1) 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 table S1) and developed clinical features of GVHD (Fig. 7D and table S1). Further, mice that received LPS developed histopathology typical of GVHD (Fig. 7, E and F). Thus, microbial components, such as LPS, are sufficient to drive GVHD in the absence of intestinal barrier loss when paired with NK cell inactivation and minor mismatch AT.

DISCUSSION

Here, 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 pretransplant conditioning–induced tissue damage. In contrast, weaker antigenic stimuli, such as minor mismatch antigens, which are common in human leukocyte antigen (HLA)–matched BMT and HSCT performed clinically, are unable to drive disease without intestinal barrier loss. Moreover, we have uncovered a regulatory role for MHC-matched recipient NK cells in

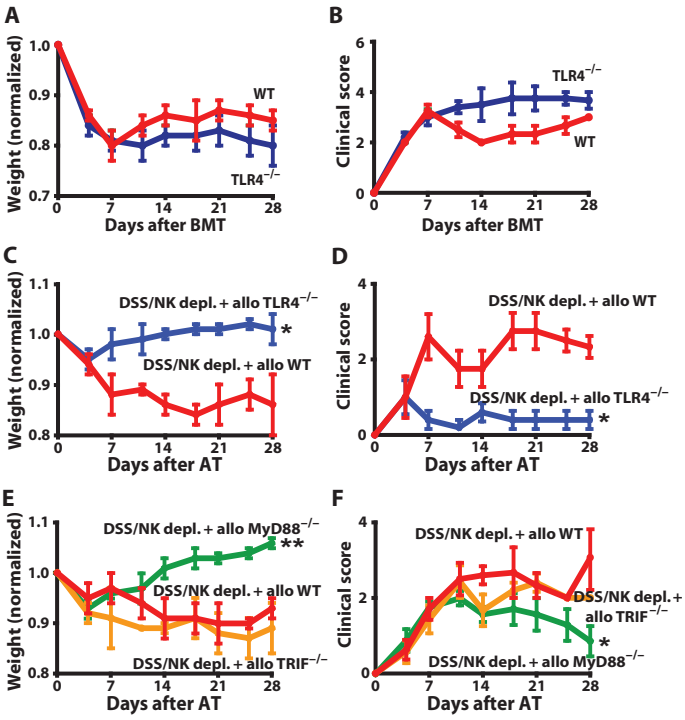

Fig. 6. Donor TLR4 and MyD88 are necessary for minor mismatch GVHD. (**A** and **B**) B6 TLR4$^{-/-}$ or WT recipients were lethally irradiated and underwent a 129 BMT (n = 4 to 5 per group). (A) Weight. (B) Clinical signs of disease. Data are pooled from two independent experiments. (**C** to **F**) 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 three independent experiments. (C) Weight and (D) clinical signs of disease (n = 4 to 5 per 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 to 8 per group). For weight, P < 0.001 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.

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 (fig. S9).

Although NK cells have long been of interest in GVHD (*24*, *25*), NK cells of donor origin, which reduce GVHD while enhancing graft antitumor 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 in a robust minor antigen mismatch GVHD model show that recipient NK cells regulate minor mismatch GVHD pathogenesis by a perforin-dependent mechanism. Recipient NK cells did not affect 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 pretransplant conditioning (*37*–*39*). However, pretransplant 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, major mismatch GVHD did develop in the absence of pretransplant 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 pretransplant 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 pretransplant conditioning. Thus, the data presented here, which 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 in mouse models of GVHD, pretransplant conditioning fulfills dual roles in disease pathogenesis by simultaneously inhibiting recipient NK cell–dependent immunoregulation of donor cells 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 systemic GVHD (fig. S9).

It remains to be determined whether the principles revealed with inbred mouse strains are operative in human GVHD. Nevertheless, the data presented here provide the rational basis for an in-depth analysis of recipient NK cell responses in patients undergoing HLA-matched BMT. In addition, although our data indicate a perforin-dependent process, the detailed mechanisms by which MHC-matched NK cells sense and regulate alloreactive T cell responses are unknown. Finally, it is important to note that the impact of recipient NK cell–dependent donor T cell immunoregulation on graft versus tumor effects, which are often critical in HSCT/BMT, remains to be determined.

Therapeutically, our results lend further support to the prophylactic use of antibiotics to limit GVHD (*45*) and also provide insight into the mechanisms underlying this effect. Additionally, our data suggest that patients may benefit from treatment with TLR4 antagonists (*46*, *47*). Finally, the most intriguing therapeutic implications of our studies

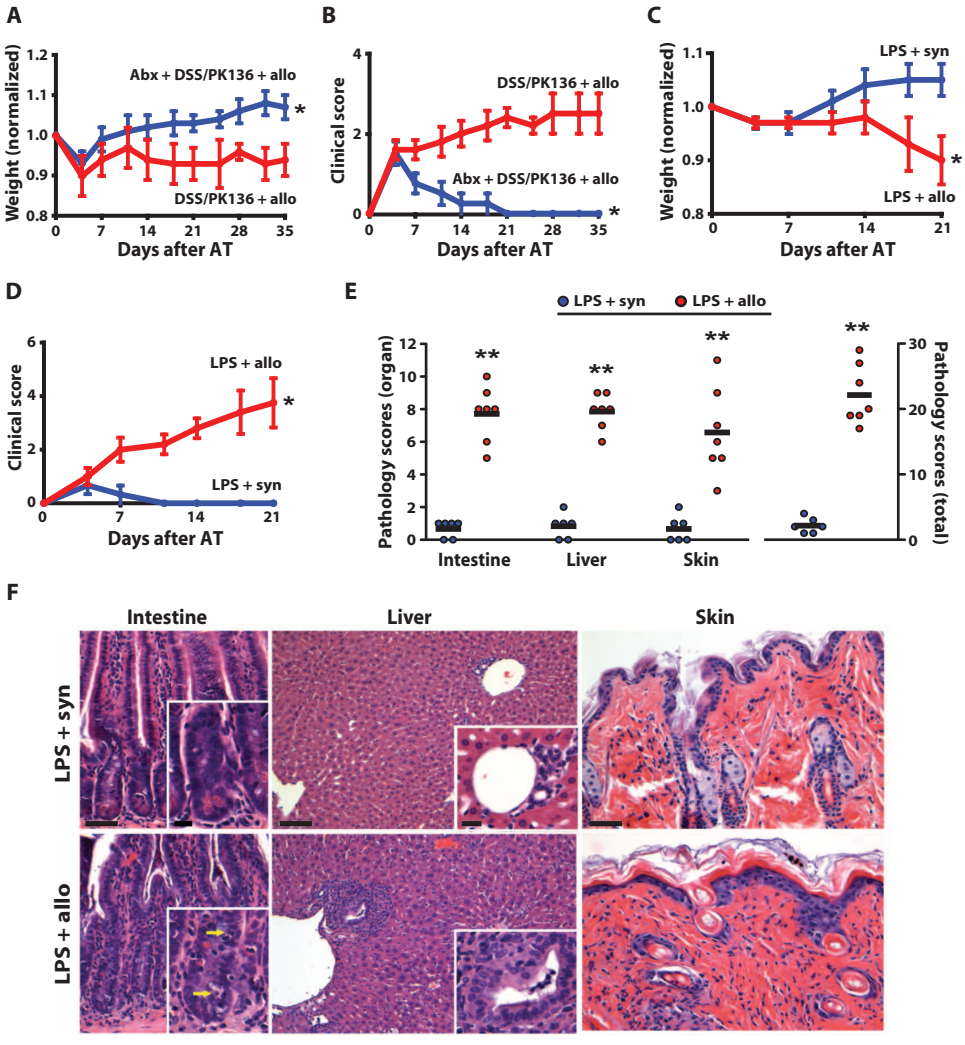

Fig. 7. Microbiota contributes to the initiation of minor mismatch GVHD. (**A** and **B**) B6 *Rag1*$^{-/-}$ mice received 3% DSS, anti-NK1.1 (clone PK136), and a minor mismatch (129) AT. One group additionally received vancomycin and imipenem from days −7 to +7 relative to AT (n = 4 to 5 per group). (A) Weight. (B) Clinical signs of disease. Data are pooled from two independent experiments. For weight, P = 0.015 by two-sided Student's t test. For clinical score, P = 0.036 by two-sided Mann-Whitney U test. (**C** to **F**) B6 *Rag2*$^{-/-}$*Pfp*$^{-/-}$ mice received an intraperitoneal injection of purified LPS (0.8 mg/kg) 2 hours before AT (n = 3 to 5 per group). (C) Weight. (D) Clinical signs of disease. Data are pooled from two 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 to 21 days after AT. Each dot represents an individual mouse. Data are pooled from two 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.

relate to the unexpected immunoregulatory role of recipient NK cells. Although further study is necessary, the data suggest that harvesting recipient NK cells before pretransplant conditioning and returning them to the patient after HSCT/BMT could arrest GVHD progression.

In sum, this study provides direct evidence that pretransplant intestinal damage not only enhances GVHD severity but also is required for clinically relevant minor mismatch GVHD. These data therefore support the hypothesis that reduction of pretransplant conditioning–induced intestinal damage or modulation of the intestinal microbiota may be effective preventative approaches in GVHD. The results also suggest that enhancing the regulatory role of MHC-matched recipient NK cells may serve as an additional approach to limit GVHD.

MATERIALS AND METHODS

Study design

The purpose of this study was to use mouse models of minor and major mismatch transplantation to evaluate the role of pretransplant conditioning in GVHD pathogenesis. Hence, we developed transplantation models that allowed for the examination of multiple variables associated with pretransplant conditioning that can affect GVHD outcome, including intestinal damage, lymphoablation, and NK cell inactivation. Mice receiving transplants were evaluated with a consistent set of parameters throughout the study. These parameters included weight loss, clinical symptomsof disease, multiple organ histopathological analysis, and cellular and molecular analysis, including intestinal permeability measurements, serum cytokine detection, and flow cytometric analysis of T cell subsets. Experimental end points (typically 25 or 35 days after transplantation) were chosen on the basis of pilot experiments that concluded that these time points allowed sufficient time for substantial GVHD to develop in our minor mismatch models. All histopathological analysis was performed by subspecialty-trained anatomic pathologists blinded to the experimental conditions. Experimental group sizes were sufficiently powered to detect statistical significance. Each transplantation experiment was independently replicated, and the numbers of experimental replicates are noted in the corresponding figure legends.

Mice

Female 129S6 (H-2^b, Taconic), Balb/c (H-2^d, Harlan), or C57BL/6J (H-2^b, 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 The 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 (IACUC) regulations at the University of Chicago and were performed under an approved protocol. Two hundred micrograms of anti-NK1.1 (clone PK136) or control IgG or 20 μl of anti–asialo GM1 (Wako Chemicals) was injected

intraperitoneally 2 days before AT. B6 *Rag1*$^{-/-}$ and *Rag2*$^{-/-}$/*Pfp*$^{-/-}$ recipients received 3% DSS in their drinking water for 4 days before AT. 129 *Rag2*$^{-/-}$ recipients received 3% DSS in their drinking water for 5 days before AT. LPS (0.8 mg/kg) from *E. coli* (Sigma) was injected intraperitoneally 2 hours before AT. Antibiotic-treated mice received vancomycin (50 mg/kg per day) and imipenem (50 mg/kg per day) in their drinking water 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 and 12 weeks were used as donors. Male B6 recipients, between the ages of 7 and 12 weeks, were lethally irradiated with 11 Gy 24 hours before BMT with 5 million bone marrow cells and 30 million splenocytes by retro-orbital injection. For AT, sex-matched B6 *Rag1*$^{-/-}$ recipients or B6 *Rag2*$^{-/-}$/*Pfp*$^{-/-}$ recipients received only splenocytes, with or without irradiation (7 Gy) 24 hours before AT. Clinical signs were scored from 0 to 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

Jejunum, liver, and skin were scored at 0.5-point intervals from 0 to 3 based on four 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 on the basis of 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 before gavage with 150 μl of FITC–4 kD dextran (80 mg/ml). 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 optimum cutting temperature compound. Frozen sections (5 μm) were fixed in 1% paraformaldehyde in phosphate-buffered saline and immunostained with 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 Alexa Fluor 594–conjugated secondary antibodies (Invitrogen), along with Hoechst 33342 (Invitrogen). Stained sections were mounted in ProLong Gold (Invitrogen) and imaged with an epifluorescence microscope with a Plan-Neofluar 40×/1.3 oil objective (Axioplan 2, Zeiss). Images were deconvolved for 10 iterations with AutoQuant X3 (Media Cybernetics) and analyzed with MetaMorph (Universal Imaging Corp.).

For immunohistochemistry, tissue sections were deparaffinized and 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 (HRP) 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 enzyme-linked immunosorbent assay

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, IFNγ (all from eBioscience), or granzyme B (BD Biosciences). T cells were stimulated with PMA/ionomycin (Sigma) for 4 hours before staining for intracellular IFNγ. Flow cytometry was performed with a FACSCanto (BD) and analyzed with FlowJo version 9.4.11 (Tree Star). Serum IFNγ, TNF, and IL-6 were quantified with Ready-SET-Go! enzyme-linked immunosorbent assay 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 or 0.1 μM), respectively. Twenty million total cells (1:1 ratio) were injected retro-orbitally. After 24 hours, splenocytes were harvested and analyzed by fluorescence-activated cell sorting. 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 μM CFSE before 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.

Statistical analysis

All data are presented as means ± SEM. Statistical significance was determined by two-tailed Student's *t* test, two-tailed Mann-Whitney *U* test, or Kaplan-Meier log-rank test. *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 MATERIALS

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Fig. S1. Typical lung histopathology develops during minor mismatch GVHD.

Fig. S2. Bone marrow is not required for minor mismatch GVHD.

Fig. S3. Tight junction–associated proteins are redistributed after irradiation and minor mismatch AT.

Fig. S4. *Rag2*$^{-/-}$/*Pfp*$^{-/-}$ recipients of mismatch AT develop features of GVHD.

Fig. S5. Major mismatch AT is associated with increases in serum cytokines.

Fig. S6. NK cell depletion and intestinal damage promote AT-induced GVHD histopathology.

Fig. S7. Minor mismatch donor cells show substantial proliferation regardless of pretransplant treatment.

Fig. S8. Perforin-deficient mice receiving allogeneic AT develop pulmonary GVHD.

Fig. S9. Pretransplant conditioning makes dual contributions to minor mismatch GVHD.

Table S1. Original data (provided as an Excel file).

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Supplementary Materials for

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

Sam C. Nalle, H. Aimee Kwak, Karen L. Edelblum, Nora E. Joseph, Gurminder Singh, Galina F. Khramtsova, Eric D. Mortenson, Peter A. Savage,* Jerrold R. Turner*

*Corresponding author. E-mail: jturner@bsd.uchicago.edu (J.R.T.); psavage@bsd.uchicago.edu (P.A.S.)

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This PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at www.sciencetranslationalmedicine.org/cgi/content/full/6/243/243ra87/DC1)

Fig. S1

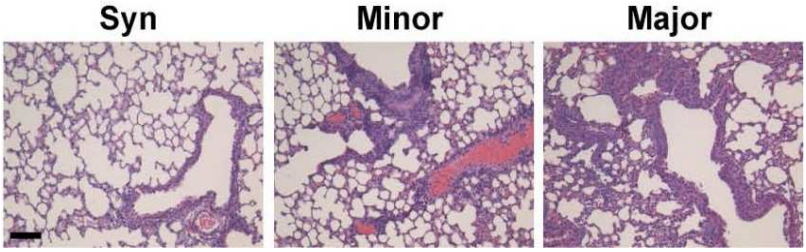

Fig. S1. **Typical lung histopathology develops during minor mismatch GVHD.** Wild-type B6 recipients were lethally irradiated and then underwent a syngeneic (syn), minor mismatch (minor, 129), or major mismatch (major, Balb/c) BMT (bone marrow and splenocytes). Tissue was harvested 5 weeks after BMT. Scale bar: 100 μm. Pathological features include peribronchial inflammatory infiltrate and damaged airway epithelium.

Fig. S2

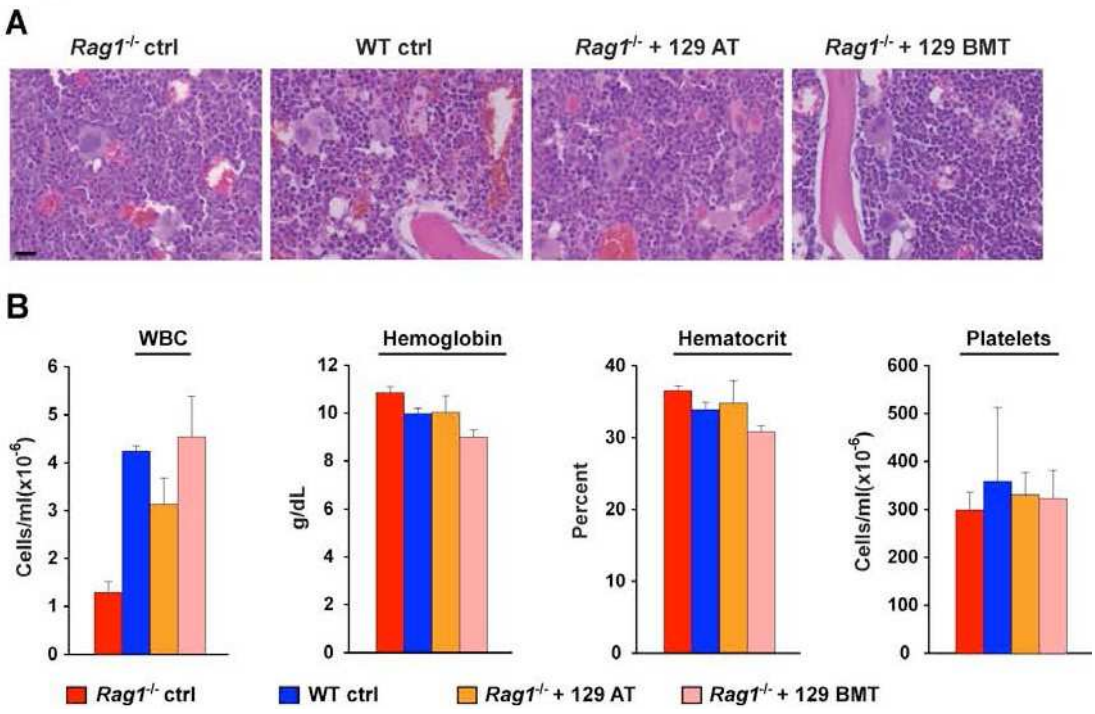

Fig. S2. **Bone marrow is not required for minor mismatch GVHD.** B6 *Rag1*$^{-/-}$ mice received 7 Gy irradiation followed by a 129 AT (splenocytes only) or 129 BMT (splenocytes and bone marrow) (n=2-3/group). Mice were sacrificed 25 days after transplantation. Controls received no treatment. (A) Bone marrow histopathology. Scale bar: 50 μm. (B) Peripheral blood cell analysis. WBC: white blood cells.

Fig. S3

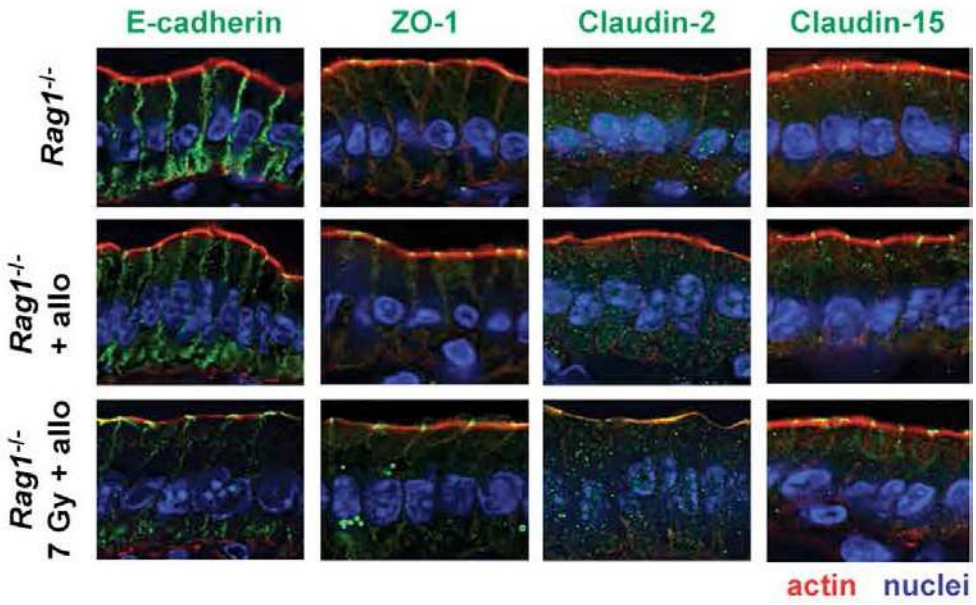

Fig. S3. **Tight junction–associated proteins are redistributed after irradiation and minor mismatch AT.** B6 *Rag1*$^{-/-}$ did or did not receive 7 Gy irradiation followed by a minor (129) mismatch AT. Tissue was harvested 7 days after AT and sections of jejunum were immunostained.

Fig. S4

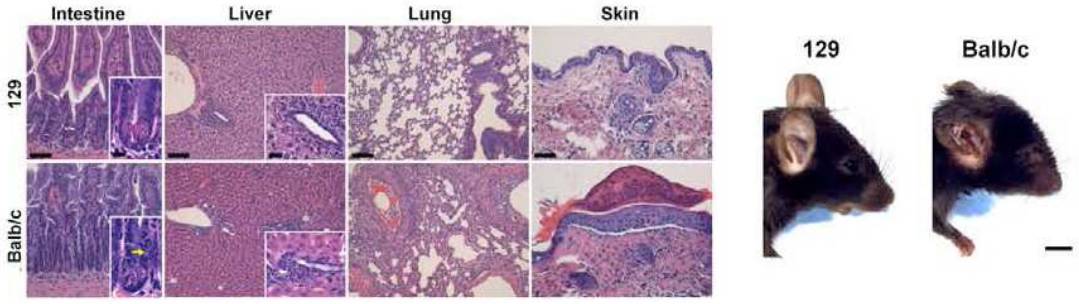

Fig. S4. ***Rag2*$^{-/-}$*/Pfp*$^{-/-}$ recipients of mismatch AT develop features of GVHD.** *Rag2*$^{-/-}$*/Pfp*$^{-/-}$ mice received a minor (129) or major (Balb/c) mismatch AT. Left: histopathology of GVHD target organs. Scale bars: intestine, 50 μm, inset, 10 μm; liver, 100 μm, inset, 20 μm; lung, 100 μm; skin, 50 μm. Yellow arrow in the intestine inset denotes an apoptotic cell. Right: skin and fur damage, 35 days after minor or major mismatch AT. Scale bar: 0.5 cm.

Fig. S5

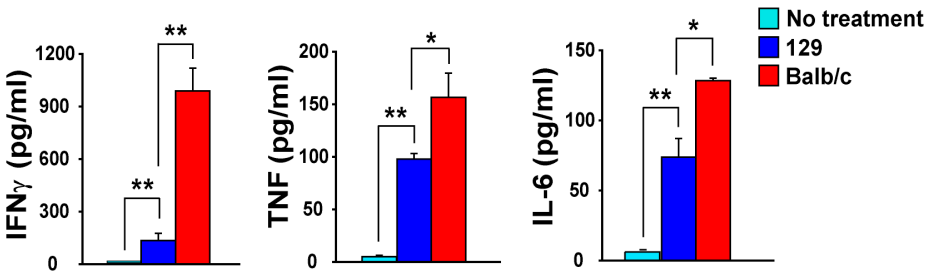

Fig. S5. **Major mismatch AT is associated with increases in serum cytokines.** *Rag2*$^{-/-}$*/Pfp*$^{-/-}$ mice received a minor (129) or major (Balb/c) mismatch AT. Serum cytokine concentrations 7 days after AT (n=2-4/group). Data are pooled from 2 independent experiments. All cytokines were evaluated by two-sided Student's t-test. IFNγ: P=<0.001 comparing mice that received no treatment vs. mice that received a 129 AT and P<0.001 comparing mice that received a 129 AT vs. mice that received a Balb/c AT. TNF: P<0.001 comparing mice that received no treatment vs. mice that received a 129 AT and P=0.0497 comparing mice that received a 129 AT vs. mice that received a Balb/c AT. IL-6: P<0.001 comparing mice that received no treatment vs. mice that received a 129 AT and P=0.019 comparing mice that received a 129 AT vs. mice that received a Balb/c AT.

Fig. S6

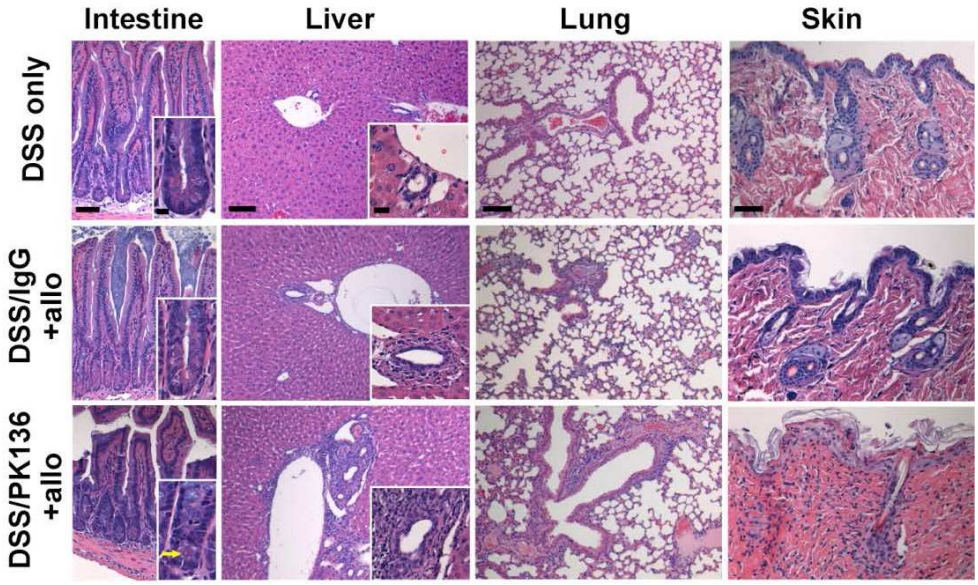

Fig. S6. **NK cell depletion and intestinal damage promote AT-induced GVHD histopathology.** B6 *Rag1*$^{-/-}$ were treated with 3% DSS + anti-NK1.1 (clone PK136) or 3% DSS + control antibody prior to minor mismatch (129) AT, or DSS only. Histopathology of GVHD target organs. Scale bars: intestine, 50 μm, inset, 10 μm; liver, 100 μm, inset, 20 μm; lung, 100 μm; skin, 50 μm. Yellow arrow in the intestine inset denotes an apoptotic cell.

Fig. S7

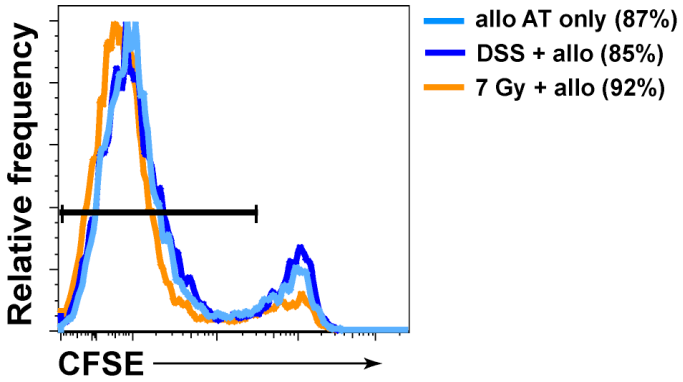

Fig. S7. **Minor mismatch donor cells show substantial proliferation regardless of pretransplant treatment.** B6 *Rag1*$^{-/-}$ were treated with 3% DSS or 7 Gy prior to minor mismatch (129) AT. In vivo proliferation of donor CD8+ T cells was measured 6 days after AT by CFSE dilution. Percent of cells that have undergone proliferation is listed. Data are representative of 3 independent experiments.

Fig. S8

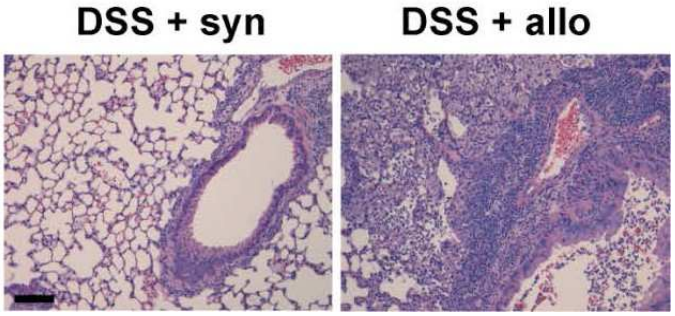

Fig. S8. **Perforin-deficient mice receiving allogeneic AT develop pulmonary GVHD.** B6 *Rag2*$^{-/-}$*Pfp*$^{-/-}$ mice were treated with 3% DSS prior to minor mismatch (129, allo) or syngeneic (B6, syn) AT. Tissue was harvested 3 weeks after AT. Scale bar: 100 μm. Pathological features include peribronchial inflammatory infiltrate and damaged airway epithelium, just as in traditional BMT models.

Fig. S9

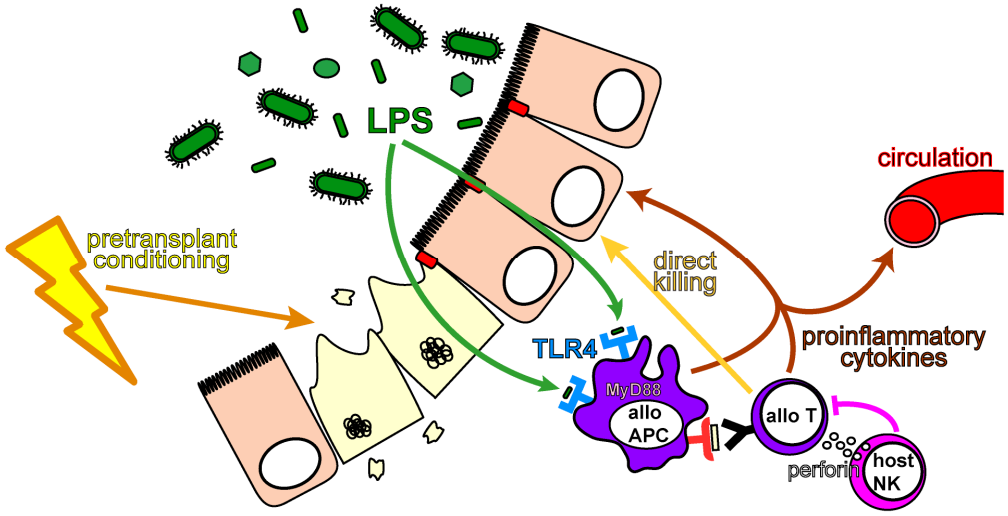

Fig. S9. **Pretransplant conditioning makes dual contributions to minor mismatch GVHD.** Pre-transplant conditioning fulfills dual roles in minor mismatch GVHD initiation by inhibiting recipient NK cell immunoregulation and inducing intestinal barrier loss. Intestinal barrier loss allows translocation of luminal microbial components, such as LPS, which triggers donor cell TLR4 and MyD88, allogeneic T cell activation and proinflammatory cytokine production, ultimately resulting in host tissue damage and GVHD.