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Host NLRP6 exacerbates graft-versus-host disease independent of gut microbial composition

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Abstract

Host NOD-like receptor family pyrin domain-containing 6 (NLRP6) regulates innate immune responses and gastrointestinal homeostasis. Its protective role in intestinal colitis and tumorigenesis is dependent on the host microbiome. Host innate immunity and microbial diversity also play a role in the severity of allogeneic immune-mediated gastrointestinal graft-versus-host disease (GVHD), the principal toxicity after allogeneic haematopoietic cell transplantation. Here, we examined the role of host NLRP6 in multiple murine models of allogeneic bone marrow transplantation. In contrast to its role in intestinal colitis, host NLRP6 aggravated gastrointestinal

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Author contributions

T.T. designed and performed the experiments, analysed the data and wrote the paper. H.F., C.R. and M.R. performed the experiments, analysed the data and wrote the paper. H.T. and C.Z. performed the experiments and analysed the data. C.L. performed the experiments and histopathological analysis. A.V.M., J.B., K.O.-W., I.M., S.B., Y.S., D.P., J.W., I.H., S.K., J.C., S.S. and S.P. performed the experiments. G.C. analysed the data, contributed reagents and wrote the paper. P.R. designed the experiments, analysed the data and wrote the paper.

Competing interests

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GVHD. The impact of host NLRP6 deficiency in mitigating GVHD was observed regardless of co-housing, antibiotic treatment or colonizing littermate germ-free wild-type and NLRP6-deficient hosts with faecal microbial transplantation from specific pathogen-free wild-type and *Nlrp6*^{-/-} animals. Chimaera studies were performed to assess the role of NLRP6 expression on host haematopoietic and non-haematopoietic cells. The allogeneic [B6Ly5.2 → *Nlrp6*^{-/-}] animals demonstrated significantly improved survival compared to the allogeneic [B6Ly5.2 → B6] animals, but did not alter the therapeutic graft-versus-tumour effects after haematopoietic cell transplantation. Our results unveil an unexpected, pathogenic role for host NLRP6 in gastrointestinal GVHD that is independent of variations in the intestinal microbiome and in contrast to its well-appreciated microbiome-dependent protective role in intestinal colitis and tumorigenesis.

The gut microbiome is sensitive to environmental, dietary and host factors and is intricately intertwined with host physiology¹. The composition and function of the gut microbiome have been associated with various disease processes. Emerging data show a strong correlation between the outgrowth of certain species with concomitant loss of others, resulting in the reduction of microbial diversity in the gastrointestinal tract, termed dysbiosis, and outcomes after human and experimental allogeneic haematopoietic cell transplantation (allo-HCT)^{1,2}. These changes have been specifically correlated to the most significant complication of allo-HCT: gastrointestinal graft-versus-host disease (GVHD)¹. Although microbial dysbiosis has become a key metric for prognosticating outcomes after allo-HCT, a direct causal role for dysbiosis in driving gastrointestinal GVHD and allo-HCT outcomes has not been demonstrated.

NOD-like receptor family pyrin domain-containing 6 (NLRP6) is highly expressed in intestinal epithelial cells (IECs) and myeloid-derived immune cells, such as dendritic cells, macrophages and monocytes. NLRP6-deficient (*Nlrp6*^{-/-}) animals demonstrate worse dextran sulfate sodium-induced colitis, water-avoidance stress-induced enteritis and colitis-associated tumorigenesis^{3,4} that was also transmitted to wild-type (WT) animals in co-housing experiments³⁻⁶. NLRP6 regulates the production of inflammatory cytokines and resistance to enteric infections⁷. Many of these data indicate a microbiome-dependent protective role for NLRP6 in various intestinal pathologies.

GVHD is a life-threatening complication of allo-HCT². The gastrointestinal tract is a major target organ of GVHD, which is the principal cause for mortality. Host tissue injuries caused by conditioning regimens release pro-inflammatory cytokines⁸, and ‘danger signals’ activate antigen-presenting cells (APCs) via the inflammasome and pattern recognition receptors and amplify gastrointestinal GVHD. Recent investigations suggested that this amplification is associated with microbial dysbiosis after allo-bone marrow transplantation (BMT)^{1,2,9,10}.

Given the aforementioned roles of NLRP6 and the microbiome, we tested the hypothesis that the absence of NLRP6 in hosts would enhance gastrointestinal GVHD. We made the surprising observation that the deficiency of NLRP6 in host target organs ameliorated gastrointestinal GVHD independent of changes in intestinal microbiota composition and the loss of microbial diversity. Our results indicate that NLRP6 has diverse functions and wields converse, microbiome composition-independent pathogenic effects in GVHD, when

compared to its microbiome composition-dependent protective role in intestinal colitis and cancer⁴.

Results

Host NLRP6 aggravates the severity and mortality of GVHD in multiple models.

NLRP6 mitigates the severity of experimental colitis^{3,6,7,11,12}. Thus, we explored the hypothesis that the absence of NLRP6 in the host would exacerbate gastrointestinal GVHD severity. Recipient B6 WT and B6 *Nlrp6*^{-/-} animals were from either syngeneic B6 or the major histocompatibility antigen disparate allogeneic BALB/c donors, as described in the Methods. Contrary to our hypothesis, allo-B6 *Nlrp6*^{-/-} animals showed a significantly improved survival compared to the allo-B6 WT animals (Fig. 1a) and reduced clinical severity of GVHD (Fig. 1b). However, donor conventional T cell (Fig. 1c and Supplementary Fig. 1a,b) and regulatory T cell (Supplementary Fig. 1c) expansion, the expression of CD69, an activation marker (Fig. 1d), the number of interferon- γ (IFN- γ)-producing T cells (Supplementary Fig. 1d), the serum levels of pro-inflammatory cytokines (IFN- γ , tumour necrosis factor- α (TNF- α) and interleukin-17A (IL-17A)) were not significantly different between the groups after allo-BMT (Fig. 1e-g). The severity of GVHD-specific histopathological scores showed significantly less severity only in the gastrointestinal tract of the B6 *Nlrp6*^{-/-} hosts (Fig. 1h,i and Supplementary Fig. 1e-g).

To eliminate strain- or model-dependent factors, we determined the effect of host NLRP6 in multiple minor antigen-mismatched C3H.sw $\rightarrow$ B6 models of GVHD. B6 *Nlrp6*^{-/-} hosts once again showed significantly reduced mortality from GVHD (Supplementary Fig. 1h) and a reduction in GVHD-specific histopathological scores only in the gastrointestinal tract, but not in other GVHD target organs (Supplementary Fig. 1i-m). Donor T cell (Supplementary Fig. 1n-p) and regulatory T cell (Supplementary Fig. 1q) expansion, CD69 expression (Supplementary Fig. 1r), IFN- γ -producing T cell counts (Supplementary Fig. 1s) and inflammatory cytokine levels (Supplementary Fig. 1t-x) were also similar in both groups.

Given the role of inflammasome-associated inflammation in the dysregulation of myeloid-derived suppressor cell (MDSC) function after allo-BMT¹³⁻¹⁵, we examined the numbers of donor-derived CD11b⁺Ly6C⁺Ly6G⁺, CD11b⁺Ly6C⁺Ly6G⁻ (monocytic MDSCs), CD11b⁺Ly6C⁻Ly6G⁺ (granulocytic MDSCs) and CD11b⁺Ly6C⁻Ly6G⁻ cells (macrophages) in the spleen and gastrointestinal tract. They were equivalent in both WT and *Nlrp6*^{-/-} hosts after allo-BMT (Fig. 2a-e). Similar results were also noted in the second model of GVHD (Supplementary Fig. 2a-e).

Nlrp6^{-/-} hosts show reduced gastrointestinal GVHD despite microbial dysbiosis.

The intestinal microbiota changes correlate with GVHD severity¹⁰. Thus, we analysed whether, in the absence of NLRP6, the indigenous host gastrointestinal microbiota at baseline, prior to BMT, was skewed towards a more salutary, tolerogenic composition. When compared to B6 WT animals, the microbiota of *Nlrp6*^{-/-} animals showed the appearance of the phyla Verrucomicrobia (genus *Akkermansia*) and Proteobacteria, increased

representation of the families Porphyromonadaceae and Prevotellaceae (members of the phylum Bacteroidetes) and decreased representation of the order Clostridiales and genus *Lactobacillus* (phylum Firmicutes) (Fig. 2f,g). This composition is similar to the shift in composition as reported¹⁶, towards a pattern that is considered to be dysbiotic (inflammatory) rather than salutary (tolerogenic)^{3,17}.

We next examined whether loss of NLRP6 was associated with reduced microbial dysbiosis after allo-BMT, thus accounting for better survival from reduced GVHD. B6 WT and B6 *Nlrp6*^{-/-} animals were allo-transplanted BALB/c donors, and their faecal microbiota was characterized 14 d post-BMT. The faecal bacterial communities of the two allogeneic groups were significantly different between the two types of host (Fig. 2h) and also significantly different when compared to their own pre-transplant faecal communities (Fig. 2g-i). These data show that gastrointestinal GVHD is reduced in the *Nlrp6*^{-/-} hosts despite the dysbiosis before or after BMT.

***Nlrp6*^{-/-} animals exhibit reduced gastrointestinal GVHD despite co-housing with WT recipients.**

We next examined whether *Nlrp6*^{-/-} animals that have been co-housed with B6 WT animals for 2 weeks at a 1/1 ratio before allo-BMT would show an altered GVHD severity and mortality after allo-BMT. There was no significant difference between the faecal bacterial communities of co-housed B6 WT animals and co-housed *Nlrp6*^{-/-} animals (Fig. 3a), but the communities from the co-housed animals tended to be distinct from B6 WT non-co-housed specific pathogen-free (SPF) mice (Fig. 3a). The faecal bacterial communities of co-housed B6 WT animals clustered together with those of their *Nlrp6*^{-/-} cage mates and were similar in composition to the faecal microbiota of *Nlrp6*^{-/-} animals prior to co-housing (Figs. 2g and 3a). Following allo-HCT, despite transfer of the microbial community from *Nlrp6*^{-/-} mice into B6 WT animals, the co-housed B6 WT animals still had greater GVHD mortality (Fig. 3b) and severity (Fig. 3c) than the co-housed *Nlrp6*^{-/-} animals, suggesting that the microbiome from *Nlrp6*^{-/-} animals did not impart protection against GVHD in the WT hosts.

***Nlrp6*^{-/-} animals demonstrate reduced GVHD after treatment with antibiotics.**

Antibiotic treatment influences the composition of the microbiota and influence GVHD-related mortality in patients¹⁸. Thus, we examined whether alterations of the indigenous intestinal microbiota with antibiotic treatment had an effect on GVHD severity in allo-*Nlrp6*^{-/-} animals. To this end, recipient B6 WT and *Nlrp6*^{-/-} animals were treated with antibiotics (see Methods) daily from day -1 until day 21 after allo-HCT. Significant differences were observed between the faecal bacterial communities of the two groups on day 7 and day 14 (antibiotic (ABX) allo-B6 WT D7 versus ABX allo-*Nlrp6*^{us/-} D7, *P* = 0.002; ABX allo-B6 WT D14 versus ABX allo-*Nlrp6*^{-/-} D14, *P* = 0.023; Fig. 3d,e). Furthermore, as expected, antibiotic treatment altered the faecal microbiome in allo-B6 WT and allo-*Nlrp6*^{-/-} hosts when compared to untreated allo-B6 WT and allo-*Nlrp6*^{-/-} hosts (*P* = 0.022; Fig. 3e). When compared to antibiotic-treated allo-B6 WT animals, the treated allo-*Nlrp6*^{-/-} animals showed reduced diversity with increased representation of Proteobacteria and *Lactobacillus*, and decreased representation of Clostridia (Figs. 2g and 3f,g). Despite the

alterations, antibiotic-treated allo-*Nlrp6*^{-/-} animals still showed greater survival than B6 WT animals (Fig. 3h).

Faecal microbial transplantation of germ-free WT and *Nlrp6*^{-/-} littermate recipients and GVHD.

To further elucidate and confirm whether the differences in the host microbiota in the *Nlrp6*^{-/-} and B6 WT animals played a role in the outcome of GVHD, we next derived B6 WT and *Nlrp6*^{-/-} animal littermates under germ-free (GF) conditions. The GF littermate B6 WT and *Nlrp6*^{-/-} animals were then colonized with the microbiome from SPF B6 WT and *Nlrp6*^{-/-} animals and were allowed 4 weeks for reconstitution of the microbial communities in the gastrointestinal tract, as described in the Methods¹⁷. Following microbial reconstitution, the GF WT and *Nlrp6*^{-/-} mice that colonized with either the B6 WT or *Nlrp6*^{-/-} SPF stool were used as recipients in BALB/c → B6 allo-BMT. The allo-[SPF B6 WT stool → GF *Nlrp6*^{-/-}] hosts showed significantly greater survival (Fig. 3i) and less GVHD severity (Fig. 3j) than littermate allo-[SPF B6 WT stool → GF B6 WT] animals, demonstrating that the absence of NLRP6 in the hosts protected them from GVHD despite the presence of the microbiome from WT SPF animals. By contrast, allo-[SPF B6 WT stool → GF WT] animals and allo-[SPF *Nlrp6*^{-/-} stool → GF B6 WT] animals showed similar mortality and GVHD severity (Fig. 3i,j), demonstrating that colonization of GF B6 WT littermate hosts with either the WT or *Nlrp6*^{-/-} stool microbiome did not affect GVHD severity.

NLRP6 deficiency in host haematopoietic-derived cells is dispensable for the reduction of GVHD.

We next explored whether the absence of NLRP6 in the host haematopoietic compartment is critical for the GVHD protective effects. The [B6 → B6Ly5.2] or [*Nlrp6*^{-/-} → B6Ly5.2] bone marrow chimaera animals were generated and utilized as recipients in a second allo-BMT 3 months after the primary BMT. As shown in Fig. 4a,b, all mouse chimaeras that received syngeneic T cells and bone marrow cells survived with no signs of GVHD, whereas the allogeneic [B6 → B6Ly5.2] and the [*Nlrp6*^{-/-} → B6Ly5.2] animals showed similar GVHD mortality and severity. We next evaluated the functional responses of haematopoietic APCs harvested from naive B6 WT and *Nlrp6*^{-/-} animals in vitro. Both B6 WT and *Nlrp6*^{-/-} macrophages showed similar responses to lipopolysaccharide (LPS) stimulation (Fig. 4c,d), and the bone marrow-derived dendritic cells (BMDCs) and splenocytes isolated from both B6 WT and *Nlrp6*^{-/-} animals showed equivalent allogeneic BALB/c T cell stimulation capacity (Fig. 4e,f). There were also similar numbers of macrophages and dendritic cells in the intestine 3 days after transplantation in both B6 WT and *Nlrp6*^{-/-} recipients (Fig. 4g). Furthermore, the host APCs, such as macrophages and dendritic cells, harvested from the colon and ileum of allogeneic B6 WT and *Nlrp6*^{-/-} recipients showed similar expression of surface markers and the ability to secrete IL-1β, TNF-α or IL-18 following ex vivo stimulation with LPS (Fig. 4g,h).

NLRP6 expression in host non-haematopoietic target tissues aggravates gastrointestinal GVHD.

Because NLRP6 is also highly expressed in IECs^{3,12}, we next evaluated whether the reduction of gastrointestinal GVHD is due to the absence of NLRP6 in host non-haematopoietic cells. To this end, the chimaeras [B6Ly5.2 → B6] or [B6Ly5.2 → *Nlrp6*^{-/-}] were generated such that NLRP6 deficiency was confined to the non-haematopoietic cell compartment and utilized as recipients in a second allo-BMT, as described in the Methods. The allo-[B6Ly5.2 → *Nlrp6*^{-/-}] animals demonstrated a significantly improved survival ($P = 0.0148$; Fig. 5a) and reduced clinical severity from GVHD (Fig. 5b) compared to the allo-[B6Ly5.2 → B6] animals. Next, we hypothesized that the reduced GVHD severity in allo-*Nlrp6*^{-/-} animals was, in part, due to decreased intestinal epithelial injury. To this end, we tested epithelial barrier function using the FITC-dextran intestinal permeability assay and found that allo-*Nlrp6*^{-/-} animals showed significantly decreased plasma FITC-dextran concentration after allo-BMT (Fig. 5c). Allo-*Nlrp6*^{-/-} animals showed increased expression of the tight junction claudin 3 (encoded by *Cldn3*) and ephrin type B receptor 3 (encoded by *Ephb3*) (Fig. 5d). Serum levels of IL-18 and IL-1 β were equivalent in allo-B6 WT and allo-*Nlrp6*^{-/-} animals, but the IL-18 production from the colon and ileum of *Nlrp6*^{-/-} recipients after allo-BMT were consistently low with similar gene expression levels of IL-18 and IL-1 β when compared to B6 WT recipients (Fig. 5e and Supplementary Fig. 3a). Taurine treatment, which stimulates the NLRP6 inflammasome, induced a greater production of IL-18 and IL-1 β from the colon and ileum of B6 WT recipients than of *Nlrp6*^{-/-} recipients. The mRNA expression of anti-apoptotic (*Bcl2* (encoding B cell lymphoma 2) or *Bclx1* (also known as *Bcl2l1*; encoding B cell lymphoma extra large)) or pro-apoptotic (*Bax* (encoding BCL-2-associated X protein) or *Bak* (encoding BCL-2 homologous antagonist/killer)) genes in the intestines of allo-*Nlrp6*^{-/-} animals were also equivalent (Fig. 5f and Supplementary Fig. 3b,c).

Absence of NLRP6 in the host enhances gastrointestinal homeostasis after allo-BMT.

We next sought to understand the processes by which the absence of NLRP6 in host intestinal target tissues contributes to ameliorating gastrointestinal GVHD. Mucus production by the goblet cells is critical for the preservation of gut homeostasis^{6,19}. The allo-B6 *Nlrp6*^{-/-} recipients showed an increased number of goblet cells in both the small and the large intestines during the early phase of allo-BMT compared to allogeneic B6 WT recipients (Fig. 6a-c). We also assessed the expression of X-box binding protein 1 (XBP-1)²⁰, aquaporin 3 (AQP3)²¹, mucin 1 (MUC1) and MUC2 (refs. ^{22,23}), which are important for intestinal homeostasis, before and after allo-HCT in B6 WT and *Nlrp6*^{-/-} animals (Supplementary Fig. 4a-d).

Next, we sought to determine changes in NLRP6 expression in the various intestinal cell subsets after BMT that have been shown to play a role in intestinal GVHD. Specifically, we sorted intestinal stem cells (CD44⁺CD24^{lo}CD166^{med}GRP78^{lo/-}), Paneth cells (CD44⁺CD24⁺CD166^{lo}) and IECs (CD44⁺CD24⁻CD166^{lo}) and determined the expression of NLRP6 in these cells by quantitative PCR^{24,25} (Supplementary Fig. 4e). IECs and intestinal stem cells showed similar expression of the *Nlrp6* gene, but Paneth cells showed higher levels of *Nlrp6* gene expression (Supplementary Fig. 4f). However, NLRP6 gene and

protein expression were significantly elevated only in the IECs on day 21 after allo-BMT (Fig. 6d,e).

To further dissect the mechanisms, we assessed the levels of a known NLRP6 agonist, taurine, that can be found in the intestinal contents, at baseline and after allo-BMT in B6 WT and *Nlrp6*^{-/-} hosts. Before BMT, taurine levels in the colon and ileum from *Nlrp6*^{-/-} hosts were not significantly different from those in the B6 WT hosts, demonstrating that the microbiome differences between them did not lead to significant changes in the metabolite taurine levels. Following allo-BMT, taurine levels increased similarly in both B6 WT and *Nlrp6*^{-/-} recipients when compared to their baseline levels (Fig. 6f). Next, to mechanistically and functionally demonstrate a role for NLRP6 agonist, we administered taurine orally (2.5 g per kg per day) to the recipients by oral gavage after BMT. There was no discernible toxicity because all syngeneic recipients survived. Oral gavages of taurine significantly shortened survival and worsened GVHD severity in the B6 WT animals compared to vehicle-treated B6 WT animals (Fig. 6g,h). By contrast, administration of taurine to allo-*Nlrp6*^{-/-} animals did not enhance GVHD severity (Fig. 6g,h). These results demonstrate that the mechanism of gastrointestinal protection from GVHD in the *Nlrp6*^{-/-} animals is in part from mitigating the amplification of GVHD caused by the metabolite taurine.

Absence of NLRP6 in the hosts maintains graft-versus-tumour activity after allo-BMT.

Because GVHD is tightly linked with the graft-versus-tumour (GVT) effect, we next explored whether deficiency of NLRP6 affects the GVT effect. Given the similar immune responses in B6 WT and *Nlrp6*^{-/-} hosts (Fig. 1), we hypothesized that, in the absence of NLRP6, immune-mediated GVT would be preserved despite a reduction in GVHD. We used the same BMT model as above, but added MBL-2 at the time of BMT. Consistent with our hypothesis, allo-*Nlrp6*^{-/-} animals showed equivalent GVT responses compared to allo-B6 WT animals (Fig. 6I,j). These data indicate that the deficiency of NLRP6 in host target tissue decreases GVHD without any effect on GVT.

Discussion

Our data collectively provide several insights. They demonstrate an unexpected, pathogenic role for NLRP6 in aggravating allogeneic GVH-mediated intestinal damage. This pathogenic role is independent of indigenous changes in the microbiome and is in contrast to the protective role of NLRP6 in mitigating damage from colitis and tumorigenesis that is dependent on the microbiome. Furthermore, our data indicate that microbial dysbiosis, as reflected by specific changes in microbiome composition and loss of diversity after allo-BMT, is not sufficient to explain the severity of GVHD in the context of host NLRP6 deficiency.

After allo-BMT, several inflammatory signals amplify GVH responses and aggravate GVHD^{2,26}. Gastrointestinal GVHD and inflammatory colitis, such as inflammatory bowel diseases, are immune-mediated diseases that share certain similarities and yet have distinct clinical and histopathological features². NLRP6 is highly expressed in IECs, and in light of its role in homeostasis and colitis^{3,4,6,7,11,12,17}, it plays an opposite role in the amplification

of disease severity in gastrointestinal GVHD. Interestingly, early on, despite the decrease in GVHD severity, *Nlrp6*^{-/-} hosts showed equivalent allogeneic donor T cell and inflammatory responses to B6 WT hosts. When taken together with the maintenance of GVT effects in *Nlrp6*^{-/-} hosts, this suggests that the reduction in GVHD is probably due to the epithelial cell-intrinsic effects of NLRP6 rather than an effect on immune responses after allo-BMT^{2,26,27}. These data will need to be explored carefully by determining the role of tumour-intrinsic NLRP6. Another possibility for improved survival after allo-BMT in the absence of NLRP6 in the hosts might be from reduced sensitivity to conditioning by radiation. Although possible, it is unlikely because (1) following syngeneic transplantation when the same lethal radiation dose was used, both B6 WT and *Nlrp6*^{-/-} mice demonstrated 100% survival (Fig. 1a) and (2) TUNEL staining of IECs following conditioning with lethal radiation on day + 7 demonstrated no significant differences in the large and small bowel epithelium between the WT and the *Nlrp6*^{-/-} hosts (Supplementary Fig. 5). Nonetheless, formal studies on radiation sensitivity independent of allo-BMT and GVHD will need to be performed to dissect the effect of radiation damage.

The role of inflammasomes is being increasingly appreciated in GVHD. Activating inflammasome pathways through NLRP3 on host haematopoietic-derived cells exacerbates GVHD¹³. In addition, dysregulation of MDSCs via inflammasome activation can exacerbate GVHD¹⁵. The expansion of donor-derived MDSC subsets and increase in IL-18 levels were similar in both allo-B6 WT and allo-*Nlrp6*^{-/-} animals, suggesting that the protection observed in *Nlrp6*^{-/-} hosts might not be due to an inflammasome-mediated effect on MDSCs. Furthermore, bone marrow chimera experiments suggested that the effects of NLRP6 was due to its expression in host non-haematopoietic tissues. Given that IECs express high levels of NLRP6 and the protective effects were confined only to the intestine, it is likely that the effects of NLRP6 on intestinal cells is crucial for the observed effects on GVHD.

Our data are consistent with the notion that NLRP6 deficiency alters the endogenous intestinal microbiota composition (increase Prevotellaceae³ and *Akkermansia*¹⁷) and that microbial dysbiosis occurs after allo-BMT and in gastrointestinal GVHD^{10,28-30}. Co-housing experiments steered the microbial composition of WT animals to that of *Nlrp6*^{-/-} animals, but did not affect the severity of GVHD. Furthermore, treatment with antibiotics aggravated GVHD in B6 WT animals, but did not affect the protective effects associated with NLRP6 deficiency in *Nlrp6*^{-/-} hosts. Similarly, conventionalization of the gastrointestinal microbial community in GF *Nlrp6*^{-/-} animals with stool from WT SPF animals also did not mitigate GVHD protection from the absence of NLRP6 in the hosts. By contrast, littermate WT GF animals that were conventionalized with stool from either NLRP6 or WT SPF animals showed similar GVHD severity. However, our data do not indicate an absence of the contributing role by the microbiome to GVHD severity; rather, just that alterations in the gut microbiome, microbial dysbiosis, from either NLRP6 deficiency, allo-BMT or antibiotic treatment are not sufficient to modulate GVHD severity in the context of host NLRP6 deficiency.

Our data show that dysbiosis in the absence of NLRP6 was not associated with significant alterations in one of its agonists, a microbial metabolite, taurine. However, exogenous

administration of taurine increased GVHD mortality in WT but not in *Nlrp6*^{-/-} hosts, demonstrating that the mechanism of NLRP6-mediated GVHD toxicity is in part from the effects of the metabolite taurine and inflammasome activation. Nonetheless, it is formally possible that the damage mediated by allogeneic T cells may lead to the release of as yet uncharacterized ligands for NLRP6 that contribute to its role in gastrointestinal GVHD. Allo-*Nlrp6*^{-/-} animals had an increased number of goblet cells, expression of MUC2 and other barrier repair proteins^{19,31}, suggesting that this might be a related or independent potential mechanism of protection after BMT. The expression of NLRP6 in various intestinal cell subsets indicates that its expression is increased after BMT only in the IECs, suggesting that its effect may be of greater consequence in these cells (Fig. 6d). However, because all other subsets express NLRP6, even if the levels do not change after BMT, the overall effects on GVHD could be a net effect on the role of NLRP6 in all of the intestinal cellular subsets, such as IECs, intestinal stem cells, Paneth cells or goblet cells. The specific, or dominant role, if any, for any one of these cells in mediating NLRP6-dependent GVHD toxicity will need to be determined in future studies. Thus, the absence of NLRP6 mitigates gastrointestinal GVHD by preserving the barrier function of epithelial cells, probably through multiple mechanisms.

Our data unveil a microbial dysbiosis-independent pathogenic role for NLRP6 in alloimmune-mediated intestinal damage, which is in contrast to its role in a microbiome-dependent protective role in other intestinal pathologies, and demonstrate that NLRP6 could be a target for mitigating GVHD without substantially compromising the beneficial GVT effects after clinical allo-BMT.

Methods

Mice.

C57BL/6 (B6, H-2^b, CD45.2⁺), B6 Ly5.2 (H-2^b, CD45.1⁺), and C3H.sw (H-2^b, CD229.1) mice were purchased from the Jackson Laboratory. BALB/c (H-2^d) mice were purchased from Charles River Laboratories. B6-background NLRP6-knockout (*Nlrp6*^{-/-}) mice (>8 generations) have been described previously¹¹. All age-matched female mice (8–12-weeks old) were used for experiments. All animals were cared for according to regulations reviewed and approved by the University Committee on Use and Care of Animals of the University of Michigan, based on the University Laboratory Animal Medicine guidelines.

Animal co-housing experiments.

Age-matched and gender-matched B6 WT and *Nlrp6*^{-/-} Age-matched and gender-matched B6 WT and *Nlrp6*^{-/-} animals were co-housed in new cages at a 1/1 ratio for 2 weeks before BMT³.

Generation of bone marrow chimaeras.

Bone marrow chimaeras ([B6 → B6Ly5.2], [*Nlrp6*^{-/-} → B6Ly5.2], [B6Ly5.2 → B6] and [B6Ly5.2 → *Nlrp6*^{-/-}]) were generated as described previously^{32,33}. Briefly, B6Ly5.2 animals received 11 Gy total body irradiation (¹³⁷Cs source) on day 1 and were transplanted intravenously with 5 × 10⁶ bone marrow and 5 × 10⁶ whole-spleen cells from B6 WT or

Nlrp6^{-/-} donor mice on day 0. To generate [B6Ly5.2 → B6] and [B6Ly5.2 → *Nlrp6*^{-/-}] chimeras, B6 WT or *Nlrp6*^{minus/-} animals received 10 Gy total body irradiation on day - 1 and were then transplanted with 5×10^6 bone marrow and 5×10^6 whole-spleen cells from B6 WT Ly5.2 donor mice on day 0. Donor haematopoietic cell engraftment was confirmed using the CD45.2 or CD45.1 monoclonal antibody (mAb) 3 months after BMT (donor type > 95.0%).

BMT.

BMTs were performed as previously described^{32,34}. Briefly, splenic T cells from donors were enriched, and the bone marrow was depleted of T cells by autoMACS (Miltenyi Biotec) utilizing CD90.2 microbeads (Miltenyi Biotec). We used well-established BMT models. B6 WT and *Nlrp6*^{-/-} animals were used as recipients and received 10–11 Gy (¹³⁷Cs source) on day - 1 and 3×10^6 CD90.2⁺ T cells along with 5×10^6 T cell-depleted bone marrow cells from either syngeneic or allogeneic BALB/c or C3H.sw donors on day 0. With antibiotic treatment, recipient animals received metronidazole (250 mg per kg; Sigma-Aldrich), kanamycin (250 mg per kg; Sigma-Aldrich), ampicillin (250 mg per kg; Sigma-Aldrich) and vancomycin (125 mg per kg; Sigma-Aldrich) by oral gavage from day - 1 to day 21 during BMT. With taurine treatment, recipients received taurine (2.5 g per kg per day; Sigma-Aldrich) by oral gavage from day 0 (ref. ³⁵). The mice were randomly assigned to syngeneic or allogeneic transplant groups and treatment groups in each experiment. No mice were excluded from analysis. No statistical methods were used to predetermine sample size. The investigators were not blinded to allocation during experiments and outcome assessment.

Faecal microbiota transplantation.

B6 WT and *Nlrp6*^{-/-} GF mice were housed and bred at the GF mouse facility, and sterility was regularly verified by aerobic and anaerobic cultures, Gram stains and quantitative PCR. GF mice were conventionalized using faeces pooled from several cages of SPF WT or *Nlrp6*^{-/-} mice. Briefly, all cages, bottles with stoppers and animal drinking water were autoclaved before being used. Food and bedding were irradiated and tested to ensure sterility prior to being used in the experiment. All GF mice received faecal microbiota transplantation from either SPF B6 WT or SPF *Nlrp6*^{-/-} donors. Whole-intestinal contents from donors were suspended in sterile PBS, and 200 µl cleared supernatants were obtained using a 100-µm strainer and gavaged into mice for 1 dose, followed by 4 weeks to allow microbiome establishment. All procedures were done under strictly anaerobic conditions in a Coy anaerobic chamber immediately after euthanasia. The conventionalized WT and *Nlrp6*^{-/-} GF mice were used as BMT recipients¹⁷.

Systemic and histopathological analysis of GVHD.

We monitored survival after allo-HCT daily and assessed the degree of clinical GVHD weekly as described previously³⁶. Histopathological analysis of the liver, gastrointestinal tract, lungs and skin, which are the primary GVHD target organs, was performed as described using a semiquantitative scoring system implemented by a single pathologist (C.L.)³⁷. After scoring, the codes were broken and the data compiled.

BMDC cultures.

To obtain BMDCs, bone marrow cells from B6 WT or *Nlrp6*^{-/-} mice were cultured with murine recombinant granulocyte-macrophage colony-stimulating factor (20 ng ml⁻¹; PeproTech) for 7 days. BMDCs were isolated using CD11c (clone N418) microbeads (Miltenyi Biotec) and autoMACS (Miltenyi Biotec). They were then utilized as a stimulator for the mixed lymphocyte reaction.

FACS analyses.

FACS analyses were performed as previously described^{38,39}. Briefly, to analyse donor T cell expansion and activation markers, splenocytes from transplanted animals were resuspended in FACS wash buffer (2% BSA in PBS) and stained with conjugated mAbs. The following antibodies were used: FITC-conjugated mAbs against mouse H2K^d (clone SF1-1.1, 116606, 1:200); PerCP-Cy5.5-conjugated mAbs against mouse CD4 (clone GK1.5, 100434, 1:200), CD8 (clone 53-6.7, 100734, 1:200) and CD11b (clone M1/70, 101228, 1:200); phycoerythrin (PE)-conjugated mAbs against mouse CD69 (clone H1.2F3, 104508, 1:200), Ly6C (clone HK1.4, 128008, 1:200) and CD45.1 (clone A20, 110708, 1:200); APC-conjugated mAbs against mouse Ly6G (clone 1A8, 127614, 1:200), CD45.2 (clone 104, 109814, 1:200) and CD326 (clone G8.8, 118214, 1:200) (BioLegend); and FITC-conjugated mouse CD229.1 (clone 30C7, 560201, 1:200, BD Biosciences). After staining, the cells were washed twice and fixed with 2% formaldehyde. The procedure was performed as described previously³⁸. For intracellular staining to detect cytokines, cells were permeabilized after fixation and stained with PE-conjugated IFN- γ (clone XMG1.2, 505808, 1:200; BioLegend) according to the manufacturer's protocol. For intracellular staining to detect XBP-1, AQP3, MUC1 and MUC2, cells were fixed with 2% formaldehyde and permeabilized with BDPhosFlow (BD Bioscience) and then stained with the non-conjugated polyclonal antibodies anti-XBP-1 (M-186, sc-7160, 1:50), anti-AQP3 (H-80, sc-20811, 1:50), anti-MUC1 (F-19, sc-6826, 1:50) and anti-MUC2 (R-12, sc-13312, 1:50) followed by staining with donkey anti-goat IgG-FITC (sc-2024, 1:100) or donkey anti-rabbit IgG-FITC (sc-2090, 1:100) (Santa Cruz Biotechnology). Cells were run on an Accuri C6 flow cytometer (BD Bioscience) or Attune NxT flow cytometer (Thermo Fisher Scientific). Analysis was performed using FlowJo v10.2.

Mixed lymphocyte reaction.

Splenic T cells from B6 WT and BALB/c WT animals were magnetically separated by MACS using CD90.2 microbeads and used as responders. B6 WT or *Nlrp6*^{-/-}-derived BMDCs were used as stimulators in a mixed lymphocyte reaction. T cells (1×10^5 per well) and irradiated (20 Gy) BMDCs (2.5×10^3 per well) were co-cultured on 96-well U-bottom plates for 96 h. The incorporation of ³H-thymidine (1 μ Ci per well; PerkinElmer) by proliferating T cells during the final 16 h of co-culture was measured by the TopCount plate reader (Perkin Elmer). When using splenocytes as stimulators, 4×10^5 per well T cells from B6 WT and BALB/c WT animals and 1×10^5 per well irradiated (30 Gy) red blood cell-lysed splenocytes from B6 WT or *Nlrp6*^{-/-} animals were co-cultured on 96-well flat-bottom plates for 72 h and analysed for proliferation following ³H-thymidine incorporation during the last 6 h of incubation.

FITC-dextran intestinal permeability assay.

Intestinal permeability was assessed by oral administration of FITC-dextran (Sigma-Aldrich), a non-metabolized carbohydrate that is used as a permeability probe. Mice were kept without food and water for 4 h and then FITC-dextran in PBS (800 mg per kg) was administered by oral gavage. Four hours later, plasma was collected and measured on a plate reader (excitation: 485 nm; emission: 535 nm). FITC-dextran concentrations were determined using a standard curve.

Epithelial cell isolation.

Small intestines and colons were digested once with 0.1 mM EDTA at 37 °C for 45 min. For intestinal cell sorting, single cells were stained with 7AAD (420404, 1:100), PerCP-Cy5.5 anti-CD45.2 (clone 104, 109828, 1:100), APC anti-CD31 (clone 390, 102410, 1:100), PE-Cy7 anti-CD44 (clone IM7, 10303, 1.5:100), Pacific Blue anti-CD24 (clone M1/69, 101820, 1.5:100), PE-conjugated anti-CD166 (clone eBioALC48; 17-1161-80, 1.25:100; eBioscience), rabbit anti-GPR78 (clone ET-21; G9043, 0.5:100; Sigma-Aldrich) and FITC antirabbit IgG (sc-2359, 1:100; Santa Cruz Biotechnology)²⁴. Stained cells were sorted with the Moflo Astrios Cell sorter.

Dendritic cell and macrophage isolation from intestines.

In brief, the colon and ileum were minced and dissociated with collagenase D (Roche) and DNase I (Roche) at 37 °C for 30 min to obtain single-cell suspensions. After filtering, the single-cell suspensions were subjected to Percoll gradient separation. To control for nonspecific binding, cells were blocked with anti-CD16/CD32 antibody (2.4G2, BD Biosciences) for 10 min at room temperature. APC anti-F4/80 antibodies (BM8, BioLegend) and anti-allophycocyanin magnetic microbeads (Miltenyi Biotec) for macrophages, and CD11c UltraPure microbeads (Miltenyi Biotec) for dendritic cells via autoMACS (Miltenyi Biotec) were used.

RNA isolation and RT-PCR.

Total cellular RNA was isolated by using TRIzol reagent (Life Technologies) and then reverse transcribed (2 µg) using Moloney murine leukaemia virus reverse transcriptase (Promega) and random hexamer primers in a total volume of 20 µl at 42 °C for 60 min. One-tenth of the reverse transcription reaction was subjected to PCR analysis using primer pairs specific for *Cldn1* forward: 5'-tcccaagccaacaccttctagt-3' and reverse: 5'-gtattcgctccaggaggatctct-3', *Cldn2* forward: 5'-gagtgaaggcagagatgagaag-3' and reverse: 5'-aggtcaggtgagggaaataga-3', *Cldn3* forward: 5'-cactaccagcagtcgatgaa-3' and reverse: 5'-gcgaggtttcttgcattc-3', occludin (*Ocdn*) forward: 5'-gggaatgtccagaacgagaaga-3' and reverse: 5'-cggtggcaatgaacaccatga-3', *Ephb2* forward 5'-atcaacactgaggtgggaag-3' and reverse: 5'-cacagcaatgaggacataca-3', *Ephb3* forward: 5'-acacacgctacacctttgag-3' and reverse: 5'-gcctggttggtggtgatatt-3', olfactomedin 4 (*Olfm4*) forward: 5'-cctctggtgcctacaataac-3' and reverse: 5'-gctggcctcagttgcataaa-3', leucine rich repeat-containing G protein-coupled receptor 5 (*Lgr5*) forward: 5'-gctttcagcatttgctga-3' and reverse: 5'-agactctccagggtggcag-3', *Nlrp6* forward: 5'-tgaccagagctccaggagt-3' and reverse: 5'-tttagcaggccaaagaggaa-3', *Bcl2* forward: 5'-ccgggagaacagggtatgataa-3' and

reverse: 5'-cccactcgtagccctctg-3', *Bcl1* forward: 5'-ggcgcacgtggccttt-3' and reverse: 5'-tccgactcaccaatacctgcat-3', Bcl-2-like protein 11 (*Bim*) forward: 5'-tggatattgacaggcacttg-3' and reverse: 5'-cataaggagcaggcacagagaa-3', *Bax* forward: 5'-actaaagtgcggagctgat-3' and reverse: 5'-atggctactgtctgccatgt-3', *Bak* forward: 5'-cagatggatgcacagagag-3' and reverse: 5'-tctgtgtaccacgaattggc-3', death-associated protein kinase 1 (*Dapk1*) forward: 5'-ctgcagatcgctcactttga-3' and reverse: 5'-ggccaagccaaagtctatga-3', cyclin-dependent kinase inhibitor 1 (*Cdkn1a*; also known as *P21*) forward: 5'-ggaagaccatgtggacctgt-3' and reverse: 5'-ccgttttcgaccctgagag-3', tumour protein p53 (*Trp53*) forward: 5'-gttccgagagctgaatgagg-3' and reverse: 5'-ttatggcgggaggtagactg-3', p53 upregulated modulator of apoptosis (*Puma*) forward: 5'-gacgacccaacgcacagta-3' and reverse: 5'-ctggtaagggcaggagtc-3', *Il18* forward: 5'-caggcctgacatcttctgcaa-3' and reverse: 5'-tctgacatggcagccattgt-3', *Il1b* forward: 5'-caaccaacaagtgatattctccatg-3' and reverse: 5'-gatccacactctccagctgca-3', and *Gapdh* forward: 5'-gtgttctaccccccaatgtg-3' and reverse 5'-catactggcagggtttctcca-3' (Integrated DNA Technologies). PCR products were resolved on 1% agarose ethidium bromide gels, bands were visualized on an AlphaImager HP System (Proteinsimple) and images were inverted for presentation to show dark bands on a light background.

Cytokine ELISA.

Concentrations of IFN- γ , IL-17A, TNF- α , IL-6, IL-1 β and IL-18 were measured in the serum or cell culture supernatant by ELISA with specific anti-mouse mAbs for capture and detection using BD OptEIA (for IFN- γ ; BD Biosciences), ELISA MAX (for IL-17A; BioLegend) and mouse IL-18 ELISA Kit (MBL International Corporation). Assays were performed according to the manufacturer's protocol and read at 450 nm using a microplate reader (Model 3550; Bio-Rad Labs). All samples and standards were run in duplicate.

Organ explant culture.

To measure cytokine production, colon and ileum sections (1 cm) were dissected from WT B6 and *Nlrp6*^{-/-} mice after allo-BMT, washed in PBS and then incubated overnight in 2 ml RPMI-1640 supplemented with 10% FCS, penicillin and streptomycin with or without 70 mM taurine¹⁹. Supernatants were collected and assayed for cytokine levels by ELISA. No eukaryotic cell lines were used in this study.

Immunohistochemistry.

MUC2 immunohistochemistry was performed by Leica BOND-III using sections (3- μ m thick) of formalin-fixed and paraffin-embedded tissues. Antigen retrieval was performed in Bond Epitope Retrieval Solution 2 (Leica) at 100 °C for 10 min. Primary rabbit polyclonal antibody against MUC2 (GTX100664, GeneTex) was applied at 1:1,000 dilution. For NLRP6 immunohistochemistry, tissues embedded in paraffin were cut into 5- μ m sections. Heat-induced antigen retrieval was performed with 10 mM sodium citrate buffer. Endogenous peroxidases were quenched with 3% hydrogen peroxide for 15 min. Primary anti-NLRP6 antibody (ABF29, Millipore) was diluted 1:1,000 in PBST containing 10% goat serum (Thermo Fisher Scientific) and incubated overnight at 4 °C. Bound antibody was detected using an anti-rabbit horseradish peroxidaselabelled polymer incubated for 30 min (EnVision+ System) and ImmPACT DAB (Vector Laboratories). Slides were then counterstained with haematoxylin, dehydrated and covered.

Histopathological evaluation of epithelial damage.

Epithelial damage was histopathologically evaluated in the tissue sections using haematoxylin and eosin staining or MUC2 immunohistochemistry. In the haematoxylin and eosin-stained tissue sections, epithelial damage was examined in terms of the loss of goblet cells and the presence of debris in the crypt. The loss of goblet cells was semiquantitatively evaluated as follows: score 0, no apparent loss; score 1, <50% loss in each tissue section; and score 2, >50% loss. The presence of debris in the crypt was semiquantitatively evaluated as follows: score 0, no apparent debris; score 1, debris in <50% of the crypts in the tissue section; and score 2, debris in >50% of the crypts. In situ cell death detection was performed by TUNEL assays (Roche).

DNA isolation and amplification.

On indicated days, faecal pellets were collected from mice and stored at -20°C . DNA was isolated from faecal samples with a PowerMag Microbiome RNA/DNA Isolation Kit (Mo Bio Laboratories) using an epMotion 5075 liquid handling system. The V4 region of the 16S rRNA gene was amplified from 1 or 7 μl DNA and sequenced with a MiSeq (Illumina) using the 500 cycle MiSeq Reagent Kit, v2 (MS-102–2003, Illumina), by the University of Michigan Microbial Systems Molecular Biology Laboratory.

Liquid chromatography-mass spectrometry.

To determine targeted taurine quantitation, samples (intestinal faecal content) from mice 21 d post-transplant were harvested, homogenized and snap-frozen in liquid N₂. Faecal content was weighed at necropsy. Samples were extracted with acetonitrile spiked with stable isotope-labelled C¹³ taurine standards. The supernatant was analysed by the Agilent 6410 mass spectrometer in a positive electrospray ionization mode after liquid chromatography using a hydrophilic interaction chromatography mode and isocratic gradient. Quantitation was performed by calibration to internal standards and expressed in M per 100 mg after normalization for weights.

Sequence processing and microbiota analysis.

The 16S rRNA gene sequence data were processed and analysed using the software package mothur (v.1.38.1 and 1.39.1) and the MiSeq SOP (v.2.6.2.1)^{40,41}. After sequence processing and alignment to the SILVA reference alignment (release 119)^{42,43}, sequences were binned into operational taxonomic units based on 97% sequence similarity using the average neighbour method^{44,45}. By calculating θ_{yc} distances (a metric that takes relative abundances of both shared and non-shared operational taxonomic units into account)⁴⁶ between communities and using analysis of molecular variance (AMOVA)⁴⁷, it was possible to determine whether there were significant differences between the microbiota of different groups. Principle coordinates analysis (PCoA) was used to visualize the θ_{yc} distances between samples. Linear discriminant analysis effect size was used to determine whether specific operational taxonomic units were differentially abundant in different groups⁴⁸. We also investigated the taxonomic composition of the bacterial communities by classifying sequences within mothur using a modified version of the Ribosomal Database Project training set (version 14)^{49,50}. Diversity metrics, including inverse Simpson, were calculated.

Statistical analysis.

Bars and error bars represent the mean and s.e.m., respectively. We performed non-survival analysis using unpaired *t*-test for statistical comparisons. The clinical and pathological GVHD scores and relative mRNA expression levels were analysed with two-tailed Mann–Whitney *U*-test. Significance was determined with $\alpha = 0.05$. We performed survival data analysis using the Wilcoxon rank test. All statistical analyses were performed using GraphPad Prism 7. The sample sizes of 3–8 per group were used for in vitro studies and 5–10 were used for individual in vivo studies. In some cases, as stated in the respective legends, data were combined from all experiments and shown collectively. These numbers were chosen based on hundreds of experiments in our laboratory (and from other published reports) for a sufficient dynamic range between the positive (allogeneic) and negative (syngeneic) control groups. All experiments, in vitro and in vivo, except for the microbiome sequencing studies, were done more than twice, in some cases more than four times.

Reporting Summary.

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The raw sequencing reads have been deposited at the NCBI Short Read Archive under BioProject ID PRJNA491725. Metabolomic data, mass spectral analytical parameters and spectral raw data from the study and metadata have been deposited in the NIH Common Fund's Data Repository and Coordinating Center under Metabolomics Workbench Project ID PR000728.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1 |. The absence of NLRP6 in hosts ameliorates GVHD in experimental BMT models. B6 WT and *Nlrp6*^{-/-} animals received 11 Gy total body irradiation on day-1 and were transplanted with 3.0×10^6 CD90.2⁺ splenic T cells along with 5×10^6 TCD-BM cells from either syngeneic B6 or allogeneic major histocompatibility (MHC)-mismatched BALB/c donors. **a,b**, Survival (**a**) and GVHD clinical score (**b**). In **a**, B6 $\rightarrow$ B6 *Nlrp6*^{-/-} data points are under B6 $\rightarrow$ B6 data points. **c**, Donor T cell (H-2k^dCD4⁺CD8⁺) expansion in the spleen on day 7 (left) and day 21 (right) after allo-BMT. **d**, CD69⁺ expression on donor CD4⁺ (left) and CD8⁺ (right) T cells in the spleen on day 7 after allo-HCT. **e-g**, Serum IFN- γ (**e**), TNF- α (**f**) and IL-17A (**g**) levels on day 7 (left) and day 21 (right) after allo-BMT. **h**, The representative figures of the small intestine (top panels) and large intestine (bottom panels) on day 7 after allo-BMT (scale bars, 200 μ m). **i**, Histopathological GVHD score in the large intestine (left) and small intestine (right) on day 7 after allo-BMT. Data in allo-B6 WT versus allo-*Nlrp6*^{-/-} animals were analysed with the Wilcoxon test (**a**), two-tailed

Mann-Whitney U -test (**b,i**) and two-tailed, unpaired t -test (**c–g**). ** $P < 0.01$ and *** $P < 0.001$ (see Supplementary Table for exact P values). Data are shown as the mean $\pm$ s.e.m. (**b–g,i**). All sample numbers are described in the figure. The representative data are from at least two independent experiments.

Fig. 2 | *Nlrp6*^{-/-} animals showed similar expansion of donor-derived myeloid cells with B6 WT animals.

a–e, The expansion of donor-derived myeloid cells on day 21 after BMT from the spleens (left graph) and small intestines (right graph) from recipients were analysed. The absolute numbers of total CD11b⁺ cells (**a**), CD11b⁺Ly6C⁺Ly6G⁺ cells (**b**), CD11b⁺Ly6C⁺Ly6G⁻ cells (**c**), CD11b⁺Ly6C⁻Ly6G⁺ cells (**d**) and CD11b⁺Ly6C⁻Ly6G⁻ cells (**e**) are shown. The representative data are from at least two independent experiments. Data are shown as the mean ± s.e.m. **f**, PCoA of distances between communities of the faecal microbiota of B6 WT mice and B6 *Nlrp6*^{-/-} mice. **g**, Community composition bar plot of the faecal microbiota of three B6 WT mice, three *Nlrp6*^{-/-} mice, three B6 WT mice 14 days after co-housing with three *Nlrp6*^{-/-} mice (CH B6 WT D14), three *Nlrp6*^{-/-} mice 14 days after co-housing with three B6 WT mice (CH *Nlrp6*^{-/-} D14), four B6 WT mice 14 days after allo-BMT (allo-B6

WT D14), four *Nlrp6*^{-/-} mice 14 days after allo-BMT (allo-*Nlrp6*^{-/-} D14), five antibiotic-treated B6 WT mice 7 days after allo-BMT (ABX allo-B6 WT D7), seven antibiotic-treated *Nlrp6*^{-/-} mice 7 days after allo-BMT (ABX allo-*Nlrp6*^{-/-} D7), three antibiotic-treated B6 WT mice 14 days after allo-BMT (ABX allo-B6 WT D14) and seven antibiotic-treated *Nlrp6*^{-/-} mice 14 days after allo-BMT (ABX allo-*Nlrp6*^{-/-} D14). **h**, PCoA of distances between faecal microbial communities of allo-B6 WT and allo-*Nlrp6*^{-/-} mice on day 14 (left). PCoA of distances between communities of the faecal microbiota of allo-B6 WT & *Nlrp6*^{-/-} D14 mice and the faecal microbiota of naive B6 WT & *Nlrp6*^{-/-} mice (right). **i**, Box plots of inverse Simpson diversity of allo-B6 WT and allo-*Nlrp6*^{-/-} on day 14 post-BMT. The box shows the first and third quartiles, the red band shows the median and the whiskers show the fifth and ninety-fifth percentiles. Two-tailed, unpaired *t*-test in allo-B6 WT versus allo-B6 *Nlrp6*^{-/-} animals was used (**a–e**). AMOVA was used in **f–h** (see Supplementary Table for exact *P* values). All sample numbers are described in the figure or caption.

Fig. 3 | *Nlrp6*^{-/-} animals showed less GVHD severity after co-housing or antibiotic treatments. B6 WT and *Nlrp6*^{-/-} animals were co-housed for 2 weeks at a 1/1 ratio before allo-BMT. **a**, PCoA of distances between communities of the faecal microbiota of B6 WT (CH B6 WT D14) mice and *Nlrp6*^{-/-} mice (CH *Nlrp6*^{-/-} D14) 14 days after co-housing (left). PCoA of distance between communities of the faecal microbiota of co-housed B6 WT and *Nlrp6*^{-/-} mice for 14 days (CH B6 WT & *Nlrp6*^{-/-} and the faecal microbiota of naive B6 WT mice (B6 WT) (right). **b,c**, Survival (**b**) and GVHD clinical score (**c**) after BMT in allo-co-housed B6 WT versus allo-co-housed *Nlrp6*^{-/-} animals. **d**, PCoA of distances between communities of the faecal microbiota of antibiotic-treated B6 WT mice (ABX allo-B6 WT D7) and antibiotic-treated *Nlrp6*^{-/-} mice 7 days after allo-BMT (ABX allo-*Nlrp6*^{-/-} D7). **e**, PCoA of distances between communities of the faecal microbiota of antibiotic-treated B6 WT mice

(ABX allo-B6 WT D14) and antibiotic-treated *Nlrp6*^{-/-} mice 14 days after allo-BMT (ABX allo-*Nlrp6*^{-/-} D14) (left). PCoA of distances between communities of the faecal microbiota of antibiotic-treated B6 WT and *Nlrp6*^{-/-} mice (ABX allo-B6 WT & *Nlrp6*^{-/-} D14) and B6 WT and *Nlrp6*^{-/-} mice 14 days after allo-BMT (allo-B6 WT & *Nlrp6*^{-/-} D14) (right). **f**, Box plots of inverse Simpson diversity of ABX allo-B6 WT D7 and ABX allo-*Nlrp6*^{-/-} D7. **g**, Box plots of inverse Simpson diversity of ABX allo-B6 WT D14 and ABX allo-*Nlrp6*^{-/-} D14. **h**, Survival with antibiotics treatment after allo-BMT. B6 → B6 (ABX) and BALB/c → B6 *Nlrp6*^{-/-} data points are under B6 → B6 data points. **i,j**, Survival (**i**) and GVHD clinical score (**j**) of allo-BMT after conventionalization of GF mice. AMOVA (**a,d,e**), Wilcoxon test (**b,h,i**) and two-tailed Mann-Whitney *U*-test (**c,j**) were used. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 (see Supplementary Table for exact *P* values). Data are shown as the mean ± s.e.m. (**b,c,j**). The box shows the first and third quartiles, the red band shows the median and the whiskers show the 5-95 percentiles (**f,g**). All sample numbers are described in the figure. The representative data are from at least two independent experiments.

Fig. 4 | Absence of NLRP6 in host haematopoietic-derived APCs is dispensable for reducing GVHD severity and mortality.

[B6 → B6Ly5.2] and [*Nlrp6*^{-/-} → B6Ly5.2] animals received 9 Gy total body irradiation and received 3×10^6 CD90⁺ T cells along with 5×10^6 TCD-BM cells from either syngeneic B6 or allogeneic MHC-mismatched BALB/c donors. **a,b**, Survival (**a**) and clinical GVHD score (**b**). In **a**, B6Ly5.2 → [B6 *Nlrp6*^{-/-} → B6Ly5.2] data points are under B6Ly5.2 → [B6 → B6Ly5.2] data points. **c,d**, TNF-α (**c**) and IL-6 (**d**) secretion by bone marrow-derived macrophages. Bone marrow-derived macrophages from either B6 WT or *Nlrp6*^{-/-} animals were stimulated with LPS (100 ng ml⁻¹) for 16h and the secretion of TNF-α (**c**) and

IL-6 (**d**) by ELISA was tested. **e,f**, In vitro mixed lymphocyte reaction. Isolated splenic CD90.2⁺ T cells from either syngeneic B6 WT or allogeneic BALB/c animals were cultured with BMDCs derived from B6 WT or *Nlrp6*^{-/-} animals for 72 h or 96 h and analysed for proliferation following ³H-thymidine incorporation during the last 16 h of incubation (**e**). Isolated splenic CD90.2⁺ T cells from either syngeneic B6 WT or allogeneic BALB/c animals were cultured with irradiated whole splenocytes from B6 WT or *Nlrp6*^{-/-} animals for 72 h and analysed for proliferation following ³H-thymidine incorporation during the last 6 h of incubation (**f**). CPM, count per minute. **g**, Total number of dendritic cells (CD11c⁺) (top) and macrophages (CD11b⁺F4/80⁺) (bottom) in the colon and ileum from WT B6 or *Nlrp6*^{-/-} recipients 3 days after allo-BMT. Surface marker expressions were analysed for CD40, CD80 and CD86 populations in CD11c⁺ dendritic cells (top) and for CD80, CD86 and MHC class II (IA/IE) in macrophages (bottom). **h**, IL-1 β , TNF- α and IL-18 secretion by isolated dendritic cells (top) and macrophages (bottom) from the colon and ileum of either B6 WT or *Nlrp6*^{-/-} animals, stimulated with LPS (100 ng ml⁻¹) for 16 h and tested by ELISA. Data were analysed in allo-B6 WT versus allo-*Nlrp6*^{-/-} animals with the Wilcoxon test (**a**), two-tailed Mann-Whitney *U*-test (**b**) and two-tailed, unpaired *t*-test (**c-h**) (see Supplementary Table for exact *P* values). Data are shown as the mean $\pm$ s.e.m. (**b-h**). All sample numbers are described in the figure. The representative data are from two independent experiments.

Fig. 5 |. Absence of NLRP6 on host non-haematopoietic cells plays an important role in ameliorating GVHD severity and mortality.

To make bone marrow chimaeras, B6 WT and *Nlrp6*^{-/-} animals were lethally irradiated with 10 Gy and infused with 5×10^6 bone marrow cells and 5×10^6 splenocytes from syngeneic B6Ly5.2 donors. Three months later, [B6Ly5.2 → B6] and [B6Ly5.2 → *Nlrp6*^{-/-}] animals were irradiated with 9 Gy and received 3×10^6 CD90⁺ T cells along with 5×10^6 TCD-BM cells from either syngeneic B6 or allogeneic MHC-mismatched BALB/c donors. **a,b**, Survival (**a**) and clinical GVHD score (**b**). [B6Ly5.2 → B6Ly5.1] versus [B6Ly5.2 → B6 *Nlrp6*^{-/-}] animals were compared at day 14 or day 21. In **a**, B6 → [B6Ly5.2 → B6 *Nlrp6*^{-/-}] data points are under B6 → [B6Ly5.2 → B6] data points. **c**, FITC-dextran intestinal permeability assay. The plasma FITC-dextran concentration was tested on day 7

after allo-BMT. **d**, The expression of multiple tissue repair-associated genes, *Cldn1/2/3*, *Ocdn*, *Ephb2/3*, *Lgr5* and *Olfm4*, in small IECs was measured by quantitative RT-PCR at day 7 after allo-BMT. **e**, Serum level of IL-18 and IL-1 β at day 7 after allo-BMT (left). IL-18 and IL-1 β levels in supernatant from the colon and ileum explants with 70 mM taurine at days 1, 3 and 7 after allo-BMT (right). **f**, The gene expression of *Bcl2*, *Bclx1*, *Bim*, *Bax* and *Bak* in small IECs was measured by quantitative RT-PCR at day 7 after allo-BMT. Wilcoxon test (**a**), two-tailed Mann-Whitney *U*-test (**b,d,f**) and two-tailed, unpaired *t*-test (**c,e**) were used. * $P < 0.05$ and ** $P < 0.01$ (see Supplementary Table for exact *P* values). Data are shown as the mean $\pm$ s.e.m. (**b–f**). All sample numbers are described in the figure. The representative data are from two independent experiments.

Fig. 6. Absence of NLRP6 in IECs enhances gut homeostasis after allo-BMT.

a,b, Representative Periodic acid-Schiff (PAS)-stained small intestine (**a**) and large intestine (**b**) sections of allo-B6 WT and *Nlrp6*^{-/-} animals at day 7 (top panels) and day 14 (bottom panels) after allo-BMT (scale bars, 200 μm). **c**, Representative MUC2 staining (top; scale bars, 200 μm) and goblet cell loss index (bottom) in the colon. **d**, The gene expression of *Nlrp6* in IECs (left), Paneth cells (middle) and stem cells (right) was measured by quantitative RT-PCR at days 0, 7 and 21 after BMT. **e**, Immunohistochemical staining of NLRP6 in colon histological sections from syngeneic and allo-B6 WT recipients at days 0, 7 and 21 after BMT (scale bars, 200 μm). **f**, Taurine levels in the stool contents from the colon (top) and ileum (bottom) of WT B6 and *Nlrp6*^{-/-} mice collected at day 0 and day 21 after

allo-BMT. Units are moles normalized to 100 mg faecal weight. **g,h**, Recipient B6 WT and B6 *Nlrp6*^{-/-} animals received taurine (2.5 g per kg per day) by oral gavage from day 0 during BMT. Survival (**g**) and the clinical GVHD score (**h**) are shown. In **g**, B6 → B6 (taurine), B6 → B6 *Nlrp6*^{-/-} (vehicle and taurine) data points are under B6 → B6 (vehicle) data points; in **h**, B6 → B6 (taurine) and B6 → B6 *Nlrp6*^{-/-} (taurine) data points are under B6 → B6 (vehicle) data points. **i,j**, B6 WT and *Nlrp6*^{-/-} animals were lethally irradiated with 10 Gy and infused with 3×10^6 CD90⁺ T cells along with 5×10^6 TCD-BM cells from either syngeneic B6 or allogeneic MHC-mismatched BALB/c donors concurrently with syngeneic 1×10^4 MBL-2 tumours at the same time as allo-HCT. Tumour growth was monitored using bioluminescence imaging at day 21 after allo-BMT (**i**). The representative data are shown. Tumour-related mortality of MBL-2 is also shown (**j**). Two-tailed Mann-Whitney *U*-test (**c,d,h**), two-tailed, unpaired *t*-test (**f**) and Wilcoxon test (**g,j**) were used. * < 0.05 and ***P* < 0.01. NS, not significant (see Supplementary Table for exact *P* values). Data are shown as the mean ± s.e.m. (**c,d,f,h**). All sample numbers are described in the figure. The representative data are from two independent experiments.