The Nlrp3 inflammasome regulates acute graft-versus-host disease

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The success of allogeneic hematopoietic cell transplantation is limited by acute graft-versus-host disease (GvHD), a severe complication accompanied by high mortality rates. Yet, the molecular mechanisms initiating this disease remain poorly defined. In this study, we show that, after conditioning therapy, intestinal commensal bacteria and the damage-associated molecular pattern uric acid contribute to Nlrp3 inflammasome–mediated IL-1β production and that gastrointestinal decontamination and uric acid depletion reduced GvHD severity. Early blockade of IL-1β or genetic deficiency of the IL-1 receptor in dendritic cells (DCs) and T cells improved survival. The Nlrp3 inflammasome components Nlrp3 and Asc, which are required for pro–IL-1β cleavage, were critical for the full manifestation of GvHD. In transplanted mice, IL-1β originated from multiple intestinal cell compartments and exerted its effects on DCs and T cells, the latter being preferentially skewed toward Th17. Compatible with these mouse data, increased levels of active caspase-1 and IL-1β were found in circulating leukocytes and intestinal GvHD lesions of patients. Thus, the identification of a crucial role for the Nlrp3 inflammasome sheds new light on the pathogenesis of GvHD and opens a potential new avenue for the targeted therapy of this severe complication.

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Abbreviations used: allo-HCT, allogeneic hematopoietic cell transplantation; Asc, apoptosis-associated speck-like protein containing a CARD; BU, Busulfan; CY, cyclophosphamide; DAMP, damage-associated molecular pattern; GvHD, graft-versus-host disease; GvL, graft-versus-leukemia; LP, lamina propria; LPL, LP lymphocyte; MFI, mean fluorescence intensity; Nlrp3, NACHT, LRR, and PYD domains-containing protein 3; PAMP, pathogen-associated molecular pattern; TBI ,total body irradiation; UA, uric acid.

Allogeneic hematopoietic cell transplantation (allo-HCT) is an established treatment option for a variety of hematological malignancies. Worldwide, allo-HCT is performed >25,000 times annually (Pasquini and Wang, 2012). Donor T cells present in the allograft contribute to the efficacy of allo-HCT, and mediate the graft-versus-leukemia (GvL) effect. Unfortunately, donor T cells can also target nonmalignant host tissues, leading to a severe complication known as graft-versus-host disease (GvHD; Ferrara et al., 2009). Acute GvHD grade 2–4 occurs in 40–50% of the allo-HCT patients and is responsible for considerable morbidity and mortality (Jacobsohn and Vogelsang, 2007). Although

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different prophylactic regimens are currently used to reduce GvHD (Ram et al., 2009), the disease remains a significant unsolved medical problem. Before allo-HCT, recipients undergo a conditioning regimen, consisting of cytotoxic drugs and γ-irradiation. Such a regimen induces tissue damage, allowing bacterial products to translocate from the skin and mucosa into the internal milieu, where they provoke a "cytokine storm" which results in inflammation in the host, activation of the recipient's antigen-presenting cells, and a subsequent donor T cell–mediated allogeneic reaction, with further amplification of the cytokine response (Shlomchik 2007). However, the molecular events governing proinflammatory cytokine production upon conditioning remain poorly understood. We have previously shown that activation of the P2X7 receptor is a critical step in the pathogenesis of GvHD (Wilhelm et al., 2010). The main endogenous ligand for P2X7 is the damage-associated molecular pattern (DAMP) adenosine-5′-triphosphate (ATP; Ferrari et al., 2006) which is released by damaged tissues upon conditioning, thereby contributing to systemic immune activation. In this regard, binding of ATP to P2X7 can cause assembly and activation of the protein 3 (Nlrp3)-inflammasome, which contains NACHT, LRR and PYD domains. The term inflammasome refers to intracellular multiprotein complexes that control activation of inflammatory caspases such as caspase-1 and -11. In recent years, several studies have reported that the Nlrp3 inflammasome is the essential platform for caspase-1 activation in response to multiple distinct exogenous and endogenous stress or danger signals (Franchi and Núñez, 2012). For caspase-1 activation, Nlrp3 utilizes the adapter protein apoptosis–associated speck-like protein containing a CARD (Asc; Davis et al., 2011; Franchi and Núñez, 2012). Full activation of the Nlrp3 inflammasome leads to cleavage of the precursor protein pro–IL-1β into its active form. As bioactive IL-1β fulfills many biological functions, including the induction of adaptive immune responses, its production by the Nlrp3 inflammasome is tightly controlled by transcriptional and post-transcriptional signals. Signal 1 can be provided by Toll-like receptors (TLRs) leading to NF-κB–mediated gene transcription, and is essential for the synthesis of the IL-1β precursor pro–IL-1β and Nlrp3. In addition, detection of the second stimulus (signal 2) triggers proteolytic processing of pro–IL-1β into mature bioactive IL-1β by the Nlrp3 inflammasome. Recently, it has been shown that microbiota induce IL-1β release via an Nlrc4-inflammasome and are essential for the development of Th17 responses in the intestine (Franchi and Núñez, 2012). Intriguingly, Th17 cells have been causally linked to instances of aggravated GvHD after allo-HCT (Fulton et al., 2012). Here, we demonstrate that the Nlrp3 inflammasome regulates GvHD by detection of DAMPs in the conditioning phase and subsequent shaping of Th17 responses in the intestines of the recipient.

RESULTS AND DISCUSSION

IL-1β affects GvHD in the early phase after allo-HCT

To study the involvement of IL-1β in GvHD development we treated mice receiving allogeneic BM and T cells (allo-HCT) with different IL-1 inhibitors. Treatment with the recombinant IL-1 receptor antagonist (IL-1RA) Anakinra starting 24 h before conditioning (day −1) significantly improved survival of mice in comparison to littermates treated with placebo, whereas there was no protection when treatment was initiated on day 5 (Fig. 1 A). Likewise, mice treated with a selective neutralizing anti–IL-1β antibody 24 h before conditioning experienced improved body weight (not depicted) and prolonged survival whereas later treatment (day 5) was not effective (Fig. 1 B). Notably, treatment with the neutralizing anti–IL-1β antibody did not affect GvL-effects or immune reconstitution (unpublished data). To assess which target cell populations are impacted by IL-1β, we quantified the expression of IL-1R in different cell populations known to have a role in GvHD development (Bryson et al., 2004; Shlomchik 2007). We observed a significant increase of IL-1R expression on $CD11c^+$ DCs, $CD4^+$ T cells, and $CD8^+$ T cells peaking on day 3 after allo-HCT (Fig. 1 C). Consequently, transplantation of allogeneic IL-1R–deficient T cells resulted in delayed GvHD-induced mortality compared with the transfer of IL-1R–competent T cells (Fig. 1 D). Furthermore, transplantation of $IL\text{-}1R^{-/-}$ DCs led to reduced GvHD mortality when compared with the transfer of $IL\text{-}1R^{+/+}$ cells (Fig. 1 E). These data support the critical role of IL-1β in the early phase of GvHD and demonstrate that IL-1β exerts its proinflammatory effects on both T cells and DCs.

The Nlrp3–inflammasome regulates acute GvHD

Asc constitutes an essential adaptor protein for caspase-1–mediated pro–IL-1β processing by distinct inflammasomes (Martinon et al., 2009). To delineate the potential involvement of Asc-containing inflammasomes in the pathogenesis of acute GvHD, we used $Asc^{-/-}$ mice as donors or recipients. In $Asc^{-/-}$ recipient mice, we observed delayed GvHD-induced mortality and a lower cumulative GvHD score compared with $Asc^{+/+}$ or $Asc^{+/-}$ littermates (Fig. 2, A and B). Conversely, Asc deficiency of the donor did not influence GvHD severity (unpublished data). Next, we used $Nlrp3^{-/-}$ mice as donors or recipients for allo-HCT to assess the involvement of the Nlrp3 inflammasome in acute GvHD. When $Nlrp3^{-/-}$ mice were used as recipients, GvHD was significantly delayed compared with WT controls at different T cell dosages, with long-term survivors in the Nlrp3-deficient group (Fig. 2, C and D). This finding correlated with decreased weight loss in $Nlrp3^{-/-}$ recipients (not depicted), a significant reduction in the histological GvHD score in the intestines of $Nlrp3^{-/-}$ allo-HCT recipients (Fig. 2 E) and was reproducible in two different GvHD models (not depicted). In contrast, Nlrp3 deficiency of the donor did not result in survival differences (unpublished data). Moreover, treatment with glibenclamide, known to inhibit the Nlrp3 inflammasome, resulted in delayed and reduced mortality of allo-HCT WT recipients comparable to IL-1RA blockade with Anakinra. These effects were seen after conditioning with total body irradiation (TBI) or fludarabine/CY (FLU/CY), demonstrating the relevance of the aforementioned results for different conditioning regimes used for human allo-HCT (Fig. 2, F and G). However, protection from GvHD-mediated mortality through blockade of the Nlrp3

inflammasome was incomplete. This can be explained by the fact that Nlrp3 inflammasome inhibition by glibenclamide was only partial, as residual amounts of IL-1β and cleaved caspase-1 were still detectable in myeloid cells (unpublished data). Furthermore, additional anti-TNF treatment starting at day 5 improved protection by the Nlrp3 inhibitor (Fig. 2 F), demonstrating an inflammasome-independent role for TNF in the pathogeneses of GvHD.

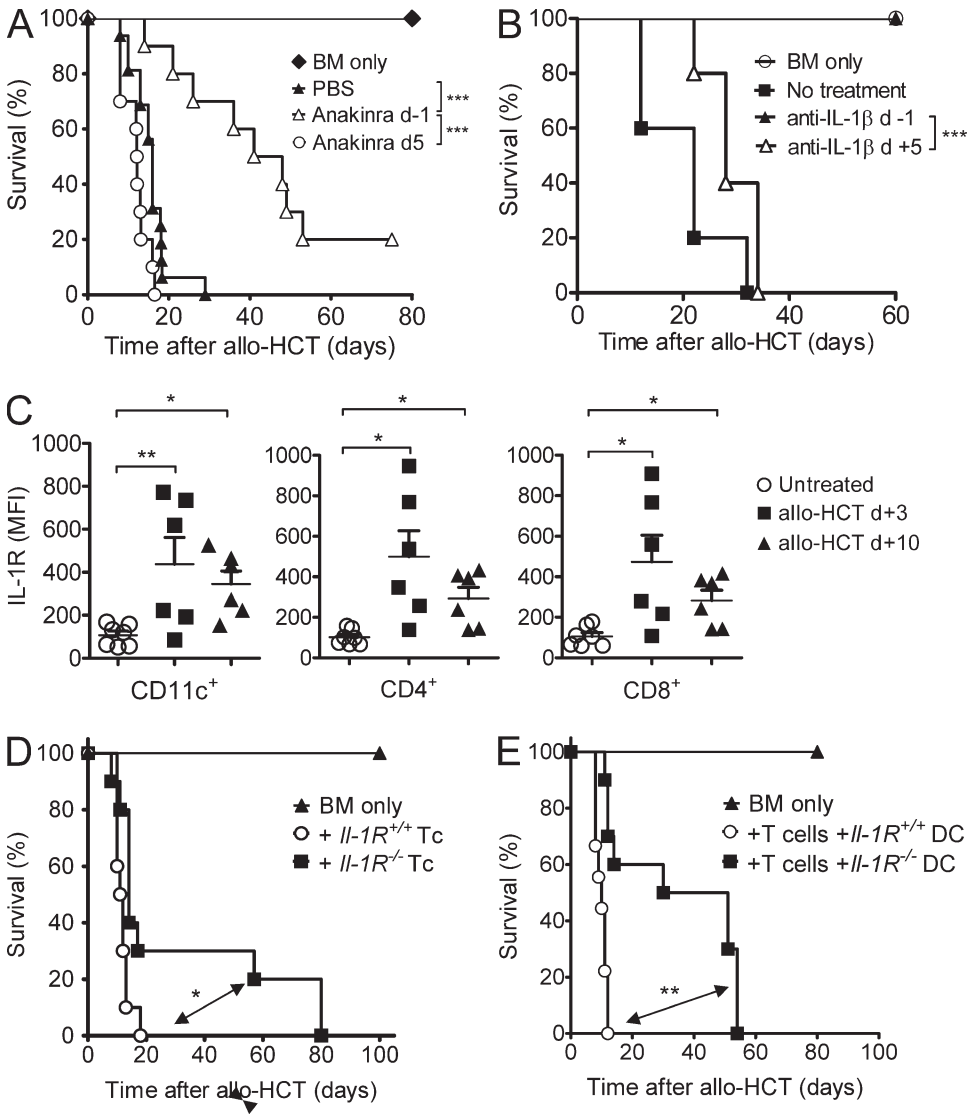

Figure 1. Proinflammatory IL-1β has effects on both T cells and DCs while receptor antagonism or depletion reduces acute GvHD related mortality. (A) BALB/c mice underwent TBI followed by transplantation with C57/BL6 BM alone (*n = 10*) or C57/BL6 BM plus T cells, and were monitored for survival. Where indicated, mice received either vehicle (PBS; *n = 16*) or Anakinra (*n = 10* per group). Treatment was started on day −1 or +5, as indicated. The experiment was performed twice and the resulting survival data were pooled. (B) C57BL/6 mice underwent TBI followed by i.v. injection of C3H BM alone or C3H BM plus T cells and were monitored for survival. Where indicated, mice received either no treatment or an antagonistic anti–IL-1β antibody on day −1 or +5. The experiment was performed twice, and the resulting survival data were pooled. Survival of mice receiving anti–IL-1β antibody on day −1 versus day +5: $P < 0.0001$; *n* = 10 per group. (C) BALB/c mice underwent TBI followed by allo-HCT with C57/BL6 BM plus T cells. Expression of IL-1R on splenic donor type (H-2K^{b+}) CD4$^+$, CD8$^+$, and CD11c$^+$ cells was analyzed by flow cytometry at the indicated time points after allo-HCT. Mean fluorescence intensity (MFI) is displayed for day 3 (filled square) and for day 10 (filled triangle) compared with the untreated group (open circle). Each data point represents an individual animal (*n* = 6 per group). The experiment was repeated twice and the resulting data were pooled. (D) BALB/c mice received TBI + C57/BL6 BM alone or with T cells from *IL-1R*$^{+/+}$ (open circle) or *IL-1R*$^{-/-}$ (filled square) donors and were monitored for survival. The experiment was performed twice and the resulting survival data were pooled. *IL-1R*$^{+/+}$ T cells versus *IL-1R*$^{-/-}$ T cells: $P = 0.0102$; *n* = 10 per group. (E) C57BL/6 mice underwent TBI followed by transplantation with BALB/c BM alone (*n = 3*) or BALB/c BM plus T cells (*n* = 10 per group). Mice that received T cells were additionally injected i.v. with DCs from *IL-1R*$^{+/+}$ C57BL/6 mice (open circle) or DCs from *IL-1R*$^{-/-}$ C57BL/6 mice (filled square) on day 0. The experiment was performed twice, and the resulting survival data were pooled. DC *IL-1R*$^{+/+}$ versus DC *IL-1R*$^{-/-}$: $P = 0.0002$. ***, $P < 0.0001$; **, $P < 0.001$; *, $P < 0.05$.

Bacterial flora and uric acid induce IL-1β

According to our data, which was obtained by neutralizing IL-1β in TBI-treated mice, we hypothesized that Nlrp3 inflammasome activation primarily occurs during the early conditioning phase before allo-HCT. However, the factors contributing to IL-1β production after conditioning are largely unknown. Yet, it is believed that translocation of bacterial products, defined as pathogen-associated molecular pattern (PAMPs), from the gastrointestinal tract through the mucosal barrier promotes the initiation of proinflammatory responses in acute GvHD (Holler et al., 2010). Therefore, we first quantified IL-1β mRNA after TBI in target tissues of GvHD. In addition to elevated pro–IL-1β levels in the intestines of irradiated mice (unpublished data), high levels of IL-1β mRNA were found in the intestinal tract and skin after TBI or BU/CY (BU/CY) conditioning (Fig. 3, A and B). Depletion of the microbial flora by antibiotic treatment diminished IL-1β mRNA levels (Fig. 3 A). The observation that neomycin, which is not absorbed in the intestines, reduced IL-1β mRNA levels in the skin supports the concept that the initiating signal for acute GvHD arises in the intestine and that influencing the bacterial microflora can prevent systemic GvHD. Moreover

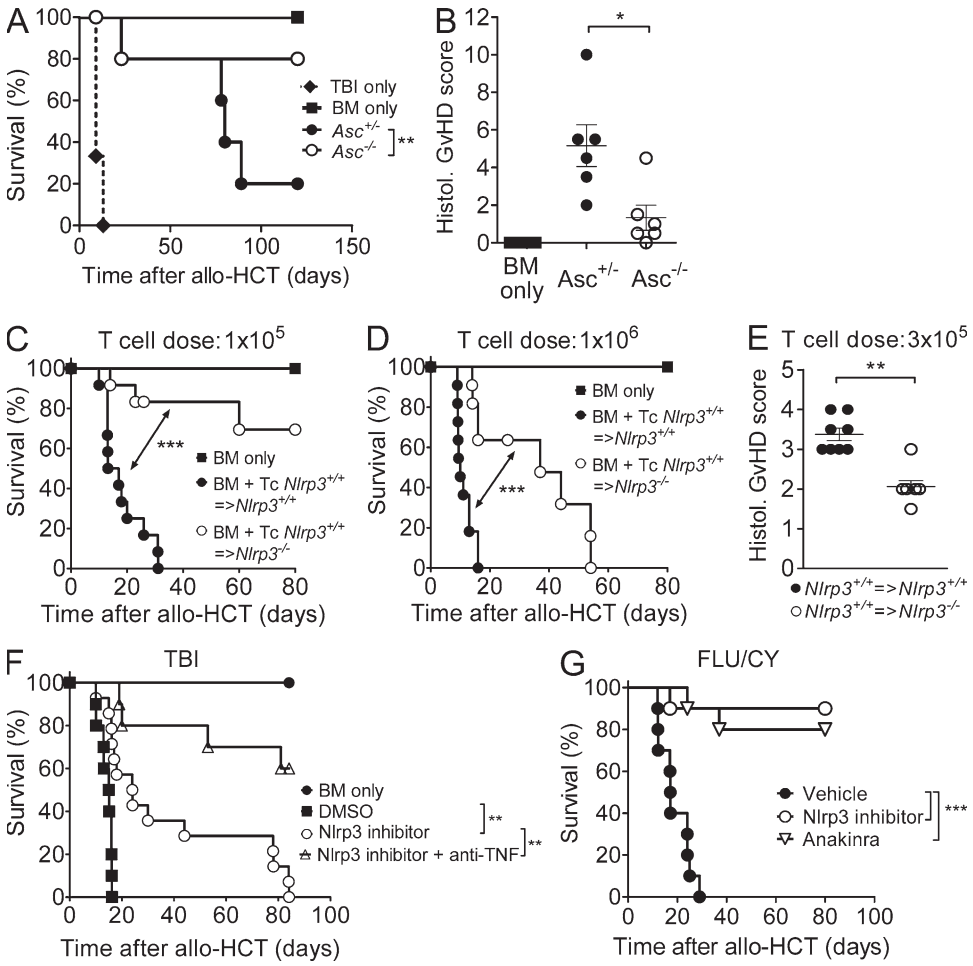

Figure 2. GvHD severity is reduced when recipients lack Asc or Nlrp3. (A and B) *Asc*$^{-/-}$ or *Asc*$^{+/-}$ C57BL/6 mice ($n = 6$ per group) underwent TBI followed by allo-HCT with C3H BM plus T cells. As control groups, WT C57BL/6 mice that received TBI alone ($n = 6$) or TBI plus C3H BM ($n = 6$) were used. Graphs show survival curves after transplantation (A) and histopathological scoring (apoptosis, inflammation) of the GvHD target organs (small intestine, large intestine and liver) on d10 after transplantation (B). The experiment was performed three times with similar results. One representative experiment is shown. (C–E) *Nlrp3*$^{-/-}$ or *Nlrp3*$^{+/+}$ C57BL/6 mice ($n = 10$–12 per group) underwent TBI followed by allo-HCT with BALB/c BM alone or BALB/c BM plus T cells. T cell doses were 10^5 (C), 10^6 (D), or 3×10^5 (E) as indicated. Graphs show survival curves after transplantation (C and D) and histopathological scoring (apoptosis, inflammation) of the GvHD target organs (small intestine, large intestine, and liver) on day 10 after transplantation (E). The experiment was performed twice, and the resulting survival data were pooled. (F) C57BL/6 mice underwent TBI, followed by transplantation with BALB/c BM alone ($n = 10$) or BALB/c BM plus T cells and were monitored for survival. Treatment was performed with PBS ($n = 10$), the Nlrp3 inhibitor glibenclamide ($n = 16$), or glibenclamide plus the TNF inhibitor Etanercept ($n = 10$) as indicated. Treatment with glibenclamide was started on day 0, and treatment with Etanercept was started on day 5. The experiment was performed twice, and the resulting data were pooled. (G) BALB/c mice were conditioned with FLU/CY based chemotherapy, followed by transplantation with C57/BL6 BM plus T cells, and then monitored for survival. Treatment was performed with vehicle (DMSO, $n = 10$), glibenclamide ($n = 10$), or Anakinra ($n = 10$). ***, $P < 0.0001$; **, $P < 0.005$; *, $P < 0.05$.

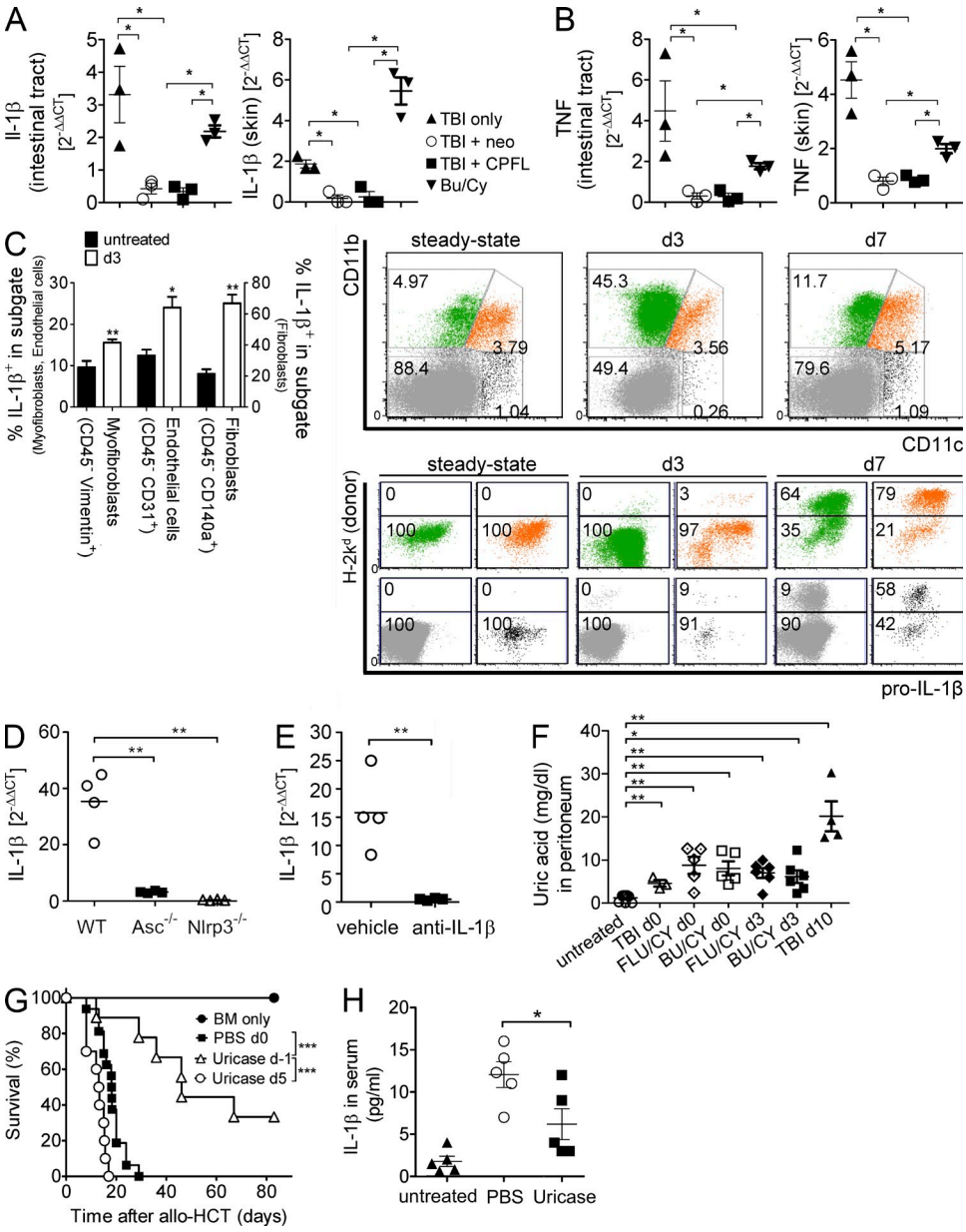

Figure 3. Effects of TBI, chemotherapy, commensal bacteria, and uric acid on IL-1β/TNF production. (A and B) RNA from the intestines and skin of mice receiving TBI (9 Gy) or chemotherapy (BU/Cyclophosphamide) was isolated 24 h after conditioning, and the expression of IL-1β and TNF was determined by quantitative PCR (qPCR). Neomycin (neo) or Ciprofloxacin (CPFL) were given starting 7 d before TBI, as indicated. Each data point represents an individual animal ($n = 3$) and the experiment was repeated 3 times. (C, left) Intestinal tract cells were isolated on d7 after allo-HCT, and the amount of intracellular IL-1β production was determined in the indicated cell populations after stimulation with PMA/ionomycin for 3 h. Shown are pooled results of six mice per group. (C, right) C57BL/6 hosts were transplanted (TBI, BALB/c BM+ T cells) and sacrificed 3 or 7 d after transplantation or left untreated (steady state). LPLs were isolated and directly analyzed for expression of CD11b, CD11c, donor/recipient markers, and intracellular expression of pro-IL-1β by flow cytometry. Top graphs show myeloid subgating of live LPLs, bottom graphs show donor H2k expression versus pro-IL-1β of myeloid subpopulations from top row. Data from one of two independent experiments with similar results and multiple time point analyses are shown. (D) RNA from the intestines of mice receiving TBI was isolated after 24 h and the expression of IL-1β was determined by quantitative PCR (qPCR). Each data point represents an individual animal ($n = 4$). The experiment was performed 3 times with similar results. (E) C57BL/6 mice received anti–IL-1β antibody or vehicle 24h before TBI or control treatment. RNA from the small intestine was isolated 40 h after TBI, and the expression of IL-1β was determined by quantitative PCR (qPCR). Each data point represents an individual animal ($n = 4$). The experiment was performed three times, with similar results. (F) BALB/c mice underwent different conditioning regimes: TBI alone (day 0; $n = 3$) or followed by allo-HCT with C57/BL6 BM plus T cells (day 10; $n = 4$); BU/CY chemotherapy or FLU/CY chemotherapy alone (day 0, $n = 5$) or followed by allo-HCT with C57/BL6 BM plus T cells (day 3, $n = 6$) as indicated. Untreated mice ($n = 9$) served as control. At the indicated time points, peritoneal fluid was isolated and UA levels were determined. Each data point represents an individual animal, and the experiment was performed twice with similar results. (G) BALB/c mice

chemotherapy-based conditioning caused increased active caspase-1 levels after allo-HCT (unpublished data). Thus, our data indicate that after irradiation the bacterial microflora in the gut provides the first signal leading to pro–IL-1β transcription. This is consistent with the known role of microbial TLR activation causing NF-κB–driven IL-1β gene transcription (Akira and Takeda, 2004). Compatible with these results, gastrointestinal decontamination protocols are applied in several transplantation centers to reduce the severity of acute GvHD (Beelen et al., 1999). Additionally, the levels of TNF, a proinflammatory cytokine known to contribute to acute GvHD pathogenesis, were significantly increased after TBI in the absence of antibiotics (Fig. 3 B). To determine the origin of IL-1β, we analyzed different hematopoietic and nonhematopoietic cell types in allo-HCT–transplanted mice and found IL-1β production in DCs, myeloid cells, myofibroblasts, endothelial cells, and fibroblasts isolated from the intestines (Fig. 3 C). Our observation suggests that multiple recipient cell subsets respond to conditioning by producing IL-1β, which targets donor DCs and T cells. Indeed, a targeted deletion of Asc in a single compartment including donor keratinocytes, DCs, or myeloid cells was not sufficient alone to influence GvHD severity (unpublished data).

To delineate which cell populations contribute to IL-1β production at early versus late time points, small intestinal lamina propria (LP) subsets were analyzed on days 0, 3, and 7 after allo-HCT. We observed that early after allo-HCT, host derived $CD11b^+$ myeloid cells were the dominant pro–IL-1β–expressing subset, whereas donor-derived DC subsets were only starting to infiltrate the LP (Fig. 3 C). At later time points (7 d after allo-HCT), however, donor-derived DC numbers increased significantly and became the main IL-1β–producing myeloid subset. T cells did not significantly contribute to IL-1β production (unpublished data). These findings might also explain why Nlrp3-deficient recipients display an early but only partial protection from GvHD.

IL-1β mRNA levels were significantly reduced in the intestines of irradiated *Nlrp3*-deficient mice (Fig. 3 D). This observation suggested a rapid positive feedback from mature IL-1β on its own pro-form synthesis. This hypothesis was confirmed with the use of an anti–IL-1β–neutralizing antibody, which also led to diminished IL-1β mRNA levels in irradiated *Nlrp3*-competent mice (Fig. 3 E).

In light of our data suggesting Nlrp3 inflammasome activation, we sought to identify DAMPs that could lead to this activation after conditioning and allo-HCT. Uric acid (UA) belongs to the class of DAMPs that potently trigger Nlrp3 inflammasome activation (Martinon et al., 2006; Gasse et al., 2009) and is known to be released from damaged cells. Hence, we assessed the role of UA in IL-1β release after TBI. We observed significantly increased UA levels in the peritoneal cavity after TBI-, BU/CY-, or FLU/CY-based conditioning and during GvHD compared with untreated mice (Fig. 3 F). Moreover, UA had a functional role in the pathogenesis of GvHD, as uricase given early (1 d before allo-HCT) resulted in improved survival (Fig. 3 G) and lower IL-1β serum levels (Fig. 3 H) compared with mice receiving placebo. However, later uricase treatment (day 5) was not protective (Fig. 3 G). Notably, uricase is used to reduce UA levels in patients with tumor lysis syndrome (Rampello et al., 2006). To assess the redundancy of multiple DAMP signaling, we adoptively transferred $P2X7^{-/-}$ or $P2X7^{+/+}$ DCs during BMT, either with or without priming with alum, an agent known to induce UA release (Kool et al., 2008). UA and ATP are both known activators of the Nlrp3 inflammasome, but with P2X7 relaying signaling only after ATP and not alum sensing. Accordingly, GvHD severity was increased when ATP-unresponsive $P2X7^{-/-}$ DCs were primed with alum, pointing to nonredundant roles of UA and ATP as GvHD-initiating DAMPs (unpublished data). Early intervention (day −1 prior TBI) with Anakinra or uricase was more effective than later treatment (day 5; Figs. 1 A and 3 G), indicating that inflammasome-mediated activation of IL-1β occurs very early in GvHD pathogenesis. Together, our data show that conditioning before allo-HCT induces the release of PAMPs and DAMPs, triggering pro–IL-1β synthesis and Nlrp3 inflammasome activation, respectively, in the recipients and promoting an inflammatory response that ultimately impacts the development and severity of acute GvHD.

The Nlrp3 inflammasome regulates Th17 responses

The effector phase of acute GvHD involves alloreactive donor T cells. Recently, IL-17A–producing $CD4^+$ T cells (Th17) cells have been shown to accumulate in the intestine in a colon inflammation model via an IL-1β–dependent mechanism (Coccia, Harrison et al., 2012). To assess whether inflammasome activation and subsequent IL-1β production in the course of GvHD had an impact on Th17 responses, we first analyzed donor T cell compartments in *Nlrp3*$^{-/-}$ allo-HCT recipients. We found reduced relative numbers of $CD4^+IL\text{-}17A^+$ T cells in the spleens of *Nlrp3*$^{-/-}$ compared with *Nlrp3*$^{+/+}$ recipients, whereas $CD4^+IFN\text{-}\gamma^+$ or $CD4^+IL\text{-}4^+$ cells (Fig. 4 A), naive T cells, effector memory T cells, or central memory T cells were unchanged (not depicted). The Nlrp3 inflammasome may also influence the differentiation of donor derived $CD4^+IL\text{-}17A^+$ T cells. To test this hypothesis in vitro, we performed an allogeneic mixed lymphocyte reaction and coincubated allogeneic splenic T cells with LPS-stimulated DCs sorted from the LP of intestines. IL-17 secretion by allogeneic T cells was significantly enhanced in the presence of

underwent TBI followed by allo-HCT with C57/BL6 BM alone ($n = 10$) or C57/BL6 BM plus T cells. Mice were treated with PBS ($n = 16$) or uricase starting from day −1 forward or from day +5 forward ($n = 10$ per group), as indicated. The experiment was performed twice and the resulting survival data were pooled. (H) On day 3 after allo-HCT, serum was isolated from BALB/c mice that received treatment with PBS or uricase from day 0 forward. Serum IL-1β levels were determined by ELISA. ***, $P = 0.0001$; **, $P < 0.01$; *, $P < 0.05$.

LPS-stimulated CD11c$^+$CD11b$^+$ DCs (Fig. 4 B), but not with LPS-stimulated CD11c$^-$CD11b$^+$ DCs. Yet, IL-17 production was completely abolished if Anakinra was added to the co-cultures containing CD11c$^+$CD11b$^+$ DCs, which is indicative of an IL-1β–dependent mechanism. A comparable reduction of Th17 commitment was observed when Anakinra was added to the same co-cultures between GMCSF-DCs and allogeneic splenic CD4 T cells (Fig. 4 C). Importantly,

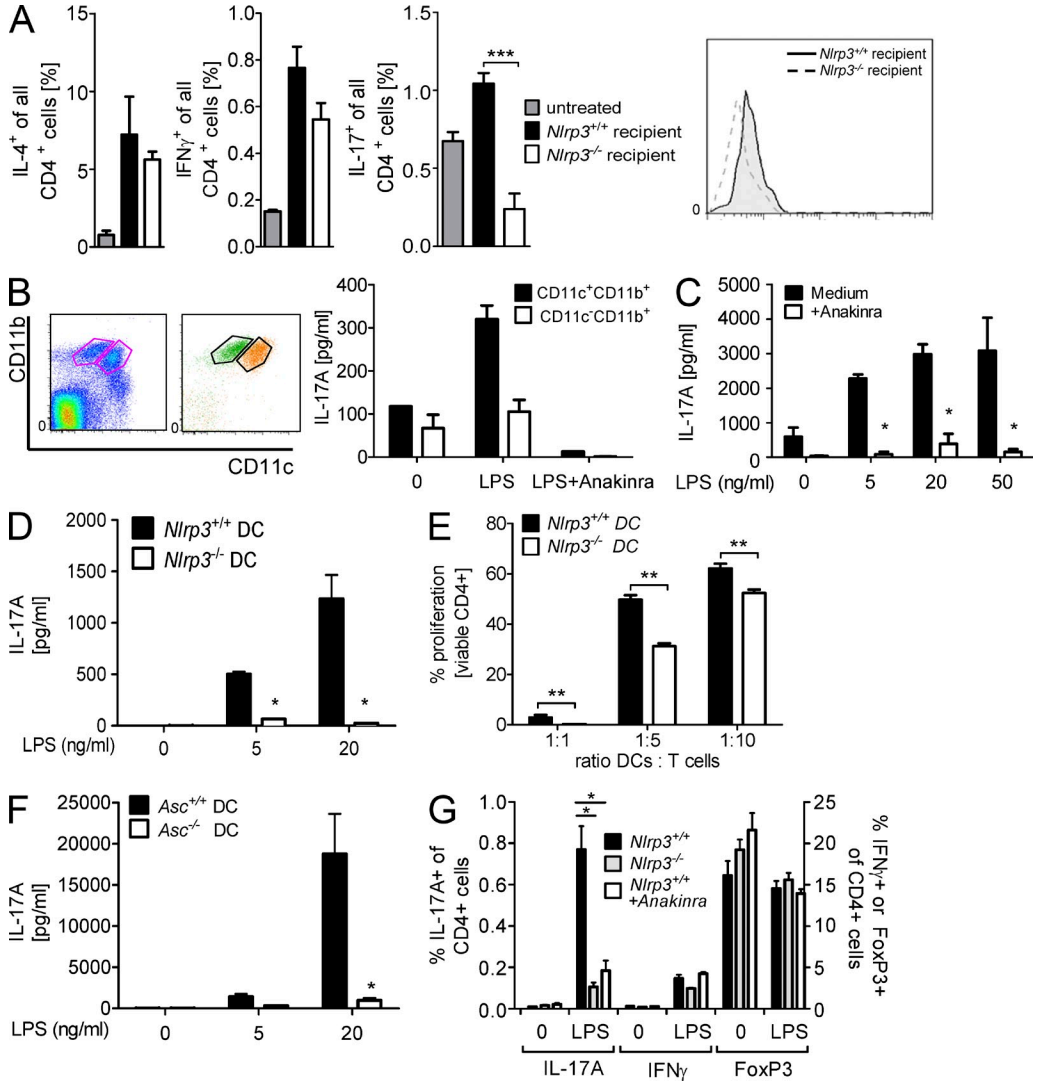

Figure 4. Nlrp3/Asc deficiency or IL-1β antagonism causes lower IL-17A production and alloreactive T cell expansion. (A) Splenocytes were isolated from untreated mice ($n = 4$) or *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ mice ($n = 6$ per group) receiving TBI+BM and T cells (BALB/c → C57BL/6 model) and analyzed for CD4$^+$ T cell cytokine production by intracellular FACS staining after PMA/Ionomycin/Brefeldin A restimulation (left). A representative histogram of IL-17A–stained cells is shown (right). The experiment was performed two times with similar results. (B) Intestinal LPL from C57BL/6 mice were sorted into CD11b$^+$CD11c$^-$ and CD11b$^+$CD11c^{mid} populations. Sorting strategy and overlaid post-sorting FACS analysis of resulting CD11b$^+$CD11c$^-$ (green) or CD11b$^+$CD11c^{mid} (orange) populations are shown on the left. Cells were stimulated with or without LPS (20 ng/ml) and Anakinra (10 µg/ml) and co-incubated with BALB/c splenic CD4$^+$ T cells. After 120 h, supernatants were analyzed for IL-17A. One representative of two independent experiments is shown. (C) BALB/c splenic CD4$^+$ T cells were co-incubated with C57/BL6 GMCSF-DCs that were preexposed to increasing LPS concentrations with or without Anakinra (10 µg/ml). After 120 h of co-culture, the supernatant was analyzed for IL-17A by ELISA. One representative of four independent experiments is shown. (D) BALB/c naive splenic CD4$^+$ T cells were incubated for 120 h with *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ GMCSF-DC that were preexposed to LPS at different concentrations and the supernatant was analyzed for IL-17A by ELISA. One representative of four independent experiments is shown. (E) CFSE-labeled BALB/c CD4$^+$ T cells were co-cultured with LPS preexposed DC from *Nlrp3*$^{+/+}$ or *Nlrp3*$^{-/-}$ mice at different ratios for 96 h. One representative of four independent experiments is shown. (F) BALB/c splenic CD4$^+$ T cells were incubated for 120 h with *Asc*$^{+/+}$ or *Asc*$^{-/-}$ GMCSF-DC that were preexposed to LPS at different concentrations and the supernatant was analyzed for IL-17A by ELISA. One representative of four independent experiments is shown. (G) T cells, as in D, were restimulated with PMA (1 µM), ionomycin (100 nM), and Brefeldin A after co-culture with unprimed or 20 ng/ml LPS-primed DCs (Nlrp3$^{+/+}$, Nlrp3$^{-/-}$, or Anakinra-treated Nlrp3$^{+/+}$) and analyzed by FACS for intracellular IL-17A, IFN-γ, and FoxP3. One representative of three independent experiments is shown. ***, $P < 0.0001$; **, $P < 0.001$; *, $P < 0.05$.

IL-6 and IFN-γ production in the GMCSF-DC co-cultures was not affected by addition of Anakinra, excluding toxic effects by the inhibitor (unpublished data). Next, we co-incubated LPS-stimulated DCs from *Nlrp3*$^{-/-}$ mice with allogeneic WT T cells. We found a significant reduction in IL-17 production and CD4$^+$ T cell expansion in the presence of *Nlrp3*$^{-/-}$ DCs (Fig. 4, D and E). Similarly, incubation of T cells with allogeneic *Asc*$^{-/-}$ DCs exposed to LPS resulted in lower IL-17A protein levels compared with those induced by WT DCs (Fig. 4 F). In contrast to the reduced number of CD4$^+$IL-17A$^+$ T cells, the numbers of CD4$^+$IFN-γ$^+$ T and T reg cells were not affected by *Nlrp3*–or *Asc*–deficiency in DCs (Fig. 4 G and not depicted). TNF and other major GvHD-related cytokines were not affected in Nlrp3-deficient recipients (unpublished data). Together, our results indicate that the Nlrp3 inflammasome regulates Th17 differentiation. These findings are compatible with data showing that IL-1R signaling is crucial for Th17 polarization of naive T cells in multiple diseases, including colitis models resembling intestinal GvHD (Coccia et al., 2012). However, we found that in addition to its requirement for Th17 induction, Nlrp3 in the APCs was also required for effective alloantigen-driven T cell proliferation, a hallmark of acute GvHD.

Cleaved caspase-1/IL-1β increase in human GvHD

Next, we evaluated inflammasome activation in a group of seven transplanted patients with and without GvHD. Cleaved caspase-1 was found in the peripheral blood leukocytes of patients with acute GvHD when stimulated with LPS, but were being almost absent in leukocytes of allo-HCT recipients without GvHD, despite stimulation (Fig. 5 A). The levels of cleaved caspase-1 were also higher in intestinal lesions

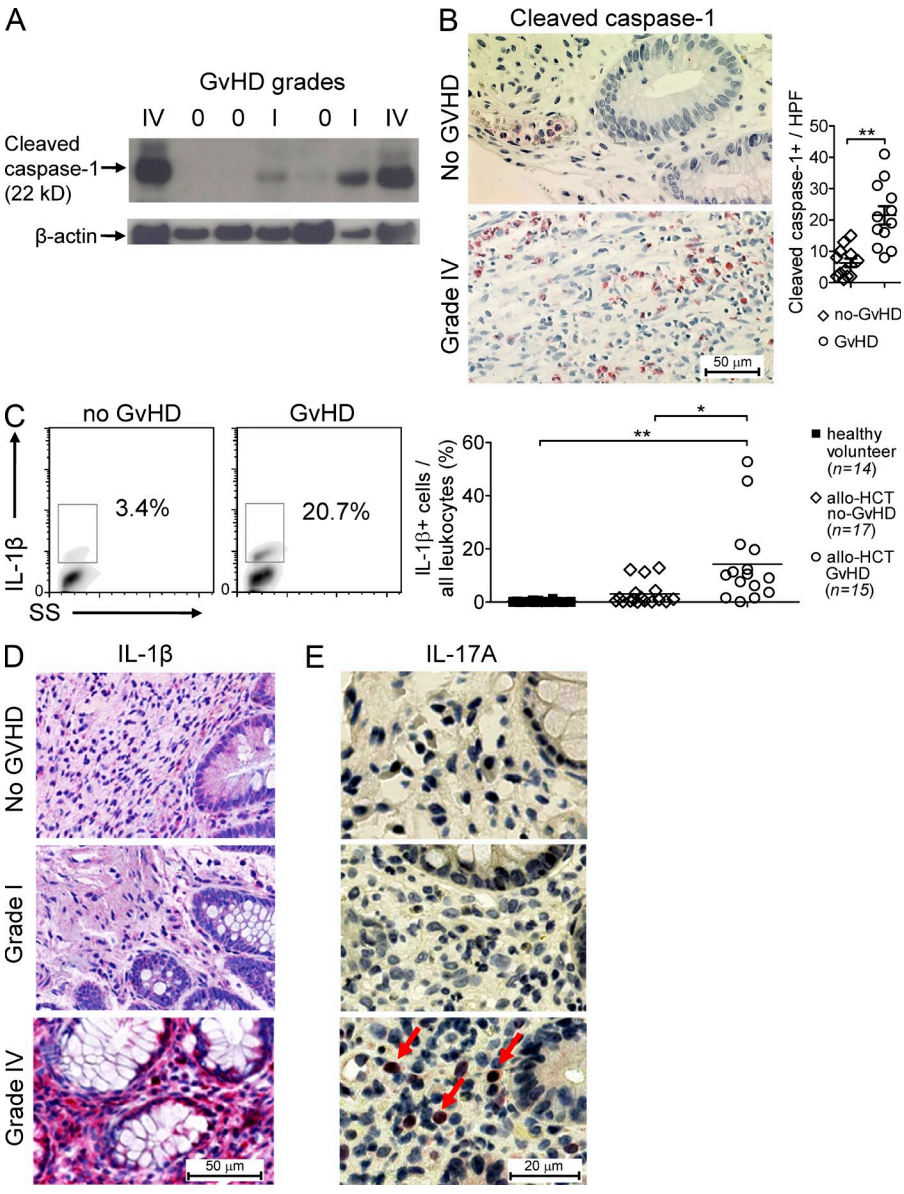

Figure 5. IL-1β and cleaved caspase-1 are found at high levels in human tissues of patients developing acute GvHD. (A) The presence of cleaved caspase-1 was determined in PBMCs from patients undergoing allo-HCT after stimulation with LPS (50 ng/ml) for 6 h. GvHD severity is indicated above the individual patient sample. (B) The amount of cleaved caspase-1 was determined by immunohistochemistry of intestinal biopsies derived from patients with or without GvHD. The cleaved caspase-1–positive cells were quantified by counting positive cells per high power field (HPF). (C) The amount of IL-1β was determined in freshly isolated PBMCs from healthy volunteers or patients undergoing allo-HCT and correlated with the incidence of acute GvHD. The left panel represents a representative flow cytometry plot; the diagram on the right shows the quantification for all individuals. Each data point represents an individual (n = 14–17 per group). (D) Immunohistochemical staining for IL-1β was performed on intestinal biopsies and correlated with the severity of acute GvHD. One representative sample is shown. (E) Immunohistochemical staining for IL-17A was performed on intestinal biopsies and correlated with the severity of acute GvHD. One representative sample is shown. **, $P < 0.005$; *, $P < 0.05$.

of GvHD patients compared with patients without GvHD (Fig. 5 B). Furthermore, we observed higher frequencies of IL-1β-producing leukocytes in the peripheral blood of patients with GvHD (Fig. 5 C), and enhanced IL-1β expression in biopsies of intestine (Fig. 5 D) and skin (not depicted) from patients with acute GvHD. Finally, IL-17A was detected in intestinal biopsies from patients with grade 4 GvHD (Fig. 5 E).

In summary, our report provides the first experimental evidence that the conditioning therapy before allo-HCT generates PAMPs and DAMPs, which can activate the Nlrp3 inflammasome during the early phase of the procedure and induce acute GvHD. We show that multiple intestinal cell subsets express pro–IL-1β after allo-HCT, underlining its broad function as a key proinflammatory cytokine. Furthermore, our data suggest that pro–IL-1β induction in the intestines of conditioned mice not only depends on signals of the enteric microflora, but also on a positive feedback loop regulated by IL-1β itself. Upon irradiation- or chemotherapy-induced tissue damage, enteric bacteria–derived PAMPs deliver the first signal needed for pro–IL-1β synthesis. The second signal, leading to inflammasome activation and subsequent secretion of bio-active IL-1β, is provided by uric acid released from damaged cells. Consistent with a very early conditioning-induced Nlrp3 inflammasome activation, depletion of IL-1β, blockade of IL-1R, or depletion of UA by uricase were significantly more efficient when started early (24 h before transplantation) rather than late (on day 5). The DAMP UA was also increased after chemotherapy-based conditioning and Nlrp3 inhibition improved survival in mice. Recent studies suggest a possible role for Th17 cells in the pathogenesis of acute GVHD (Kappel et al., 2009; Yu et al., 2011), but the innate signaling molecules driving this response were not defined. In this study, we identify an Nlrp3 inflammasome and IL-1β–dependent mechanism for allogeneic Th17 differentiation that may contribute to the development of acute GVHD. Collectively, our observations emphasize the role of a conditioning therapy–mediated insult to the intestinal mucosa which leads to PAMP/DAMP-mediated Nlrp3 inflammasome activation and IL-1β release, with subsequent intestinal priming of an allogeneic Th17 response that may contribute to systemic severe GvHD. Moreover, our findings are of major importance for the future design of prophylactic strategies targeting IL-1β. Our data suggest that directed therapeutics targeting the Nlrp3 inflammasome or depletion of the DAMP uric acid could reduce the consequences of intestinal damage not only in the setting of allo-HCT but also in patients suffering from infection or undergoing chemotherapy and radiotherapy.

MATERIALS AND METHODS

Human subjects. We collected all samples after approval by the ethics committee of the Albert-Ludwigs University (Freiburg, Germany; Protocol number: 267/11) and after written informed consent. Intestinal tissue biopsies, skin biopsies, and peripheral blood samples were collected in a prospective manner from individuals undergoing allo-HCT with or without GvHD. GvHD grading was performed on the basis of histopathology. Staining for IL-1β of the human biopsies was performed according to standard protocols. Human PBMCs were isolated by Ficoll density gradient separation and used for intracellular IL-1β staining or protein isolation and Western blot for cleaved caspase-1.

Mice. C57BL/6 (H-2K^b, Thy-1.2), BALB/c (H-2K^d, Thy-1.2), and C3H/He mice were purchased from Charles River or Harlan and from the local stock of the animal facility at Freiburg University Medical Center. Mice were used between 6 and 12 wk of age. Only gender-matched combinations were used for transplant experiments. All animal protocols were approved by the Committee on the Use and Care of Laboratory Animals at Freiburg University at Munich University and by the veterinary authorities of Zürich. IL-1R$^{-/-}$ mice were provided by M. Freudenberg (Max-Planck Institute for Immunobiology and Epigenetics, Freiburg, Germany). Nlrp3$^{-/-}$ and Asc$^{-/-}$ mice were provided by J. Tschopp (University of Lausanne, Lausanne, Switzerland). The floxed ASC$^{f/f}$ line was previously described (Drexler et al., 2012) and carried ASC flanked by two loxP sites (C57BL/6J background). ASC$^{f/f}$ mice were crossed with mice transgenic for K14-Cre orLysM-Cre, or with CD11c-Cre mice (Jackson ImmunoResearch Laboratories) on a C57BL/6 background.

BM-derived DC (BMDC) isolation. Single-cell suspensions from the BM of C57BL/6J or BALB/c mice were isolated, and DC maturation was achieved by addition of GM-CSF (10 ng/ml; R&D Systems) to the cultures for 7 d, as previously described (Reichardt et al., 2008).

BM transplantation model and histopathology scoring. Allogeneic BM transplants were performed as previously described (Zeiser et al., 2008). Recipients were given 5 × 10^6 BM cells after TBI with 9 Gy (BALB/c) or 10–11 Gy (C57BL/6). Where indicated, mice received BU (20 mg/kg/d from day 7 to 4; Busilvex; Pierre Fabre Pharma) and CY (100 mg/kg/d from day −3 to −2; Endoxan; Baxter Healthcare) i.p. as previously reported (Sadeghi et al., 2008). For reduced intensity conditioning, mice received fludarabine (200 mg/kg/d from day −8 to −4; Genzyme) and CY (60 mg/kg/d from day −3 to −2) i.p., as reported (Turner et al., 2008). Day of stem cell injection was assigned as day 0, days before stem cell injection are numbered backward. T cell doses (CD4/CD8 MACS enrichment; Miltenyi Biotech) varied depending on the transplant model C57BL/6 → BALB/c (3 × 10^5), BALB/c → C57BL/6 (3 × 10^5 or 10^5 or 10 × 10^5 when indicated) or C3H → C57BL/6 (2 × 10^6). Sections of small and large bowel samples collected on day 10 were stained with hematoxylin/eosin and scored by an experienced pathologist blinded to the treatment groups. GvHD was scored on the basis of a previously published histopathology scoring system (Kaplan et al., 2004; Wilhelm et al., 2010).

Generation of chimeric recipients with P2X7R deficiency of hematopoietic or nonhematopoietic tissues. WT/C57BL/6 recipients were given 5 × 10^6 WT or P2X$_7$R$^{-/-}$ BM cells (C57BL/6) intravenously after TBI with 9Gy (2 × 4.5Gy). The following donor/recipient pair was combined: P2X7R$^{-/-}$ → P2X7R$^{+/+}$ (hematopoietic system: P2X$_7$R$^{-/-}$). The second HCT (allogeneic; BALB/c → C57BL/6; T cell dose, 3 × 10^5) was performed at day 40 after the first HCT (syngeneic). To reconstitute chimeras with recipient type DCs, we infused 3 × 10^6 DCs intravenously per mouse on day 1 after TBI with 9 Gy (2 × 4.5 Gy). Where indicated, DCs had been exposed to media alone or media and alum (240 μg/ml, exposure for 2 h).

B cell lymphoma model. To investigate GvL activity of transferred donor T cells, we used luciferase transgenic A20 (A20^{luc}) B cell leukemia that had been previously demonstrated to migrate primarily to the BM, with secondary infiltration of the spleen and other lymphoid organs when injected intravenously at the time of BMT (Zeiser et al., 2006). Animals were injected with A20^{luc} cells (BALB/c background, 5 × 10^5) 2 d before administration of the T cells from C57BL/6 donors.

Peritoneal sample collection and UA measurement. A total of 3 ml of PBS (Invitrogen) was injected i.p. using an 18-gauge × 1.5 needle (B/Braun)

after the mouse had been sacrificed. Of this amount, 1 ml was recollected and immediately processed on ice for UA measurement. UA measurement was by QuantiChrom Uric Acid Assay kit (peak absorbance at 590 nm; BioAssay Systems) according to the manufacturer's instructions.

Drug treatment. Mice received Uricase (10 µg) in 100 µl of PBS i.p. on days 1, 0, 1, 4, and 8. Anakinra was given at a dose of 200 µg in 100 µl of PBS i.p. on day 1, 0, 1, 2, 4, 6, and 8. A monoclonal antagonistic anti–mouse IL-1β antibody was injected at a dose of 200 µg in 200 µl of PBS i.p. 24 h before TBI or 5 d after TBI. The mice were injected i.p. with 500 µg/kg glyburide (Nlrp3 inhibitor) or the formulation vehicle (DMSO) in 100 µl PBS on day 0, 1, 4, and 8. Etanercept (Enbrel, TNF inhibitor) was given at a dose of 1 mg/kg on days 5, 8, 11, 13, 15, 18, 21, and 24. For gastrointestinal decontamination, C57BL/6 mice were given 2.5 mg/ml neomycin or 1 mg/ml ciprofloxacin in drinking water 7 d before TBI.

Western blot. For human samples and murine splenic samples, cell pellets were resuspended using lysis buffer containing RIPA, protease (Santa Cruz Biotechnology, Inc.), and phosphatase (Sigma-Aldrich) inhibitor cocktail, and then incubated on ice (10 min) and centrifuged down (10,000 rpm, 4°C, 10 min). The supernatants were collected and stored at −20°C. Protein concentration was determined by BCA assay (Thermo Fisher Scientific) following the manufacturer's instructions.

Total cell protein was separated in 4–12% SDS-PAGE gels (Invitrogen), transferred on polyvinylidene fluoride (PVDF) membranes (GE Healthcare), and probed with specific antibodies. Immunoreactive polypeptides were detected by chemiluminescence using Electro-Chemi Luminescence reagents (GE Healthcare). For detection of cleaved caspase-1, we used an anti–cleaved caspase-1 rabbit mAb (D57A2; Cell Signaling Technology) for human samples or an anti-cleaved caspase-1 p10 rabbit mAb (sc-514; Santa Cruz Biotechnology, Inc.; 1:500) for murine samples. As secondary antibodies, we used HRP-conjugated anti–rabbit antibody (Cell Signaling Technology) for detection of the anti-cleaved caspase-1 antibodies (1:10,000) and HRP conjugated anti–mouse antibody for detection of the anti–β-actin Ab (1:2750). For murine tissue analysis, terminal ileum was isolated, cleaned of fecal content, and lysed in NP-40 buffer. After snap-freezing in liquid nitrogen and thawing, tissue lysates were analyzed by Western Blot using standard SDS PAGE protocols with polyclonal anti–IL-1β antibody (AF401; R&D Systems) and an ECL-based ChemoCam System (Intas).

qPCR of the intestines and skin for IL-1β and TNF. Total RNA was extracted from intestine and skin using QIAGEN RNA isolation kits according to the manufacturer's instructions. Quantitative RT-PCR was performed with SYBR-green on an ABI prism 7700 sequence detection system (Applied Biosystems). For each gene, the results were normalized to ribosomal protein L27 (RPL27) and given as $2^{-\Delta\Delta Ct}$ values normalized to intestine and skin from control animals. The results are represented as mean ± SD of three independent experiments. The detailed information about oligonucleotide primers and conditions used are available upon request.

Flow cytometry. The following antibodies were used for flow cytometry analysis: anti-CD11c (Pacific blue, clone, N418; BioLegend), anti-CD3e (FITC, clone, 145-2C11; eBioscience), anti-CD4 (Pacific blue, clone, RM4-5; BioLegend; PE-Cy5, clone, GK1.5; eBioscience), anti-CD8 (FITC, clone, 53–6.7; BD), anti–IL-1R (Alexa Fluor 647, clone, JAMA147; ABD Serotec), anti-CD62L (clone, MEL-14; BioLegend), anti-TCRβ (FITC, clone, H97-597; eBioscience). For intracellular FoxP3-staining of splenocytes and for staining of co-cultured allogeneic T cells, FoxP3 staining kit (eBioscience) was used and cells were stained with anti-FoxP3 (clone FJK-16s; BD), IFN-γ (clone XMG1.2; eBioscience), anti-IL17A (PE-Cy7, clone eBio17B7; eBioscience; Brilliant Violet 421, clone TC11-18H10; BioLegend), and anti-IL-1β (fluorescein, clone, 166931). For intracellular cytokine staining of splenocytes, Cytofix/Cytoperm kit (BD) was used and cells were stained with anti-IL17A (clone TC11-18H10; BD), anti–IL-4 antibody (clone 11B11; eBioscience), or IFN-γ (clone XMG1.2, eBioscience). Annexin V (BD) and propidium iodide (eBioscience) were used to differentiate between viable, necrotic, and apoptotic cells. The following definitions were used to analyze T cell differentiation: T_{EM}, $CD62L^{low}/CD44^{+}$; T_{CM}, $CD62L^{+}/CD44^{+}$; T_{N}, $CD62L^{+}/CD44^{-}$. Data were acquired with a CyanADP (Beckman Coulter) or a FACSCanto II (BD), and then analyzed with FlowJo 7/8 software (Tree Star).

Immune reconstitution. To analyze the kinetics of immune reconstitution, BM-only recipient mice were sacrificed on days 40 and 70 after allo-HCT, and the spleens were stained for the donor marker and the respective lineage marker.

T cell proliferation/polarization assay. For analysis of T cell proliferation in vitro, $CD4^{+}$ T cells from BALB/c mice were purified by positive selection with anti-CD4 magnetic microbeads (Miltenyi Biotec), labeled with 1 µM CFSE (Molecular Probes) as previously described (Zeiser et al., 2007) and exposed to LPS-exposed allogeneic BMDCs from C57BL/6 mice for 96 h. For cytokine production assays, $CD4^{+}$ T cells were isolated by negative selection and with subsequent CD62L-positive selection (both from Miltenyi Biotec) as indicated and co-cultured with GMCSF-DCs preexposed to LPS (20 ng/ml) or control for 120 h.

IL-1β, IL-6, IFN-γ, and IL-17A ELISA. A mouse IL-1β ELISA kit (OptEIA; BD) was used to determine the serum levels in mice according to the manufacturer's instructions from 50 µl of serum. Mouse IL-17A (eBioscience), IL-6 (BD), and IFN-γ (BD) ELISA kits were used to determine the cytokine levels in the co-culture supernatants.

Serum cytokine measurements by bead array. The levels of IFN-γ, TNF, IL-6, IL-10, IL-12p70, and MCP-1 were analyzed from serum of recipient mice on day 7 after allo-HSCT with the CBA Inflammation kit (BD).

Conventional and immunofluorescence microscopy. Tissues were embedded in OCT and cryopreserved at −80°C. 5-µm-thick fresh frozen sections were mounted on positively charged, precleaned microscope slides (Superfrost/Plus; Thermo Fisher Scientific) and stored at −80°C. For morphological assessment, tissues were stained with hematoxylin/eosin according to standard protocols. Evaluation of the stained tissue sections was performed on an Axioplan 2 (Carl Zeiss) microscope. Standard objectives used were 20×/numerical aperture 0.45 and 40×/numerical aperture 0.60. Microscopic photos were obtained using a Spot digital camera.

Reagents. Anakinra (Kineret; Amgen) was diluted with PBS and used at a concentration of 10 µg/ml in vitro. Uricase (MP Biomedical), 10 µg/100 µl of PBS (Invitrogen), and 5 mg of glibenclamide (InvivoGen) was dissolved in 200 ml of DMSO and then diluted with PBS. LPS was obtained from InvivoGen (Ultrapure LPS-EK from *E. coli* K12). The blocking anti–mouse IL-1β monoclonal antibody was provided by H. Gram and T. Jung (Novartis).

Isolation of LP lymphocytes (LPLs). Mice were sacrificed and intestines were flushed with $PBS^{-Ca/-Mg}$ after dissection of fat and mesenteric tissue and excision of Peyer's patches. 1-cm pieces of longitudinally opened and $PBS^{-Ca/-Mg}$-washed intestine were then incubated with HBSS containing 2 mM EDTA, 10 mM Hepes, 5% FCS, 1% P/S, and 1 mM DTT. After 15 min of incubation on a shaker at 37°C, intraepithelial lymphocytes and epithelium were separated via 100-µm filters. EDTA incubation and filter separation was repeated once before intestine pieces were washed with $PBS^{-Ca/-Mg}$ and incubated in $PBS^{+Ca/+Mg}$ with FCS (5%), Collagenase II (300 u/ml; Gibco), and DNase I (0.1 mg/ml; Roche) on a shaker at 37°C. Cells in suspension were then purified on a 40/80% Percoll gradient (Biochrom). For FACS sorting, cells were stained for CD11c (APC, clone N418; eBioscience) and CD11b (APC-eFluor780, clone M1/70; eBioscience) and sorted on a BD FACSAria device. DC subsets were then plated in complete RPMI in 96-well plates. For intracellular IL-1β staining, cells were fixed as described above and stained with anti–pro-IL1β (eBioscience; clone NJTEN3).

Statistical analysis. Data were analyzed using the two tailed Student's *t* test of the arithmetic mean. Differences in animal survival (Kaplan-Meier survival curves) were analyzed by log-rank test. A p-value <0.05 was considered statistically significant.

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