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A TLR5 Agonist Enhances CD8+ T Cell-Mediated Graft-versus-Tumor Effect without Exacerbating Graft-versus-Host Disease

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Allogeneic hematopoietic cell transplantation is an established treatment for hematologic and nonhematologic malignancies. Donor-derived immune cells can identify and attack host tumor cells, producing a graft-versus-tumor (GVT) effect that is crucial to the effectiveness of the transplantation therapy. CBLB502 is a novel agonist for TLR5 derived from Salmonella flagellin. On the basis of the TLR5-mediated immunomodulatory function, we examined the effect of CBLB502 on GVT activity. Using two tumor models that do not express TLR5, and thereby do not directly respond to CBLB502, we found that CBLB502 treatment significantly enhanced allogeneic CD8+ T cell-mediated GVT activity, which was evidenced by decreased tumor burden and improved host survival. Importantly, histopathologic analyses showed that CBLB502 treatment did not exacerbate the moderate graft-versus-host disease condition caused by the allogeneic CD8+ T cells. Moreover, mechanistic analyses showed that CBLB502 stimulates CD8+ T cell proliferation and enhances their tumor killing activity mainly indirectly through a mechanism that involves the IL-12 signaling pathway and the CD11c+ and CD11b+ populations in the bone marrow. This study demonstrates a new beneficial effect of CBLB502, and suggests that TLR5-mediated immune modulation may be a promising approach to improve GVT immunity without exacerbating graft-versus-host disease. The Journal of Immunology, 2012, 189: 4719–4727.

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allogeneic bone marrow transplantation (allo-BMT) is an established treatment for leukemia, lymphoma, and other hematologic and nonhematologic malignancies (1). Donor-derived immune cells were found to be able to identify and attack tumor cells in the host, producing a graft-versus-tumor (GVT) effect (2). Initially, GVT effect was regarded as a beneficial by-product of graft-versus-host disease (GVHD) (3). It has since been recognized as a unique immune response that is critical for the therapy of hematologic and other malignancies (4, 5). Multiple cell types such as NK cells, CD4+, and CD8+ T cells have been implicated in the GVT mechanisms (6–8). Among them, CD8+ T cells were shown to be a major contributor for GVT activity because of the strong alloantigen specificity (9, 10).

Therefore, it remains critical to modulate CD8+ T cell response in allo-BMT to enhance GVT activity whereas limiting GVHD. TLRs can recognize pathogen-associated molecular patterns of bacterial or viral origin. TLR-mediated recognition of “danger signals” allows the immune system to rapidly respond to pathogens via innate immune cells and subsequently may activate adaptive immune responses (11). So far, 13 members of the TLR family have been identified in mice (12). Because of their immune-modulating function, various TLR ligands have been investigated as adjuvants to induce favorable immune responses (13–15). Flagellin, the structural component of bacterial flagellum, is a natural ligand for TLR5 (16). TLR5 binding by flagellin was shown to convert tolerogenic dendritic cells (DCs) into activating APCs that preferentially induce Th1 responses (17). Flagellin was also shown to promote Th2 and humoral immune response through direct stimulation of TLR5-expressing CD11c+ cells (18–20). However, there have been conflicting reports regarding the ability of flagellin to promote CD8+ T cell response (21–25). It was shown that flagellin was not able to stimulate CD8+ T cells when administered separately from Ag (24). In contrast, flagellin was able to promote CD8+ T cell response when fused with OVA protein, SIINFEKL peptide, or viral Ags (21–25). In addition, flagellin has been reported to modulate antitumor immune responses (26). However, studies with tumor models have yielded conflicting results with regard to whether flagellin actually promotes or suppresses tumor growth (26–28).

Recently, a pharmacologically optimized form of flagellin, CBLB502, was generated and shown to be as efficacious as flagellin in inducing NF-kB activation. CBLB502 demonstrated powerful radioprotection to lethally irradiated hosts in both mouse and monkey models (29). Because of its lower toxicity and lower immunogenicity, CBLB502 emerges as a more attractive TLR5 agonist and is currently undergoing phase I clinical trial. Mean-
while, preclinical studies with mouse models have shown that CBLB502 can inhibit acute renal ischemic failure and can protect mice from dermatitis caused by local cancer radiotherapy (30, 31). These reports support a notion that CBLB502 may be an effective agent in modulating beneficial immune responses. Therefore, the goal of this study is to test the hypothesis that CBLB502 can improve CD8⁺ T cell response in the context of allo-BMT and whether it affects the balance between GVAM and GVHD.

Materials and Methods

Mice and cells

C57BL/6 (H-2b) mice were purchased from The Jackson Laboratory. BALB/c (H-2d) and DBA/2 (H-2b) mice were purchased from National Cancer Institute. A20 lymphoma, derived from BALB/c strain, and P815 mastocytoma cells, derived from DBA/2 strain, were transduced to express luciferase as described previously (32). SCCVII, derived from C3H/HEN (H-2b) mice, is a squamous cell carcinoma tumor line that is known to express TLR5 (31). All mice were maintained in specific pathogen-free housing, and all experiments were conducted in accordance with the animal care guidelines for Roswell Park Cancer Institute, using protocols approved by the animal studies committee.

Reagents and Abs

CBLB502 was derived from flagellin as described previously (29). L-glutamin was used as the luciferase substrate for bioluminescence imaging to determine the tumor burden in vivo. Kits or microarrays used for the isolation of CD8⁺ T cells (Pan T cell kit), T cell-depleted bone marrow (TCD-BM) cell (CD90.2 beads), CD11b⁺ cell (CD11b⁺ beads), and CD11c⁺ cells (CD11c⁺ beads) were purchased from Miltenyi Biotec. Flow cytometry Abs, including anti-CD8 (53.67), anti-CD3 (145-2C11), anti-CD4 (RM4-4), anti-CD4 (RM4-5), H-2Kb (A6F-88.5.5.3), anti-CD11b (M1/70), and anti-CD11c (N418), were purchased from eBioscience.

RT-PCR

For analysis of TLR5 mRNA expression, total RNA from A20, P815, and SCCVII were extracted using TRizol reagent according to manufacturer’s instructions (Invitrogen, Carlsbad, CA). To eliminate any eventual contamination with genomic DNA, we treated isolated RNAs (1 μg) with DNaseI (Invitrogen, Carlsbad, CA). cDNAs were synthesized using SuperscriptTM II Reverse Transcriptase and oligo(dT)12-18 primer (Invitrogen), according to manufacturer’s protocol. A total of 40 ng cDNA was amplified by PCR using primer specific for TLR5 (GenBank accession number NM_016928.2) gene (forward, 5′-AGTCCCCCAGCTCCAGTTTC-3′; reverse, 5′-GGGCCCCCTACGTTGGTG-3′). The RT-PCR conditions were 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 35°C for 20 s, and 72°C for 1 min, and 1 cycle of 72°C for 10 min (for final extension). Primer specificity for GAPDH (forward, 5′-ACCAAGTCCTAG CCATCAC-3′; reverse, 5′-TCCACACCCATGTTGGCTA-3′) was used as a control. The RT-PCR conditions for GAPDH were 95°C for 2 min, followed by 20 cycles of 95°C for 30 s, 50°C for 20 s, and 72°C for 1 min, and 1 cycle of 72°C for 10 min (for final extension).

Preparation of CD8⁺ T cells

CD8⁺ T cells (purity 85–90%) were purified with autoMACS from the spleens by using Pan T isolation kit II combined with biotin-conjugated anti-CD4 according to the protocols (Miltenyi). TCD-BM cells were prepared by using anti-CD90.2 microbeads. For CD11c⁺ bone marrow (BM) cells, positive selection was applied to total BM cells with CD11c microbeads (Miltenyi) (purity ∼80%). For CD11b⁺ BM cells, positive selection was applied to total BM cells with CD11b microbeads (Miltenyi) (purity > 99%).

GVMT model

BALB/c (H-2d) host mice were irradiated with 9 Gy from [137Cs] sources at Roswell Park Cancer Institute. DBA/2 (H-2b) host mice were irradiated with 14 Gy by two separate doses with 24-h interval. Two days (for BALB/c mice) or 1 d (for DBA/2 mice) after the last irradiation, all host mice were inoculated with 1.0 × 10⁶ A20 tumor cells or 1.0 × 10⁵ P815 tumor cells i.v. followed by another i.v. injection of 2.0 × 10⁶ TCD-BM cells only or combined with 0.3 × 10⁶ CD8⁺ T cells isolated from C57BL/6 (H-2Kb) mice. CBLB502 was given s.c. by 1 μg/dose per mouse at days 1, 3, 5, 7, and 9 after allo-BMT. Bioluminescence imaging was performed to monitor tumour burden as described previously (32–34). Tumor burden was expressed as photon flux (photons/s).

MLR

A standard one-way MLR was performed in 1 ml media on a 48-well plate with mixing total splenocytes (1.0 × 10⁶) derived from C57BL/6 mice (responders) with irradiated (100 Gy) BALB/c or DBA/2 splenocytes (1.0 × 10⁶) as stimulators. For a modified one-way MLR, 0.2 × 10⁶ CD8⁺ T cells, purified from C57BL/6 mice, were cultured with 1.0 × 10⁶ irradiated BALB/c or DBA/2 splenocytes (stimulators) in the presence or absence of 0.5 × 10⁶ C57BL/6-derived TCD-BM cells. For in vitro tumor burden assay, tumor cells were added after 4 d of MLR and then cocultured for another 12 h before bioluminescence imaging.

CFSE-based proliferation assay

Purified CD8⁺ T cells (1.0 × 10⁶/ml) were stained with CFSE at a final concentration of 0.3 μg/ml for 5 min at 37°C. A total of 0.2 × 10⁶ CFSE-labeled CD8⁺ T cells were plated as MLR responders in 1 ml media on 48-well plate with or without 0.5 × 10⁶ TCD-BM cells in the presence of irradiated BALB/c splenocytes treated with either CBLB502 or PBS. After culturing for 3 or 5 d, cells were harvested and stained with mouse H-2Kb and CD8 Abs. CFSE dilution on the 7-aminoactinomycin D (7-AAD) H-2Kb⁺CD8⁺ cells was analyzed with FCS express software (De Novo Software).

Histopathologic analysis of GVHD target organs

Mice were euthanized on day 31 after allo-BMT. Large and small intestines were removed, formalin fixed, sectioned, and stained with H&E. Intestine tissues were examined for the presence of crypt epithelial cell apoptosis, crypt loss, surface colonocyte vacuolization, surface colonocyte attenuation, lamina propria inflammatory cell infiltrate, mucosal ulceration, luminal sloughing of cellular debris, leukocyte infiltration of the lamina propria, and villous blunting. A previously established semiquantitative scoring system was used to assess abnormalities associated with GVHD (35). The scoring system is designated 0 as normal, 0.5 as focal and rare, 1.0 as focal and mild, 2.0 as diffuse and mild, 3.0 as diffuse and moderate, and 4.0 as diffuse and severe.

Results

CBLB502 enhances CD8⁺ T cell-mediated GVMT effect

This study is designed to determine the effect of CBLB502 on GVMT activity that is mediated by donor-derived immune cells. Previously, CBLB502 demonstrated potent radioprotection effect to lethally irradiated animals if given before or immediately after irradiation exposure (29). CBLB502 did not show detectable radioprotection effect if administered 2 d after lethal irradiation. To exclude any radioprotection effect that may interfere with the GVMT activity, we established a 2-d delayed allo-BMT model. The BALB/c (H-2d) host mice were lethally irradiated (9 Gy) 2 d before being transplanted with TCD-BM cells derived from the C57BL/6 (H-2b) donor mice. In addition, to exclude any direct response to CBLB502 by tumor cells, we have used two tumor models that do not express TLR5 and therefore do not directly respond to CBLB502 (Fig. IA). Specifically for the A20 lymphoma model, the BALB/c (H-2d) host mice were lethally irradiated 2 d before an i.v. inoculation with 1.0 × 10⁶ A20 cells, followed by an i.v. injection of 2.0 × 10⁶ TCD-BM cells only or combined with 0.3 × 10⁶ CD8⁺ T cells purified from C57BL/6 donors. These mice were then treated with CBLB502 (1.0 μg in 0.1 ml PBS per mouse) or PBS via s.c. route for five times at 1, 3, 5, 7, and 9 after allo-BMT. To determine the effect of CBLB502 on GVMT activity, we performed bioluminescence imaging to measure tumor burden. Compared with PBS control, CBLB502 treatment showed no significant effect on tumor burden in the hosts receiving TCD-BM only (Fig. IB), suggesting that CBLB502 did not affect tumor growth in the absence of donor T cells. Transplant of CD8⁺ T cells in addition to TCD-BM significantly reduced tumor burden (p < 0.0001), indicating that donor CD8⁺
T cells are responsible for tumor eradication. Notably, CBLB502 treatment significantly enhanced the ability of donor CD8+ T cells to control tumor growth (p = 0.009). To further examine the impact of transplant and CBLB502 treatment on the tumor-bearing mice, we monitored survival (Fig. 1C). As expected, all of the hosts receiving TCD-BM only died of tumor outgrowth, and CBLB502 treatment had no significant effect on their survival. Transplant of CD8+ T cells significantly prolonged host survival (p < 0.0001) but failed to rescue the hosts from death caused by tumor growth. CBLB502 treatment combined with CD8+ T cell transplant rescued 60% (4/7) of the tumor-bearing hosts (p = 0.0219), which were found tumor free at day 60 and gained >10% of weight compared with their initial body weight. To rule out a tumor model-specific or host strain-specific effect, we inoculated DBA/2 (H-2d) mice with the syngeneic P815 mastocytoma cells and performed similar transplantation with C57BL/6 donor mice. CBLB502 treatment and tumor burden measurement were performed as described earlier. Representative data from one of two independent experiments are shown as mean ± SD, with three to four mice in each group. The p value was acquired by two-way ANOVA analysis.

FIGURE 1. CBLB502 treatment enhances CD8+ T cell-mediated GVT effect, reduces tumor burden, and improves host survival. (A) TLR5 mRNA expression was not detected in A20 and P815 tumor cells. SCCVII tumor cell line, expressing TLR5, was used as a positive control. GAPDH mRNA expression is used as an internal loading control. (B) Two days after lethal irradiation, BALB/c mice were i.v. inoculated with 1.0 × 10^6 luciferase-expressing A20 tumor cells and then transplanted with 2.0 × 10^6 TCD-BM cells alone or combined with 0.3 × 10^6 CD8+ T cells harvested from C57BL/6 donor mice. These mice were then treated with CBLB502 (1.0 μg in 0.1 ml PBS/mouse) or PBS via s.c. route for five times at 1, 3, 5, 7, and 9 d after allo-BMT. Tumor burden was measured by bioluminescence imaging. Representative data from one of three independent experiments are shown as mean ± SD, with three to four mice in each group. The p value was acquired by two-way ANOVA analysis. (C) Kaplan–Meier survival curves of the BALB/c hosts are summarized from two independent experiments as described in (B). (D) One day after receiving total 14 Gy irradiation split into two separate doses with 24-h interval, DBA/2 mice were inoculated with 1.0 × 10^6 luciferase-expressing P815 tumor cells and then transplanted with 2.0 × 10^6 TCD-BM cells alone or combined with 0.3 × 10^6 CD8+ T cells harvested from C57BL/6 donor mice. CBLB502 treatment and tumor burden measurement were performed as described earlier. Representative data from one of two independent experiments are shown as mean ± SD, with three to four mice in each group. The p value was acquired by two-way ANOVA analysis.

CBLB502 enhances allogeneic CD8+ T cell-mediated tumor killing in vitro and in vivo

To recapitulate the in vivo GVT effect and to test what cellular compartments are responding to CBLB502, we moved onto a
modified MLR system (36). We used $1.0 \times 10^6$ irradiated BALB/c (H-2b) splenocytes to stimulate $0.2 \times 10^6$ CD8+ responder T cells in the absence or presence of $0.5 \times 10^6$ TCD-BM cells purified from C57BL/6 mice (H-2b). After 4 d of culture, $0.2 \times 10^6$ A20 tumor cells were added as target and incubated for another 12 h. Tumor burden was then measured by bioluminescence imaging. In the absence of TCD-BM cells, CD8+ T cells did not show significant killing activity (Fig. 2A). CBLB502 treatment appeared to slightly increase the killing activity of CD8+ T cells ($p = 0.0135$). However, this system might not be stringent enough to conclude whether CBLB502 directly stimulate CD8+ T cells, because the purity of the isolated CD8+ T cells is typically 85–90% and contamination by other cell types (Supplemental Fig. 1) could mediate an indirect activation. Nevertheless, when TCD-BM cells were added in the MLR culture, the killing activity of CD8+ T cells was significantly increased ($p = 0.0046$), probably because BM-derived APCs augmented CD8+ T cell activation in this system. Moreover, CBLB502 treatment in the presence of TCD-BM further enhanced the killing activity of CD8+ T cells ($p = 0.0101$). Finally, it is important to emphasize that the presence of TCD-BM cells caused the highest stimulation effect of CBLB502 on the killing activity CD8+ T cells ($p < 0.0001$), suggesting that BM-derived APCs may be the principal responder to CBLB502. We also performed equivalent experiments with the P815 tumor model and observed similar results (Fig. 2B). Together, these results demonstrate that, in this modified MLR system, CBLB502 treatment significantly enhances the tumor-killing activity of allogeneic CD8+ T cells, and that some BM-derived cells, probably APCs, can respond to CBLB502 and mediate this stimulation effect.

In addition, to examine whether CBLB502 enhances the in vivo cytotoxic activity of donor CD8+ T cells, we transplanted C57BL/6-derived TCD-BM and CD8+ T cells into BALB/c hosts. These mice were then treated with CBLB502 or PBS five times as described earlier. At day 10 after allo-BMT, we harvested the spleen cells from the hosts. These in vivo activated cells were then coincubated in 96-well plates with luciferase-expressing A20 tumor cells for 20 h. Tumor burden was then measured by bioluminescence imaging. As shown in Fig. 2C, spleen cells harvested from BM-only recipients showed no tumor-killing activity because their tumor burden was the same as the control wells seeded with only tumor cells. In contrast, spleen cells harvested from the hosts receiving both BM and CD8+ T cells showed significant tumor-killing activity. CBLB502 treatment clearly enhanced the tumor-killing activity of these CD8+ T cells (Fig. 2C).

CBLB502 enhances the proliferation of allogeneic CD8+ T cells in vitro and in vivo

Flagellin was previously reported to enhance CD8+ T cell response only when it is fused with a peptide or protein Ag (22, 24). To investigate whether CBLB502 can promote CD8+ T cell response in this allo-BMT model, we used a CFSE dilution assay to measure CD8+ T cell proliferation in the modified MLR system.

**FIGURE 2.** CBLB502 treatment enhances CD8+ T cell-mediated tumor-killing activity in vitro and in vivo. (A and B) A modified one-way MLR was set up in 1 ml culture in triplicate on 48-well plate. Purified CD8+ T cells (0.2 $\times$ 10⁶; responders), derived from C57BL/6 mice, were cultured with irradiated BALB/c or DBA/2 splenocytes (1.0 $\times$ 10⁶; stimulators) in the presence or absence of C57BL/6-derived TCD-BM cells (0.5 $\times$ 10⁶) and treated by CBLB502 (100 ng) or PBS. Four days later, A20 (0.2 $\times$ 10⁶) (A) or P815 (0.1 $\times$ 10⁶) (B) target tumor cells were added and incubated for another 12 h. Bioluminescence imaging was performed to measure tumor burden in each well. Triplicate wells cultured with irradiated stimulators alone without adding TCD-BM or CD8+ T cells were used as the control. All tumor burden data were normalized relative to the average tumor burden in the control wells. Data were representative of three independent experiments. (C) Allo-BMT and CBLB502 treatment were performed as described in Fig. 1B. At day 10 after allo-BMT, spleen cells were harvested from the host. A total of $1 \times 10^6$ spleen cells from each host were coincubated with $2.5 \times 10^4$ luciferase-expressing A20 tumor cells in 96-well plates with triplicate samples for 20 h. Tumor burden was then measured by bioluminescence imaging. All tumor burden data were normalized relative to the average tumor burden in the control wells cultured with $2.5 \times 10^4$ A20 tumor cells alone. Data were representative of two independent experiments. The $p$ value was acquired by Student $t$ test.
brief, purified CD8+ T cells were labeled with CFSE and added into the MLR culture as outlined earlier. After 3 or 5 d, the cultured cells were harvested and stained with Abs for cell-surface markers. As a marker for active proliferation, CFSE dilution on the 7-AAD-H-2Kb+CD8+ T cells was analyzed by flow cytometry. Only slight CD8+ T cell proliferation was observed at day 3, whereas significant proliferation was observed at day 5 in the MLR (Fig. 3A). CBLB502 enhanced CD8+ T cell proliferation, and so did the presence of TCD-BM cells. Notably, CBLB502 treatment in the presence of TCD-BM cells promoted CD8+ T cell proliferation to the maximum level, correlated with the highest killing activity of CD8+ T cells under this condition. These data clearly indicate that CBLB502 can augment CD8+ T cell proliferation in vitro through BM-derived cells.

In addition, to examine whether CBLB502 can augment CD8+ T cell proliferation in vivo, we transplanted C57BL/6-derived TCD-BM and CD8+ T cells into BALB/c hosts inoculated with A20 tumor cells. These mice were then treated with CBLB502 or PBS for five times as described earlier. At day 9 after allo-BMT, we harvested the spleen cells from the hosts and used flow cytometry to analyze these cells. Although we transplanted only 0.3 \times 10^6 CD8+ T cells into each host, we harvested an average of nearly 3 \times 10^6 H-2Kb+CD8+ donor T cells from each host spleen, indicating that the CD8+ donor T cells underwent significant expansion (Fig. 3B). Notably, CBLB502 treatment increased not only the frequency of CD8+ donor T cells, but also the total cell number in the spleen. Overall, CBLB502 treatment resulted in an expansion of donor CD8+ T cells that was \times 2-fold that in the PBS-treated hosts.

CBLB502 increases CD8+ T cell response through CD11c+ and CD11b+ BM cells

Previous studies using RT-PCR and a polyclonal Ab suggested that TLR5 may be expressed on many cell types, including DCs, macrophages, and epithelial cells (37, 38). However, because of the lack of a reliable mAb, the cellular expression pattern of TLR5 remains poorly defined. To explore which cell type in the BM...
responds to CBLB502 and stimulates CD8+ T cell response in our model, we first tested whether DC could mediate the CBLB502 effect. CD11c+ cells were purified from fresh BM by positive selection (purity ~80%). We used 0.1 × 10^6 CD11c+ cells to replace the 0.5 × 10^6 TCD-BM cells in the modified MLR system. Although CD11c+ cells alone did not show any direct tumor-killing activity (Fig. 4A), they exhibited a T cell activation effect similar to that of TCD-BM cells. In response to CBLB502 treatment, CD11c+ cells were able to significantly enhance the killing activity of CD8+ T cells. Because CD11b+ population is the most frequent myeloid population (39), we tested whether CD11b+ cells could also mediate the effect seen with CBLB502 treatment. We purified CD11b+ cells from BM (purity > 99%) and added them into the modified MLR culture. As shown in Fig. 4B, CD11b+ cells alone did not show any killing activity but displayed a T cell activation effect similar to that of CD11c+ cells. In response to CBLB502 treatment, CD11b+ cells were also capable of enhancing the killing activity of CD8+ T cells. Together, these results indicate that both CD11c+ and CD11b+ cells in the BM can respond to CBLB502 and stimulate CD8+ T cell function in this allo-BMT model.

CBLB502 promotes allogeneic CD8+ T cell response through IL-12 signaling pathway

TLR5 binding initiates a signal transduction cascade that leads to the activation of NF-κB, which subsequently promotes the expression of a variety of inflammatory cytokines (40–42). Among them, IL-12 is known to be produced by DCs and macrophages, and to play a critical role for T cell activation (43). To examine the role of the IL-12 signaling pathway in CBLB502-mediated activation of T cell function, we used two MLR systems. First, in a standard one-way MLR, we used total splenocytes harvested from C57BL/6 WT and IL-12Rβ2-/- mice as stimulators. The MLR cultures were treated with CBLB502 (100 ng) or PBS in the beginning. After 4 d of MLR, A20 or P815 tumor cells were added as targets and cocultured for another 12 h, followed by bioluminescence imaging to measure tumor burden. Compared with the stimulator-only control in which no responder splenocytes were added, both WT and IL-12Rβ2-/- responders exhibited significant tumor-killing activity. CBLB502 treatment of the MLR significantly enhanced the tumor-killing activity of WT responders, whereas IL-12Rβ2-/- responders showed a significantly decreased response to CBLB502 treatment (p < 0.01). To mimic the in vivo allo-BMT model, we used purified TCD-BM cells and CD8+ T cells as responders to perform a modified MLR as described earlier, which yielded very similar results (Fig. 5B). Taken together, these results demonstrate that the IL-12 signaling pathway is involved in CBLB502-mediated stimulation of allogeneic T cell function.

CBLB502 does not exacerbate GVHD in the tumor-bearing mice

We have shown earlier that CBLB502 treatment enhances GVT effect in the allo-BMT model. To explore any potential effect on GVHD in the tumor-bearing mice, we collected tissue samples at 31 d after transplantation, a critical period when some of the host mice started to die of tumor growth. We performed histopathologic analyses of the skin, liver, and small and large intestines. No evidence of GVHD was observed in the skin. The liver samples did not yield conclusive results because of various amounts of tumor cell infiltrates making it difficult to distinguish GVHD damage from tumor-induced damage. Nevertheless, we observed reliable evidence of GVHD in both large and small intestines that were free from tumor infiltration. We used an established scoring system to evaluate GVHD severity (35). As shown in Fig. 6A, transplant of CD8+ T cells induced mild to moderate condition of GVHD. However, CBLB502 treatment did not cause any significant histopathologic difference in the tumor-bearing mice. Body weight was also monitored, and CBLB502 administration did not cause significant weight loss. Together, these results indicate that the posttransplant administration of CBLB502 did not exacerbate GVHD in the tumor-bearing mice.
CBLB502 does not affect donor cell engraftment or CD8\(^+\) T cell differentiation

In allo-BMT model, donor cell engraftment is a critical aspect that can impact GVT effect and host survival. To determine whether CBLB502 treatment influences donor cell engraftment, we used flow cytometry to measure the frequency of H-2K\(^b\) donor cells in host spleens at day 31 after allo-BMT. As shown in Supplemental Fig. 2A and 2B, 95% of host spleen cells were found to be H-2K\(^b\) donor derived, and CBLB502 treatment did not cause any significant change. These results indicate that donor cells engrafted successfully, and that our CBLB502 treatment scheme did not affect donor cell engraftment. To further check whether CBLB502 treatment could cause any change in donor T cell phenotype, we determined the frequencies of naive (CD44\(^-\)CD62L\(^+\)), central memory (CD44\(^+\)CD62L\(^+\)), and effector memory (CD44\(^+\)CD62L\(^-\)) cells in the host spleens at days 10 and 31 after allo-BMT. As shown in Supplemental Fig. 2C and 2D, CBLB502 treatment did not cause any significant change for these phenotypes. These data indicate that this scheme of CBLB502 treatment does not change the phenotypic differentiation of donor T cells.

Discussion

Using an allo-BMT model, we show that a novel TLR5 agonist CBLB502 significantly enhances CD8\(^+\) T cell-mediated GVT immunity. CBLB502 improves the proliferation and function of allogeneic CD8\(^+\) T cells mainly indirectly through a mechanism that involves donor BM-derived CD11c\(^+\) and CD11b\(^+\) cells and the IL-12 signaling pathway. As a result, CBLB502 treatment leads to reduced tumor burden, prolonged survival for the tumor-bearing mice, and tumor-free survival for about half of hosts prechallenged with tumor cells. Importantly, CBLB502 treatment induces the improved GVT effect without causing more severe GVHD.

A great challenge in allo-BMT is to identify novel approaches that can separate the beneficial GVT effect from the undesirable GVHD damage. Therefore, it is a significant finding that CBLB502 treatment enhances GVT effect but does not cause more severe GVHD. However, our results are not entirely consistent with a recent report, which used tumor-free hosts to show that peri-transplant administration of flagellin reduced GVHD in an allo-BMT model whereas enhancing donor-derived antiviral immunity (44). The discrepancy may be because of several factors, besides the difference between flagellin and CBLB502. We used BALB/c hosts, whereas the previous report used C57BL/6 hosts. For the GVT study, we transplanted 0.3 \times 10^6 CD8\(^+\) T cells that the hosts could tolerate without developing lethal GVHD, whereas the previous report transplanted 5 \times 10^6 total splenocytes to study lethal GVHD. In addition, the different administration scheme may be another factor. In the previous report, flagellin was given at 3 h before irradiation and 24 h after transplantation. In our study, CBLB502 treatment initiated 3 d after irradiation and 1 d after transplantation, based on a rationale that its potent ra-
dioprotection effect could interfere with donor cell engraftment. As shown in Supplemental Fig. 2, our treatment scheme successfully excluded any undesirable effect on donor cell engraftment, which is a prerequisite for donor-derived GVT effect. Furthermore, this scheme induces transient proliferation and activation of donor T cells without changing their long-term phenotypic differentiation, which may explain why CBLB502 enhances GVT effect without exacerbating long-lasting GVHD damage.

Flagellin is a highly effective adjuvant for the CD4+ T cell and humoral immune responses (17–20). However, it is controversial whether flagellin can promote the CD8+ T cell response (21–25). A recent report showed that flagellin was not able to stimulate CD8+ T cells when administered separately from Ag (24). Our results indicate that CBLB502 can significantly enhance allogeneic CD8+ T cell function in vivo and in vitro. CBLB502 is derived from flagellin by deleting its high-V region, whereas keeping the N and C terminals that are essential for TLR5 binding (16, 29). The high-V region represents the most immunogenic component of flagellin, which may cause toxic effects or induce humoral immune response against flagellin itself. CBLB502 is smaller and less immunogenic, which may facilitate binding with TLR5 and subsequently more efficient NF-κB activation.

Previous studies have also shown conflicting results regarding TLR5 expression. Studies using RT-PCR and a polyclonal Ab suggested that TLR5 may be expressed on many cell types, including DCs, macrophages, and epithelial cells (37, 38). In contrast, another study showed that TLR5 was expressed mainly on intestinal CD11c+ lamina propria cells, but not on conventional DCs or macrophages (45). Because of the lack of a reliable Ab, this study did not attempt to describe an accurate TLR5 expression pattern. Instead, we used an MLR-based in vitro functional assay to show that TCR-BM cells or BM-derived CD11c+ and CD11b+ cells are capable of responding to CBLB502, and thus enhancing their ability to modulate CD8+ T cell function in the allo-BMT setting.

In summary, this study shows that CBLB502 can significantly improve GV immunity by modulating CD8+ T cell response mainly through donor-derived CD11b+ and CD11c+ BM cells. As a pharmacologically optimized TLR5 agonist currently undergoing phase I clinical trial, CBLB502 is superior to flagellin because of lower toxicity and lower immunogenicity. Using an allo-BMT model, this study demonstrates a new beneficial effect of CBLB502 and also provides evidence to support a rational scheme of CBLB502 administration that may be a promising approach to improve the effectiveness of allo-BMT without exacerbating GVHD.

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Disclosures

L.G.B. and A.V.G. are paid consultants of Cleveland BioLabs, Inc. A.V.G. is a shareholder of Cleveland BioLabs, Inc. The other authors have no financial conflicts of interest.

References


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Supplement Figure 1. Purity check of MACS-sorted CD$^8^+$ T cells before transplantation.

CD$^8^+$ T cells were purified with auto-MACS from C57BL/6 donor spleen cells by using Pan T isolation kit II combined with biotin-conjugated anti-CD4 according to the protocols (Miltenyi). The purified cells were stained with fluorescently conjugated antibodies including anti-CD3, anti-CD4, anti-CD8, anti-CD11b, anti-CD11c, CD19 and anti-NK1.1 and analyzed by flow cytometry. The table below is summarized from 5 independent purifications and shows the percentages (mean ± standard deviation) of CD8$^+$CD3$^+$ T cells, CD4$^+$CD3$^+$ T cells, CD11c$^+$ dendritic cells, CD19$^+$ B cells, NK1.1$^+$CD3$^+$ NK cells, NK1.1$^+$CD3$^+$ NKT cells, and CD11b$^+$ monocytes or macrophages. Representative flow cytometry plots are also shown below. The purity of the real CD8$^+$CD3$^+$ T cells is typically 85-90%. However, the samples also contain >2% dendritic cells and <1% B cells, NK cells, NKT cells and monocytes or macrophages.

<table>
<thead>
<tr>
<th></th>
<th>CD8$^+$CD3$^+$</th>
<th>CD4$^+$CD3$^+$</th>
<th>CD11c$^+$</th>
<th>CD19$^+$</th>
<th>NK1.1$^+$CD3$^+$</th>
<th>NK1.1$^+$CD3$^+$</th>
<th>CD11b$^+$</th>
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<tbody>
<tr>
<td></td>
<td>88.1 ± 2.4</td>
<td>&lt;0.05</td>
<td>2.2 ± 0.2</td>
<td>0.8 ± 0.3</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>0.7 ± 0.1</td>
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Supplement Figure 2. CBLB502 does not affect donor cell engraftment and donor CD8+ T phenotype. (A) 31 days after tumor inoculation and allo-BMT, spleen cells were harvested from the BALB/c host mice for flow cytometric analyses. Representative dot plots to show the percentage of H-2Kb+ and CD3+ donor cells in the host spleen. (B) Summary data of H-2Kb+ donor cells in the host spleens were shown, with three mice in each group. (C) Representative dot plots to show the percentages of naïve (CD44-CD62L+), central memory (CD44+CD62L+) or effector memory (CD44+CD62L-) phenotypes on the gated H-2Kb+CD8+ donor T cells in the host spleens. (D) Summary data to show the naïve, central memory and effector memory populations in the donor CD8+ T cells in the host spleens at days 10 and 31 after allo-BMT, with 3-4 mice in each group.