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Mannan binding lectin promotes murine graft versus host disease by amplifying lipopolysaccharide-initiated inflammation

David Heja,

Dongchang Zhao,

Evan Cody,

Arun Cumpelik,

Pik Chin Lim,

Mariano Prado-Acosta,

Liv Palma,

Sergio Dellepiane,

Nicholas Chun,

James Ferrara,

Peter S. Heeger*

Translational Transplant Research Center, Tisch Cancer Institute, Precision Immunology Institute, Tisch Cancer Institute and Department of Medicine, Icahn School of Medicine at Mount Sinai, NY, NY

Abstract

Background.—Conditioning regimens used for hematopoietic stem cell transplants (HCT) can escalate the severity of acute, T cell-mediated, graft-versus-host disease (GVHD) by disrupting gastrointestinal integrity and initiating lipopolysaccharide-(LPS)-dependent innate immune cell activation. Activation of the complement cascade has been associated with murine GVHD, and previous work has shown that alternative pathway complement activation can amplify T cell immunity. Whether and how mannan binding lection (MBL), a component of the complement system that binds mannose as well as oligosaccharide components of lipopolysaccharide (LPS) and lipotechoic acid, affects GVHD is unknown.

Objective: Our objective was to test the hypothesis that MBL modulates murine GVHD and to determine mechanisms.

Study design.—We adoptively transferred C3.SW bone marrow (BM) cells ± T cells into irradiated wild type (WT) or MBL-deficient C57Bl/6 (B6) recipients with or without inhibiting MBL-initiated complement activation using C1-esterase inhibitor (C1INH). We analyzed the

***Crresponding author:** Peter S Heeger, MD; Translational Transplant Research Center, Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, USA; peter.heeger@mssm.edu; Phone: 212 241 6324.

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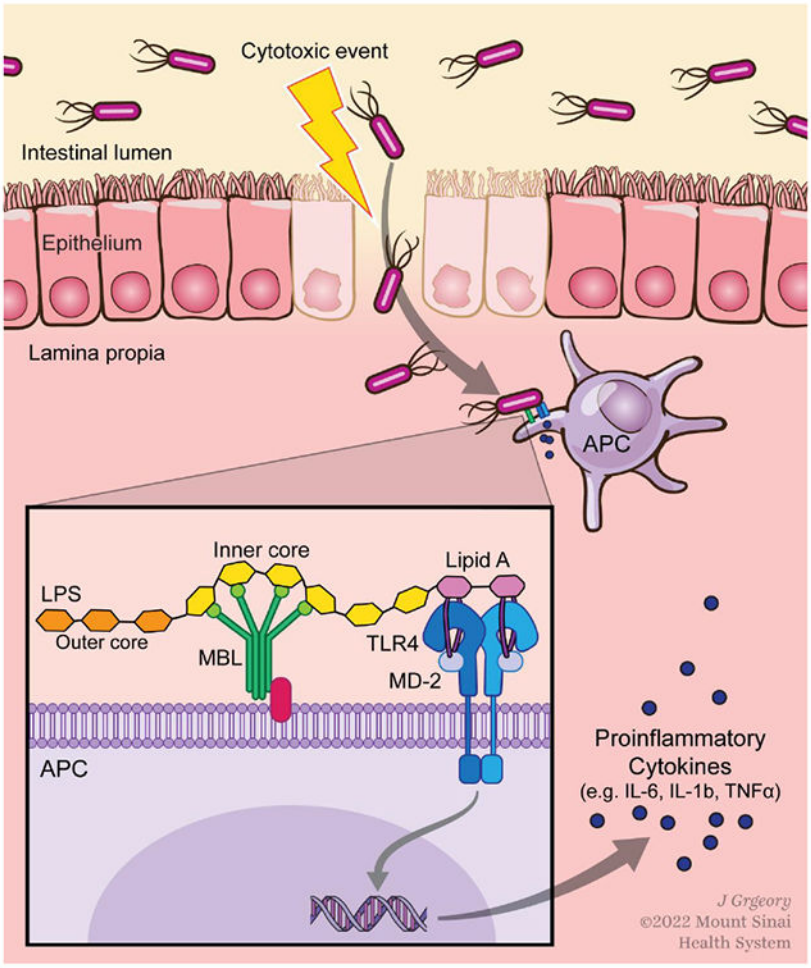
clinical severity of disease expression and analyzed intestinal gene and cell infiltration. In vitro studies assessed MBL expression on antigen presenting cells (APCs) and compared LPS-induced responses of WT and MBL-deficient APCs.

Results: MBL-deficient recipients of donor BM ± T cells exhibited significantly less weight loss over the initial 2 post-transplant weeks ($p<0.05$) vs B6 controls, with similar donor engraftment between groups. In recipients of C3.SW BM + T cells, the clinical expression of GVHD was less severe ($p<0.05$) and overall survival better ($p<0.05$) in MBL-deficient vs. WT recipients. On day-7 post-transplant, analyses showed MBL-deficient recipients exhibited less intestinal Il1b, Il17 and Il12 p40 gene expression ($p<0.05$ for each) and fewer infiltrating, intestinal $CD11c^+$, $CD11b^+$, $F4/80^+$ cells $TCR\beta^+$, $CD4^+$, $CD4^+IL17^+$ and $CD8^+$ T cells ($p<0.05$ for each). Ovalbumin or allogeneic cell immunizations induced equivalent T cell responses in MBL-deficient and WT mice, demonstrating MBL-deficiency does not directly impact T cell immunity in the absence of irradiation conditioning. Administration of C1INH did not alter the clinical expression of GVHD in preconditioned WT B6 recipients, suggesting that MBL amplifies clinical expression of GVHD via a complement-independent mechanism. WT, but not MBL-deficient, antigen presenting cells (APCs) express MBL on their surfaces. LPS-stimulated APCs from MBL-deficient mice produced less pro-inflammatory cytokine ($p<0.05$) and induced weaker alloreactive T cell responses ($p<0.05$) vs. WT APCs.

Conclusion: Together, the data show that MBL modulates murine GVHD, likely by amplifying complement-independent, LPS-initiated, gastrointestinal inflammation. The results suggest that devising strategies to block LPS/MBL ligations on APCs has potential for reducing the clinical expression of GVHD.

Graphical Abstract



MBL amplifies GVHD via a complement-independent mechanism. Disruption of the GI epithelial lining by irradiation conditioning results in translocation of luminal bacteria, a subset of which express lipopolysaccharide (LPS). LPS binds to mannan binding lectin (MBL) bound to surfaces of antigen presenting cells (APC). The MBL thereby facilitates LPS binding to APC-expressed TLR4 (distinct regions of LPS bind MBL and TLR4) resulting in APC activation that enhances early weight loss and promotes acute GVHD. The illustration depicts only LPS-expressing bacteria for clarity, noting that the gut microbiome is highly heterogeneous and that it contains many species that lack LPS.

Keywords

acute GVHD; complement; mannan binding lectin; LPS

Introduction

Myeloablative conditioning that initiates innate immune cell activation amplifies the expression of acute graft-versus-host disease (GVHD), a major cause of morbidity following hematopoietic stem cell transplantation (HCT).(1) Within the gastrointestinal tract, studies

from murine models showed that myeloablative conditioning disrupts intestinal epithelial integrity and facilitates translocation of intestinal microbes.(1-3) Pathogen associated molecular patterns (PAMP) including lipopolysaccharide (LPS) activate lamina propria antigen presenting cells (APC) via ligating pattern recognition receptors, including Toll-Like Receptor 4 (TLR4).(4–7) Subsequent production of pro-inflammatory cytokines including TNFα amplify gastrointestinal inflammation that results in weight loss and diarrhea typical of GVHD.(2, 8–10) Antibiotic therapy or LPS antagonism limits early post-transplant inflammation and improves GVHD outcome. (11–13)

The complement system, traditionally considered a component of innate immunity, can be initiated via the classical pathway, the mannan binding lectin (MBL) pathway and the alternative pathway, all of which converge to form C3 convertases. Downstream activation results in assembly of a C5 convertase required for C5b-9 membrane attack complex formation. Multiple groups have provided evidence that complement activation participates in the pathogenesis of GVHD (14–18). Accumulating evidence further showed that complement activation products, including C3a and C5a, crucially mediate differentiation and function of alloreactive T cells (19, 20) and B cells (21, 22). Specifically, previous work in murine GVHD models showed that signaling via C5a receptor (C5ar1) and C3a receptor (C3ar1) on T cells and antigen presenting cells (APCs) play pathogenic roles in murine GVHD and in xenogeneic human anti-mouse GVHD.(23–26) While these studies linking the complement system to acute GVHD implicate alternative pathway and classical pathway complement activation, whether and how MBL affects acute GVHD has not been addressed.

MBL is a member of the C-type lectin superfamily that functions as a pattern recognition receptor (PRR) (27–29). The mature protein is an oligomer of three subunit protomers each composed of 30kDa polypeptide chains with a structure that includes a cysteinerich N-terminal domain, a collagen-like stalk and a C-terminal carbohydrate recognition domain (CRD). MBL's CRD binds to mannose, mannan, and fucose as well as oligosaccharide components of lipopolysaccharide (LPS) and lipoteichoic acid (LTA).(30, 31) Whereas human MBL derives from a single gene (MBL2), mice express two forms of MBL, MBL-A and MBL-C (encoded by *Mbl1* and *Mbl2* genes, respectively) which have distinct but overlapping binding specificities and expression patterns.(28, 29, 32) Upon binding to one of its ligands, human and murine MBL undergo a conformational change that activates mannose-associated serine protease-1 and -2 (MASP1 and MASP2, respectively) to initiate cleavage of C4 and C2 and result in formation of C3 convertases.(33, 34) Beyond its ability to initiate complement activation, MBL's collagen stalk can bind to surface-expressed CD35/CR1, a complex of calreticulin and CD91, as well as to CD14 on dendritic cells and macrophages, among other cell types. (35–39) Functions of surface bound MBL are incompletely understood but studies by several groups showed that ligation of surface bound MBL facilitates complement-independent pathogen opsonization.(27, 40, 41) Building upon previous work linking the complement system to GVHD (23–26), we tested the hypothesis that MBL participates in the pathogenesis of acute murine GVHD.

Materials and methods

Mice

We purchased C57BL/6 (*B6; H-2^b*), BALB/c (*H-2^d*) and C3H.SW (*H-2^b*), *Tlr4$^{tm1.2Karp}$* (*H-2^b, Tlr4$^{-/-}$*) from The Jackson Laboratory (Bar Harbor, ME) and maintained colonies at the Mount Sinai Center for Comparative Medicine. We obtained B6 *Mbl1$^{-/-}$* and *Mbl2$^{-/-}$* mice (42), a gift from Greg Stahl, Harvard University Boston MA, and intercrossed them to produce *Mbl1$^{-/-}$Mbl2$^{-/-}$* animals. All animals were housed in the Center for Comparative Medicine and Surgery at the Icahn School of Medicine at Mount Sinai under Institutional Animal Care in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International approval IACUC-2018-0014. Experiments were performed in compliance with ethical guidelines under IACUC approval. Experiments with groups of age- (6-12 weeks) and sex-matched mice, using littermates or animals maintained in the same room and cohoused within the same cages for >2 weeks, to limit potential effects of microbiome differences.

Acute graft versus host disease model

We used a minor antigen disparate C3H.SW → B6 acute GVHD model as previously described (43). Briefly, we isolated bone marrow cells from the femurs and tibiae of C3H.SW donor mice and isolated splenic T cells by positive selection using CD90.2 magnetic beads (Miltenyi Biotec cat. 130-121-278) according to the manufacturer's instructions. Groups of age and sex matched WT B6 or congenic *Mbl1$^{-/-}$Mbl2$^{-/-}$* recipients (co-housed to limit microbiome effects) received 800-1,050 cGy total-body irradiation from an x-ray source (RS 2000 Biological Research Irradiator) on day −1, and then injected intravenously with donor BM with or without T cells (5×10^6/mouse each) in 200 μl PBS on day 0. In selected experiments recipients were treated with C1-inhibitor (C1-INH, 0.4 IU/g body weight daily, beginning day −1 through day 5, CSL Behring, King of Prussia, PA). Survival was monitored daily, and clinical GVHD was assessed weekly by a previously described scoring system (13).

Antibodies

Viability dye (cat. L34968) and CFSE (cat. 65-0850-84) were both purchased from Invitrogen. The following monoclonal antibodies (mAbs) were also purchased from Invitrogen: H-2Kd APC (clone SF1-1.1.1, cat. 17-5957-82), CD8a PE-Cy7 (clone 53-6.7, cat. 25-0081-82), MHCII Pacific Blue (clone M5/114.15.2, cat. 48-5321-8), and CD11c PE-Cy7 (clone N418, cat. 12-7177-82). The following mAbs were purchased from Biolegend: CD4 APC/cy7 (clone GK1.5, cat. 100414), CD11b APC (clone M1/70, cat. 101212), CD86 PECy7 (clone GL-1, cat. 105014). B220 APC (clone RA3-6B2) was purchased from BD Pharmingen (cat. 553092). We obtained anti-CD5.1 (Ly5.1)-FITC, clone H11-86.1, from BD Pharmingen (cat 557487).

Cell isolations

Spleens were passed through a 40μM strainer and lysed with ACK red blood cell lysis buffer to generate single cell suspensions. T cell depletion, isolation of naïve $CD4^+$ T cells, naïve

$CD8^+$ T cells or total T cells/APCs were accomplished using the appropriate magnetic beads (see below).

Cell culture experiments

Enriched CD45+ APCs (negative fraction of EasySep Mouse CD90.2 kit II, STEMCELL cat. 18951) from untreated B6 mice were stimulated *in vitro* ± LPS (E.coli 0111:B4, Invivogen cat. tlrl-eblps) and then co-cultured with total (EasySep Mouse T cell isolation kit, STEMCELL cat. 19851) or naïve allogeneic BALB/c T cells (EasySep Mouse Naïve $CD4^+$ T cell isolation kit, STEMCELL cat. 19765, or EasySep Mouse Naïve $CD8^+$ T cell isolation kit, STEMCELL cat. 19858) labeled with or without CFSE as indicated. Cells were incubated in complete medium (RPMI + 10% FCS + L-Glutamine + sodium pyruvate + nonessential amino acids + Pen/Strep + β-mercaptoethanol) at 37°C. Cell proliferation (CFSE dilution) was assessed by flow cytometry on day 3-4.

To assess intracellular cytokines after a 4-day mixed lymphocyte response, cells were washed, stained for surface molecules, fixed, permeabilized and stained for intracellular IFNγ and IL-17. Analysis was performed by a FacsLyric flow cytometer (BD Biosciences, Franklin Lakes, NJ) and data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). In some experiments splenic APCs cultured ± LPS for 48 h were phenotyped for surface markers by flow cytometry and generated culture supernatants were analyzed for cytokines by ELISA.

To assess MBL binding, spleen cells isolated from WT or $Mbl1^{-/-}Mbl2^{-/-}$ mice were incubated without serum or with fresh pooled serum obtained from B6 or $Mbl1^{-/-}Mbl2^{-/-}$ mice and diluted to 30% with 1x Annexin V buffer (BD Biosciences, 556454) for 30 minutes at room temperature. After washing the cells, we detected surface bound MBL with rat anti-mouse Mbl-A or Mbl-C mAbs (MBL-A clone 2B4, Hycult cat. HM1036 and MBL-C Hycult clone 14D12, cat. HM1038), and a FITC-conjugated goat anti-rat secondary antibody (BD Biosciences, cat. 554016). Antibodies were diluted in 1x Annexin V buffer. Mbl surface binding was analyzed on major splenic immune cell population by flow cytometry. The signal obtained with $Mbl1^{-/-}Mbl2^{-/-}$ serum was considered as background.

Serum and culture media cytokine detection

We collected blood from mice by submandibular venipuncture. Blood was incubated for 30 minutes at room temperature, then centrifuged at 2,000 x g for 15 minutes and subsequently stored at −80°C until use. Serum levels of cytokines were measured using Mesoscale (Rockville, MD) multiplex assays as per manufacturers protocols. ELISAs were performed on culture supernatants with ELISA MAX™ Standard Set Mouse TNF-α (Biolegend, cat. 430904) and ELISA MAX™ Deluxe Set Mouse IL-6 (Biolegend, cat. 431304) per manufacturer instructions.

IFNγ ELISPOT assays

Three weeks after immunizing groups of WT and $Mbl1^{-/-}Mbl2^{-/-}$ mice with chicken egg ovalbumin (Albumin from chicken egg white, Sigma cat. A5503) mixed in complete Freund's adjuvant (Sigma-Aldrich, St. Louis MO) or 2 weeks after i.v. injection of $10x10^6$,

T cell depleted allogeneic BALB/c spleen cells, we quantified splenic antigen specific IFNγ-producing T cells by ELISPOT as previously described (20, 44–46).

LPS ELISA

We quantified LPS in serum and from spleen cell lysates using mouse LPS ELISA kit (Biomatik, Kitchener, Ontario, Canada, cat EKC37294) as per manufacturer's instructions.

C3 deposition assay

We performed a C3 deposition assay as previously described.(33, 34) Briefly, high binding microtiter plates (Nunc Maxisorp, ThermoFisher cat. 44-2404-21) were coated with 10 μg/ml mannan (Sigma cat. M3640) in PBS overnight at 4C and blocked with 1% BSA in TBS (50mM Tris, 150mM NaCl, pH7.4). Serum collected from the mandibular vein was diluted 1:60 in TBS, 5mM $MgCl_2$, 5mM $CaCl_2$, 0.05% Tween buffer and incubated on the plates at 37C for 30 minutes. Complement activation was stopped by washing the plates 3X with TBS, 0.05% Tween. C3 deposition was detected with HRP-conjugated goat anti-mouse C3 IgG (1:4000; Cappel's, MP Biosystems cat. 55557) in TBS, 5mg/ml BSA, 0.05% tween (1h RT). After washing, the plates were developed with TMB substrate (Sigma cat. T0440). Following acidification of the wells with 1N HCl, absorbance signal was detected at 450nm with a BioTek μQuant Spectrophotometer (BioTek, Winooski VT).

Real time polymerase chain reaction

RNA isolation was performed using Trizol (Thermofisher, Waltham MA) and cDNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Waltham MA) as per the manufacturer's instructions. RT-PCR (TaqMan probes; Applied Biosystems) was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules CA). All the mouse PCR primers were purchased from Life Technologies. PCR products were normalized to the control gene (GAPDH) and expressed as fold increase compared with unstimulated cells using the ΔΔCt method.

Statistics

Statistical significance was determined by Student's t test (unpaired, two-tailed), two-way ANOVA (with Bonferroni post-tests to compare replicate means) or by log-rank (Mantel-Cox) test performed in GraphPad Prism with a significance threshold values of $p<0.05$. All experiments were repeated at least twice. Data are presented as mean values with SD. Error bars indicate mean± SEM and ns indicates $p>0.05$, not significant.

Results

To test whether MBL modulates the development and/or severity of acute GVHD, we irradiated WT B6 and co-housed, congenic, *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ B6 mice with 1050 cGy and adoptively transferred bone marrow (BM) plus $5x10^6$ T cells from minor antigen-(mH)-disparate C3H.SW donors (Figure 1A–C). Following this conditioning regimen, we observed increased survival of *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ recipients, as well as less weight loss and lower clinical scores relative to the WT controls. When we repeated the experiment using a lower radiation dose (800 cGy) and the same number of donor T cells ($5x10^6$, Figure 1D–F),

we observed 30-day survival of >90% for all animals without a difference between groups. Nonetheless, the *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ mice rapidly regained their initial weight loss, while the WT controls did not ($p<0.05$ through day 30), and this was associated with lower clinical scores in the *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ recipients over this same time period. When we irradiated groups of mice with 800 cGy and administered donor BM without T cells (Figure 1G), we observed less weight loss over the initial 3 weeks in the *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ recipients. In separate WT and *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ recipients of allogeneic BM without T cells, analysis of day 5 serum LPS levels (WT mean 0.108 ± 0.004 ng/ml vs. *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ mean 0.322 ± 0.187 ng/ml $p=0.12$, n=3 animals/group) or spleen cell lysate LPS levels (WT mean 0.189 ± s.d. 0.087 ng/ml vs. *Mbl1*$^{-/-}$Mbl2$^{-/-}$ mean 0.228 ± s.d. 0.093 ng/ml, $p=0.7$, n=3 animals/group) showed no differences between groups.

C3.SW and B6 animals express the same MHC haplotype (*H-2*^{b}). To quantify donor cell engraftment, we analyzed expression of CD5 polymorphisms by flow cytometry. CD5 is predominantly expressed on murine T cells. B6 mice (WT and *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$) express the CD5.2 (Ly5.2) polymorphism while C3.SW mice express CD5.1 (Ly5.1). (47) Groups of WT and *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ mice underwent 800 cGy irradiation plus adoptive transfer of C3.SW BM (without T cells). Six weeks post-transplant, analyses showed no significant differences in donor CD5 expressing splenic CD4^{+} or CD8^{+} T cells (Figure 1H–I), supporting the conclusion that the absence of recipient MBL does not adversely affect donor BM engraftment. Control flow cytometric analyses of spleen cells from naïve WT B6 and *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ mice (Supplemental Fig S1) also showed no significant differences in frequencies of CD45^{+} lymphocytes or in total CD4^{+} and CD8^{+} T cells, B cells, monocyte/macrophages or dendritic cell subsets between genotypes.

To begin to address mechanisms, we induced GVHD in WT and *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ mice with 1050 Gy donor BM and donor 5x10^{6} T cells, and performed *ex vivo* analyses of splenic and intestinal immune cells on day 7 post-transplant (Fig 2A, **schematic**), confirming greater weight loss in the WT recipients at that time point (Supplemental Fig S2). These analyses showed significantly fewer intestinal CD45^{+} leukocytes in the *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ mice (Fig 2B) but showed no differences in splenic CD45^{+} cells between groups (Fig 2B). Within the intestinal CD45^{+} population we observed fewer CD11c^{+}, CD11b^{+}, and F4/80^{+} cells, as well as fewer TCRβ^{+}, CD4^{+}, CD4^{+}IL17^{+} and CD8^{+} T cells in the *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ mice (Fig 2C).

Gene expression analysis of the intestines (day 7, Fig 3A) revealed lower expression of the pro-inflammatory cytokines *Il1b, Il12b, Tnfa*, and *Il17* in the *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ mice, without differences in *Il6* and *Ifng* between groups (Fig 3A). Control PCR studies confirmed expression *Mbl1* in the WT B6 mice, without detectable expression in the intestines of *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ mice (data not shown). Analyses of serum cytokines showed no differences in IL-1β, IL-6, IL-12, TNFα or IFNγ between groups (Fig. 3B). Together the data are consistent with the conclusion that MBL deficiency specifically limits early local post-transplant intestinal inflammation in this GVHD model.

Our previous studies showed that in models of immunization or solid organ transplant rejection (no irradiation), immune cell-derived, alternative pathway-initiated complement

activation results in C3a/C3ar1 and C5a/C5ar1 signaling during cognate T cell/APC interactions to amplify T cell responses.(19, 20, 23, 24, 48-50) In contrast to these published results, MBL-deficiency did not affect the strength of antigen-specific T cell responses following ovalbumin immunization (Fig 4A). Similarly, recipient MBL-deficiency did not affect the size of the polyclonal alloreactive T cell response induced by i.v. injection of allogeneic spleen cells (Fig 4B). These data support the conclusion that MBL deficiency has no effect on antigen-induced T cell responses in these models that lack irradiation preconditioning.

To specifically test if MBL's effects on GVHD require complement activation, we irradiated groups of WT B6 mice (1050 Gy), adoptively transferred donor BM and T cells as above, and treated them with C1 esterase inhibitor (C1-INH, Fig 5A, **schematic**), a serine protease that dissociates/inactivates MASP1/2 from MBL and dissociates/inactivates C1r/s from C1q, thereby preventing MBL (and classical) pathway complement activation. We administered the drug for 7 days (during the response to conditioning) at a dose previously shown to fully block MBL-initiated complement activation *in vivo* (51) and we verified inhibition of MBL-dependent complement activation in the serum for >24 h after administration (Fig 5B). Administration of C1-INH did not improve survival, weight loss or clinical scores compared to vehicle-treated controls (Fig 5C–E). This result together with the previous experiments demonstrating improved outcomes in MBL-deficient recipients suggest that the effects of MBL on early weight loss and GVHD severity are independent of MBL-initiated complement activation.

We next analyzed spleen cells for surface-expressed MBL by flow cytometry (Figure 6A) and detected MBL on $B220^+$ B cells, CD11c+$CD11b^-$ DCs and $CD11c^-CD11b^+$ myeloid cells, but not on $CD4^+$ or $CD8^+$ T cells from WT mice incubated with WT serum. Control experiments showed no MBL on the surfaces of $Mbl1^{-/-}Mbl2^{-/-}$ cells (Fig 6B). Incubation of $Mbl1^{-/-}Mbl2^{-/-}$ spleen cells with serum from WT mice restored MBL-surface expression (Fig 6B) while control studies showed no MBL staining on the same populations of immune cells upon incubation with $Mbl1^{-/-}Mbl2^{-/-}$ serum.

LPS, which can bind MBL (30), has been mechanistically linked to GVHD (2, 8, 13). Our above described experiments showed that MBL a) binds to antigen presenting cell surfaces (Fig 6) and b) modulates GVHD in a complement-independent manner (Fig 1, 5). Together these observations raised the hypothesis that APC-expressed MBL facilitates LPS interactions with APC-expressed TLR4 to amplify post-conditioning, LPS-dependent, immune cell activation. To test this hypothesis, we cultured WT and $Mbl1^{-/-}Mbl2^{-/-}$ APCs with LPS, and analyzed cytokine production in culture supernatants and activation markers on cell surfaces 48h later. These assays showed that while LPS treatment of WT APCs resulted in significant, dose-dependent increases in IL-6 and TNFα, the effects were significantly reduced in cultures containing $Mbl1^{-/-}Mbl2^{-/-}$ APCs (Fig 7A). Control experiments using poly I:C (which does not ligate MBL) induced equivalent increases in cytokine production in cultures containing WT and $Mbl1^{-/-}Mbl2^{-/-}$ APCs (Fig 7B). LPS also induced greater upregulation of surface-expressed co-stimulatory molecules CD40 and CD86 on WT APCs compared to LPS-stimulated $Mbl1^{-/-}Mbl2^{-/-}$ APCs (Fig 7C). We next co-cultured WT BALB/c T cells with WT or $Mbl1^{-/-}Mbl2^{-/-}$ MHC-disparate

B6 APCs ± LPS, and quantified $CD4^+$ and $CD8^+$ T cell proliferation (CFSE-dilution Fig 7D). These assays showed blunted LPS-induced augmentation of T cell proliferation in cultures containing *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ vs. WT B6 APCs. Control assays showed similar T cell proliferation induced by poly I:C stimulated WT and *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ APCs (Fig 7E), and confirmed that LPS did not alter T cell proliferation in cultures using TLR4-deficient (MBL^+) APCs (Supplemental Fig 3), together supporting the conclusion that the above noted effects are dependent upon an MBL-LPS interaction.

Discussion

Increasing evidence supports pleiotropic immune functions of MBL beyond its ability to initiate MASP-dependent complement activation. Among its previously reported activities, a number of laboratories showed that MBL enables pathogen opsonization, ligates apoptotic cells to facilitate their clearance, and amplifies cytokine production in response to bacteria by interacting with TLR2, all of which are innate immune functions.(29, 31, 40, 52) Whether and how these additional functions apply to *in vivo* disease models has not been previously addressed. Herein we add to this literature by newly demonstrating that recipient MBL modulates the clinical, histological and molecular severity of acute intestinal GVHD that occurs following a standard murine conditioning regimen.

We observed that MBL-deficient recipients of donor BM lost less weight over the initial 2 weeks post-HCT whether or not they were adoptively transferred with donor T cells. These results support the conclusion that MBL-deficiency indirectly alters the clinical expression of murine GVHD by limiting the innate immune cell activation triggered by the irradiation conditioning regimen. Dysregulation of inflammatory cytokine cascades initiated by HCT-conditioning regimens are known to crucially influence the clinical expression of disease, particularly of gastrointestinal GVHD.(1, 3, 9, 53) HCT conditioning regimens disrupt gastrointestinal integrity thereby facilitating LPS translocation into the systemic circulation where it triggers mononuclear cell cytokine release (including TNFα and IL-6). This mechanism contributes to GVHD morbidity: transplantation with donor cells resistant to LPS stimulation, decontamination of the gut microflora, or treatment with an LPS antagonist, each reduced the clinical expression of acute GVHD. (6, 8, 10–13, 54)

While reduced disease severity in MBL-deficient mice could potentially be caused by a BM engraftment defect in the absence of MBL, our analyses of donor CD5 polymorphism expression on T cells 6-weeks post-transplant show no differences between groups, lowering the probability that MBL regulates engraftment to account for the observed differences. Nonetheless, we acknowledge that the restricted cellular expression of CD5 and the lack of other available markers to distinguish donor and recipient cells in this strain combination, limits our ability to fully exclude an effect. While MBL deficiency could also theoretically increase infectious risk, our observation that HCT outcomes are better in MBL-deficient vs. WT recipients does not support a role for infection contributing to outcomes in this system.

Previous work by our research laboratory, among others, demonstrated a crucial role for complement in activation and differentiation of murine and human effector T cell differentiation and function, including T cells that mediate acute and chronic GVHD (24, 26,

48). These studies showed that effects on T cells required immune cell-derived complement (not serum, liver-derived complement) activated by the alternative pathway (19, 20, 23) and/or was intracellular (55) and activated via cathepsin L-mediated cleavage. This body of work mechanistically linked C3a/C3ar1- and C5a/C5ar1-signaling on T cells and APCs to disease activity.

This previous work and our observations showing reduced GVHD in MBL-deficient recipients (Figure 1) raised the possibility that MBL-initiated complement activation could also activate alloreactive T cells to augment the clinical expression of GVHD in this model. Administration of C1INH, which blocks MBL's ability to activate the complement cascade without altering LPS binding, had no effect on GVHD outcome and T cell immunity to immunizations were not different between WT and MBL-deficient mice. Together, the results support the conclusion that MBL's effects on GVHD are complement-independent. Thus, while inhibitors of MASP-2 (required for MBL-dependent complement activation) are being tested in humans (56), our data do not support testing these agents for prevention of acute GVHD.

Our observation that MBL-deficiency did not alter the number of antigen-reactive, IFNγ-producing T cells induced to ovalbumin or allogeneic cell immunization (performed in the absence of irradiation conditioning), also support the conclusion that MBL does not directly affect the strength Th1 T cell immunity. We have not fully excluded the possibility that MBL could additionally alter T cell cytokine profiles. Our previous work and studies by others in preclinical models (24, 26, 57) implicate C5aR and/or C3aR signaling as regulating T immunity that causes GVHD, supporting the need to alternatively test whether blocking C5aR with Avacopan [FDA approved for use in humans with anti-neutrophil cytoplasmic antibody vasculitis (58, 59)] is a viable complement target for prevention/treatment of GVHD.

The cumulative results support the interpretation that the MBL, which we showed binds to APC surfaces, amplifies LPS-induced APC activation and pro-inflammatory cytokine release, secondarily enhancing LPS-induced, alloreactive T cell proliferation. Our documentation that irradiated MBL-deficient recipients of donor BM plus T cells show lower pro-inflammatory cytokine gene expression in the gut and reduced numbers of gut infiltrating immune cells support this contention. It is additionally possible that the MBL influences immune cell migration into the gut. We note that previous work by others (16) showed unimpeded GVHD in mice deficient in both MBL and C1q (MBL-deficiency alone was not tested). C1q binds to DCs and provides inhibitory signals, preventing DC maturation (60, 61). Thus, C1q deficiency could theoretically enhance DC activation independent of the absence of MBL in animal lacking both C1q and MBL, providing a potential explanation to account for the observed differences.

As *Tlr4*$^{-/-}$ APCs express MBL, but do not respond to LPS, the data suggest that surface-expressed MBL interacts with TLR4 to mediate the effects, rather than an alternative mechanism wherein MBL directly transmits an activating signal to the APC. The receptors known to bind MBL's collagen stalk (yielding surface expression on immune cells) include CR1/CD35, a complex of calreticulin and CD91 and CD14,(35–39, 52) receptors that

are not known to transmit APC activating signals, but rather promote noninflammatory opsonization and apoptotic cell clearance. Our results, and the previous documentation that MBL and TLR4 bind distinct sites on LPS (30, 62), together suggest that immune cell-expressed MBL potentially functions by facilitating ligation of LPS to TLR4 on cell surfaces (see Graphical Abstract). Previous work by others implicated an analogous cooperative interaction between MBL and TLR2/6 in amplifying innate immune responses to Staph aureus-derived lipoteichoic acid.(29, 31) Our newly documented effect of MBL on acute GVHD is distinct from observations by others implicating complement activation (14, 18) and potentially the MBL/MASP pathway (63, 64) as pathogenically mediating post-HCT, chronic, thrombotic microangiopathy in humans.

Within this context, our *in vitro* data implicate cell-surface-bound MBL as an amplifier of LPS/TLR4 induced release by APCs that in turn augments expansion and differentiation of pathogenic alloreactive, cytokine-secreting T cells. Our observations that MBL-deficiency reduces innate cytokine gene expression and blunts accumulation of IL-17-producing T cell infiltrates specifically in the gut (Fig 3), without effects on T cell immunity initiated by multiple distinct stimuli (Fig 4), are further consistent with a local LPS-dependent mechanism. This mechanism can explain why MBL deficiency similarly limited early (within the initial 2 weeks) post-conditioning, weight loss even in the absence of co-transferred donor T cells.

Our data provide proof-of-principle that MBL can amplify LPS-driven inflammation *in vivo* and reveal a protective effect of MBL deficiency in a controlled murine GVHD system. MBL deficiency is prevalent in 10-25% of humans and may increase the risk of invasive fungal infections following HCT (41, 65–67). While there is only inferential data implicating LPS as a pathogenic mediator of human GVHD [reviewed in (53)], one small, single center analysis of a pediatric population showed less severe GVHD in MBL-deficient HCT recipients (in the absence of increased infectious disease complications), a finding that is consistent with our results (68).

In summary, our observations newly show that recipient MBL is a complement-independent amplifier of murine GVHD, that likely functions by facilitating LPS-induced APC activation post-conditioning. The findings raise the possibility that developing new therapeutic strategies aimed at blocking MBL's ability to bind LPS could reduce early post-transplant inflammation and potentially lower the risk of developing acute GVHD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- GVHD severity is reduced in mice lacking mannose binding lectin
- The effects of absent mannose binding lectin on GVHD are complement independent
- Mannose binding lectin is expressed on antigen presenting cell surfaces
- Absence of mannose binding lectin reduces activation of dendritic cells by LPS
- Blocking mannose binding lectin/LPS ligations can be tested to prevent GVHD

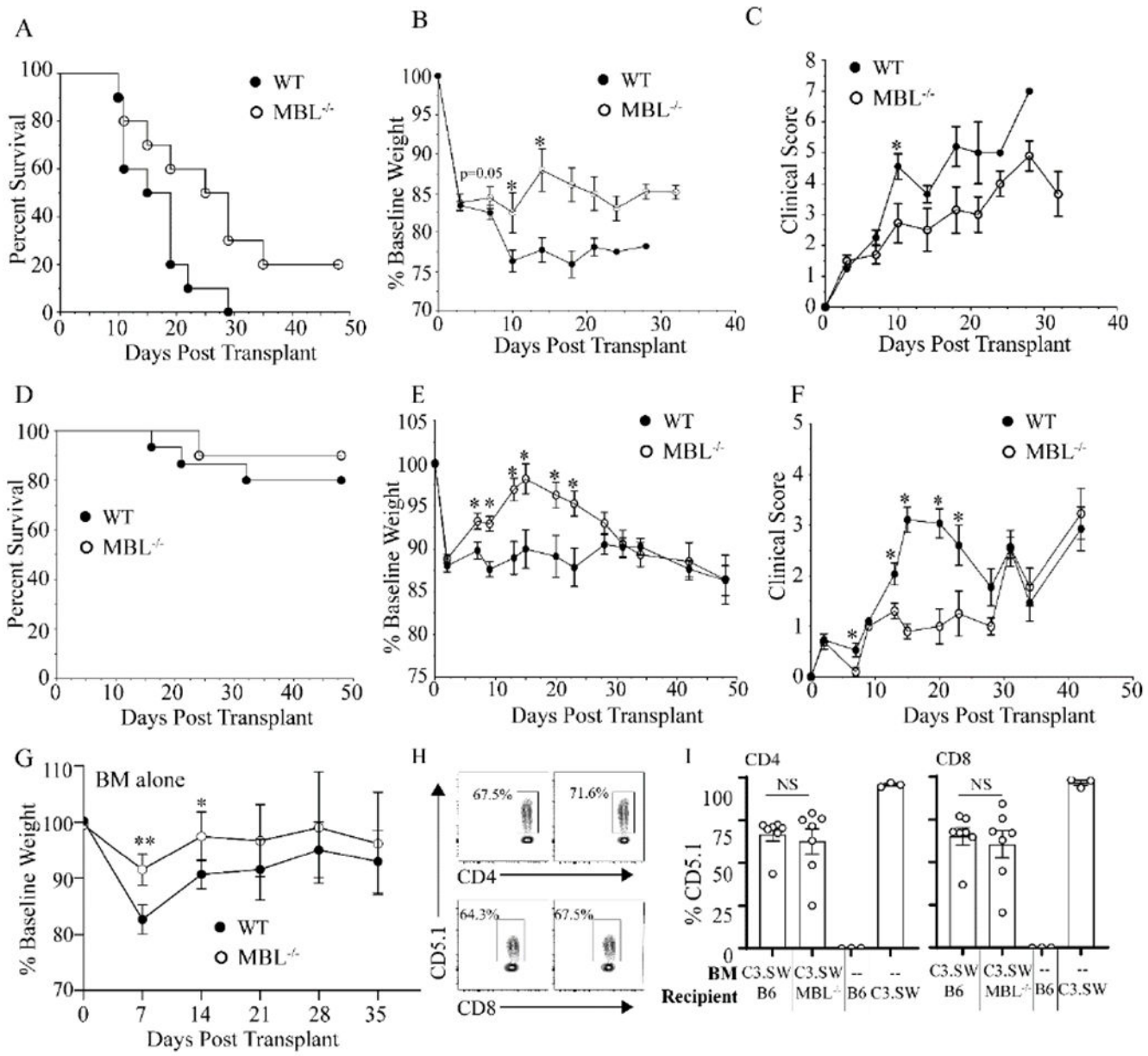

Figure 1. MBL-deficiency limits acute GVHD severity.

A-C. Survival rates (A, n=10/grp), serial weights (B, n=10/grp) and clinical scores (C, n=10/grp) for B6 WT and *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ B6 mice exposed to 1050 cGy conditioning radiation followed by C3H.SW bone marrow and 5x10^6 T cell transplantation. D-F. Survival rates (D, n=10-15/grp), serial weights (E, n=10-15/grp) and clinical scores (F, n=10-15/grp for WT and *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ B6 (MBL$^{-/-}$) mice exposed to 800 cGy conditioning radiation followed by bone marrow and 5x10^6 T cell transplantation (*p<0.05). Survival statistics calculated by log-rank mantel cox test. Weight and clinical scores were calculated using student's t-test comparing WT and *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ B6 mice at each specified time point. A p-value <0.05 considered statistically significant for all calculations. G. Serial weights (n=7/group) of B6 WT and *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ B6 mice exposed to 800 cGy conditioning radiation followed

by C3H.SW bone marrow without T cells. Statistics performed by one-way ANOVA with individual comparisons at each time point. *p<0.05, **p<0.01 H-I. Assessment of donor BM cell engraftment. Representative flow plots CD5.1 (C3.SW donor) expression gated on splenic $CD4^+$ T cell or $CD8^+$ T cells (H) and quantitation (I) WT or *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ B6 (CD5.2) recipients of C3.SW BM (without T cells, n=7/group) 6-weeks post-transplant. Spleen cells from WT (no transplant) B6 and C3.SW (n=3/group) are including in the graphs as controls. NS=not significant.

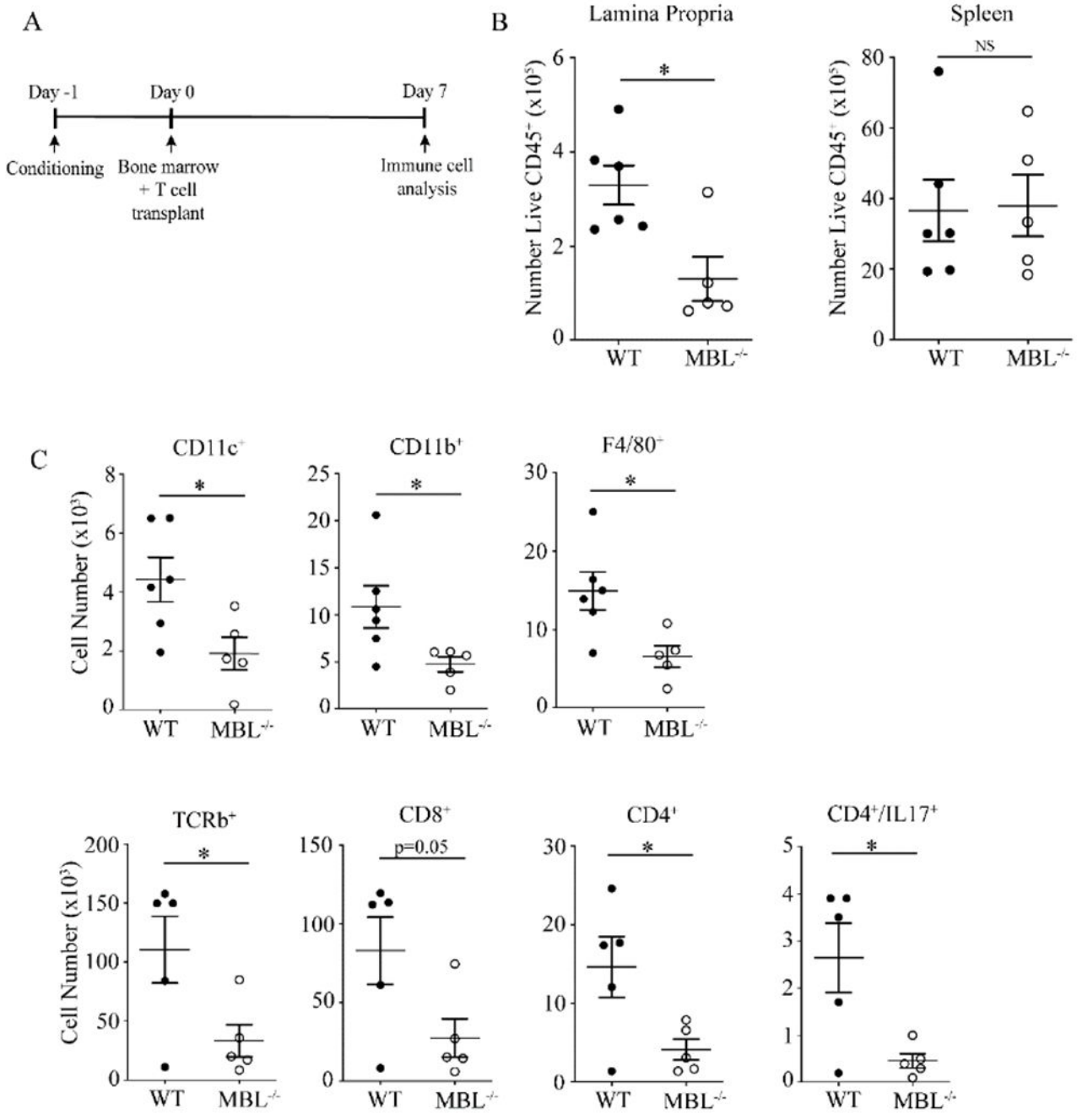

Figure 2. MBL promotes intestinal immune cell infiltration after induction of acute GVHD.

A. Schematic of experimental design. B. Quantification of $CD45^+$ leukocytes in the lamina propria (left panel) and spleen (right panel) of *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ ($MBL^{-/-}$) and WT B6 mice at 7d post-GVHD induction (n=5-6/grp, *p<0.05). C. Characterization and quantification of lamina propria immune cell infiltrate 7 d post-GVHD induction by flow cytometry. (n=5-6/grp, *p<0.05). Statistics calculated by student's t-test with a p-value <0.05 considered statistically significant.

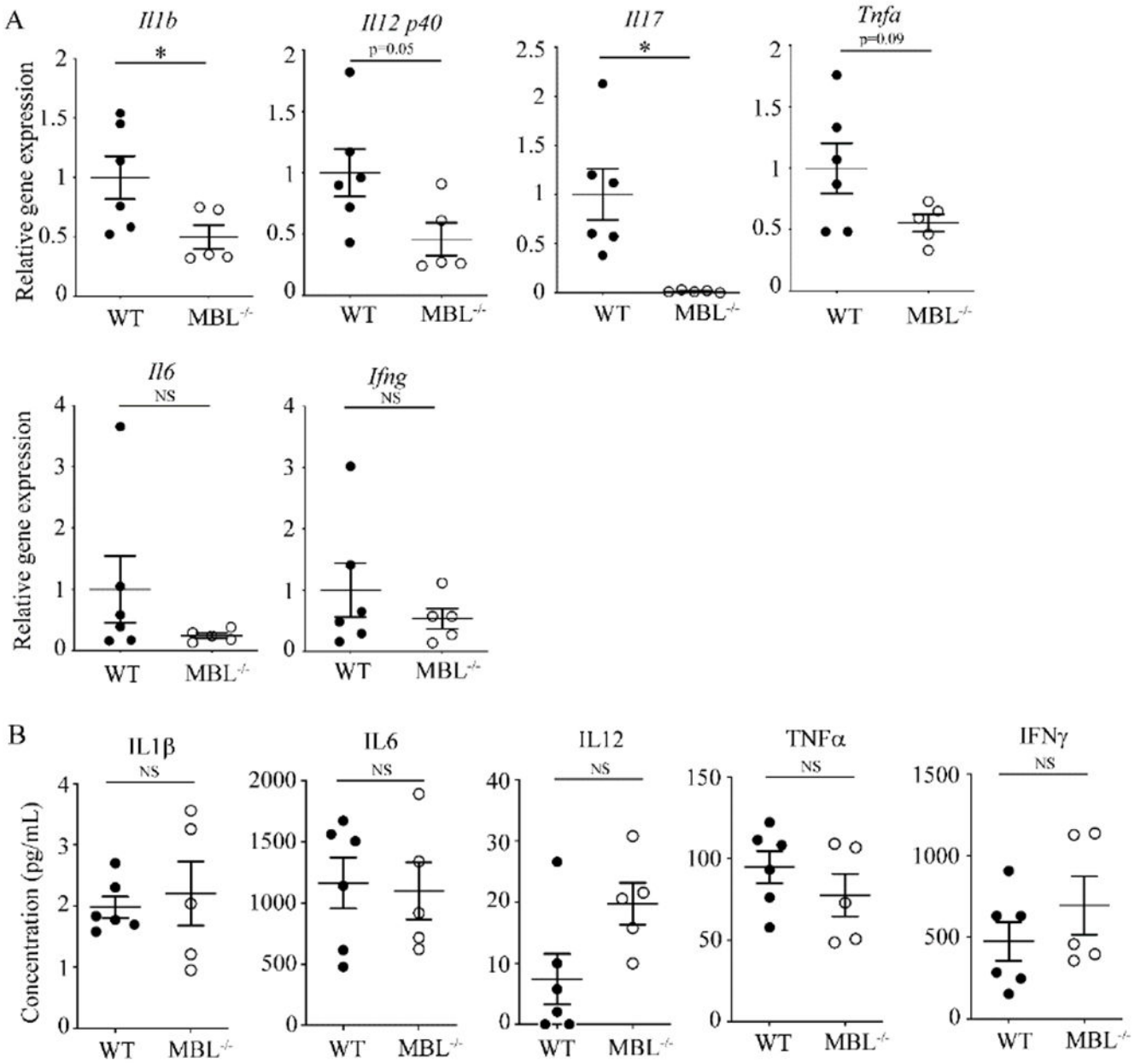

Figure 3. Local intestinal cytokine production after acute GVHD induction is MBL-dependent.

A. Intestinal RNA isolated from WT and *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ (MBL$^{-/-}$) mice at 7 d post-GVHD induction analyzed by qPCR for gene expression of inflammatory cytokines. Results are expressed as fold change relative to the mean of the WT condition. (n=5-6/grp, *p<0.05, NS=not significant). B. Serum cytokine levels from WT and *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ mice at 7d post-GVHD induction (n=5-6/grp, NS=not significant). Statistics calculated by student t-test with a p-value <0.05 considered statistically significant.

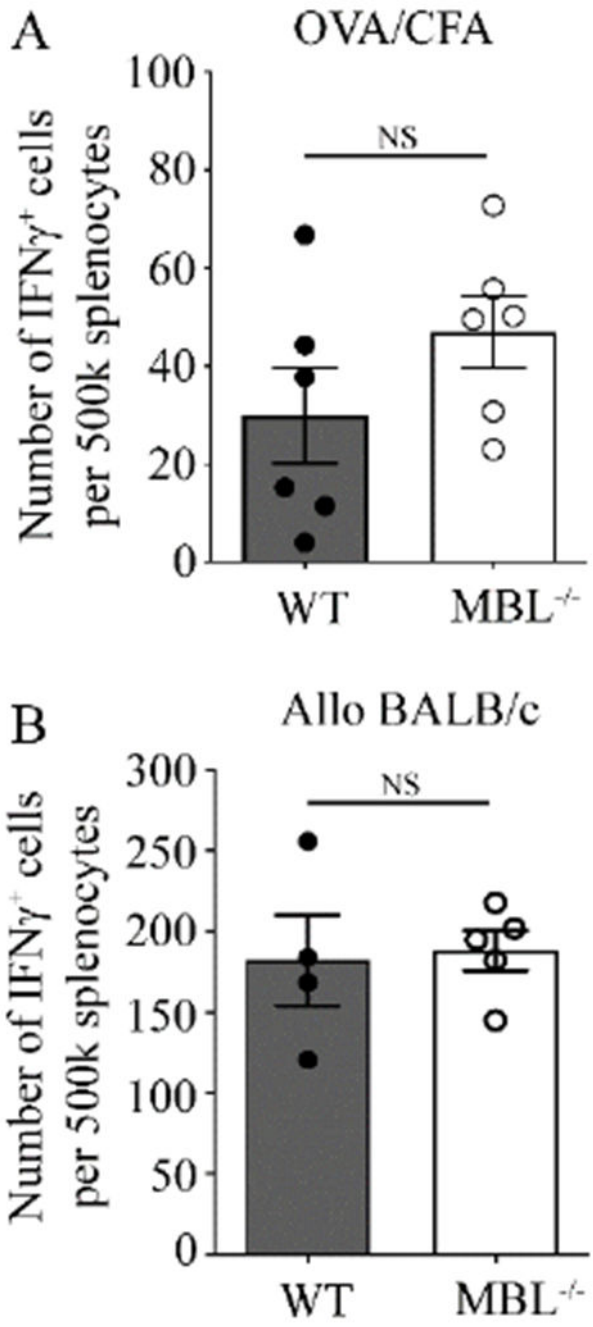

Figure 4. MBL deficiency does not alter strength of immunization-induced T cell responses.

A. Quantification (ELISPOT) of IFNγ-producing Ova-antigen reactive T cell response 21 d after subcutaneous immunization with OVA-CFA (n=6/grp. NS=not significant. Statistics calculated using student's t-test). B. Quantification (ELISPOT) of IFNγ-producing polyclonal allo-reactive B6 T cell response 21 d after intravenous immunization with allogeneic WT BALB/c splenocytes (n=6/grp. NS=not significant. Statistics calculated using student's t-test).

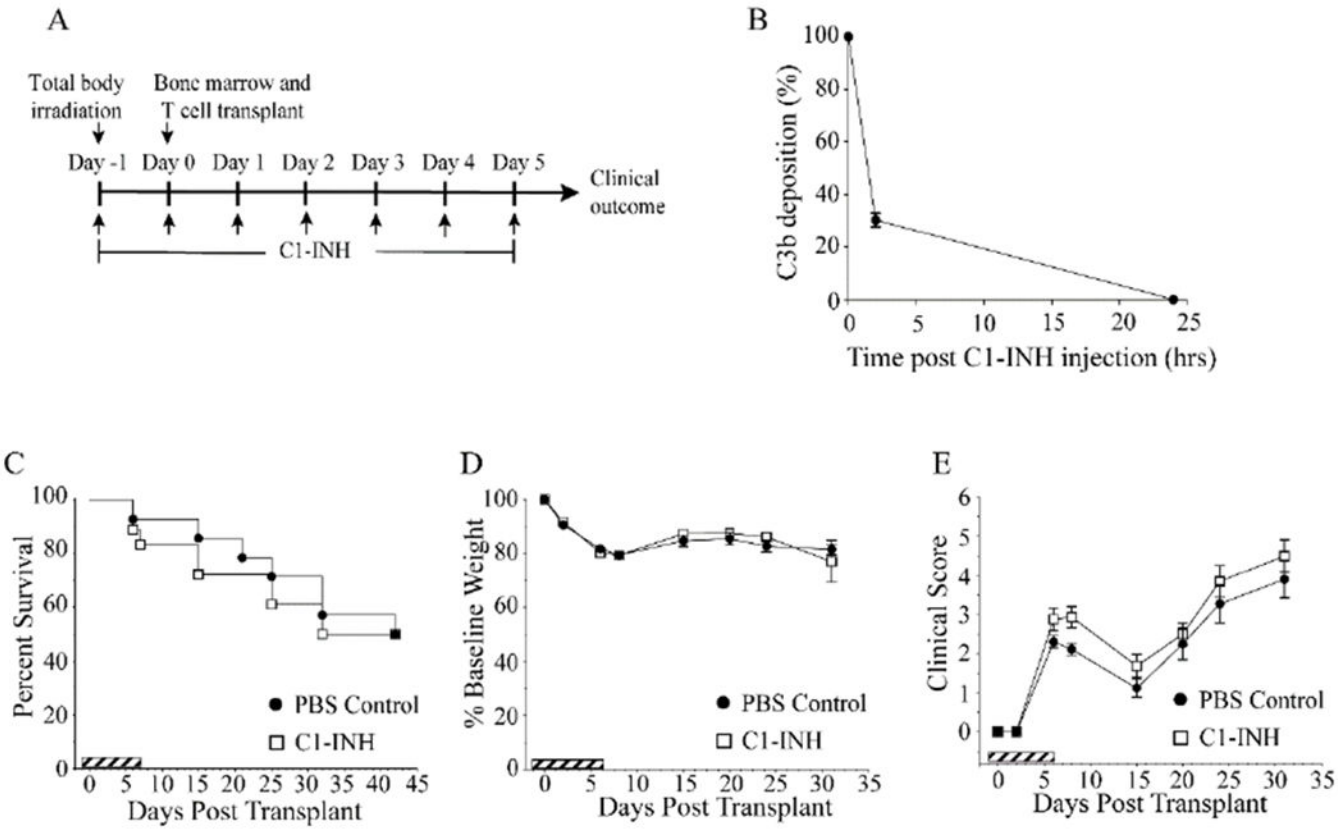

Figure 5. Administration of C1INH has no effect on GVHD outcome.

A. Schematic of experimental design. B. Serum obtained from WT mice before and serially after administration of a single dose of C1 INH was tested *in vitro* for mannan initiated C3b production. (C-E) WT B6 mice exposed to 1050 Gy conditioning radiation followed by bone marrow and $5x10^6$ T cell transplantation and treated with C1-inhibitor (0.4 IU/g body weight on days −1 through 5, striped bar) or control PBS and assessed for (C) survival (n=15-18), (D) weight (n=15-18), (E) clinical score (n=15-18). Survival statistics calculated by log-rank mantel cox test. Weight and clinical scores were calculated using student's t-test comparing WT and *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ B6 mice at each specified time point. A p-value <0.05 considered statistically significant.

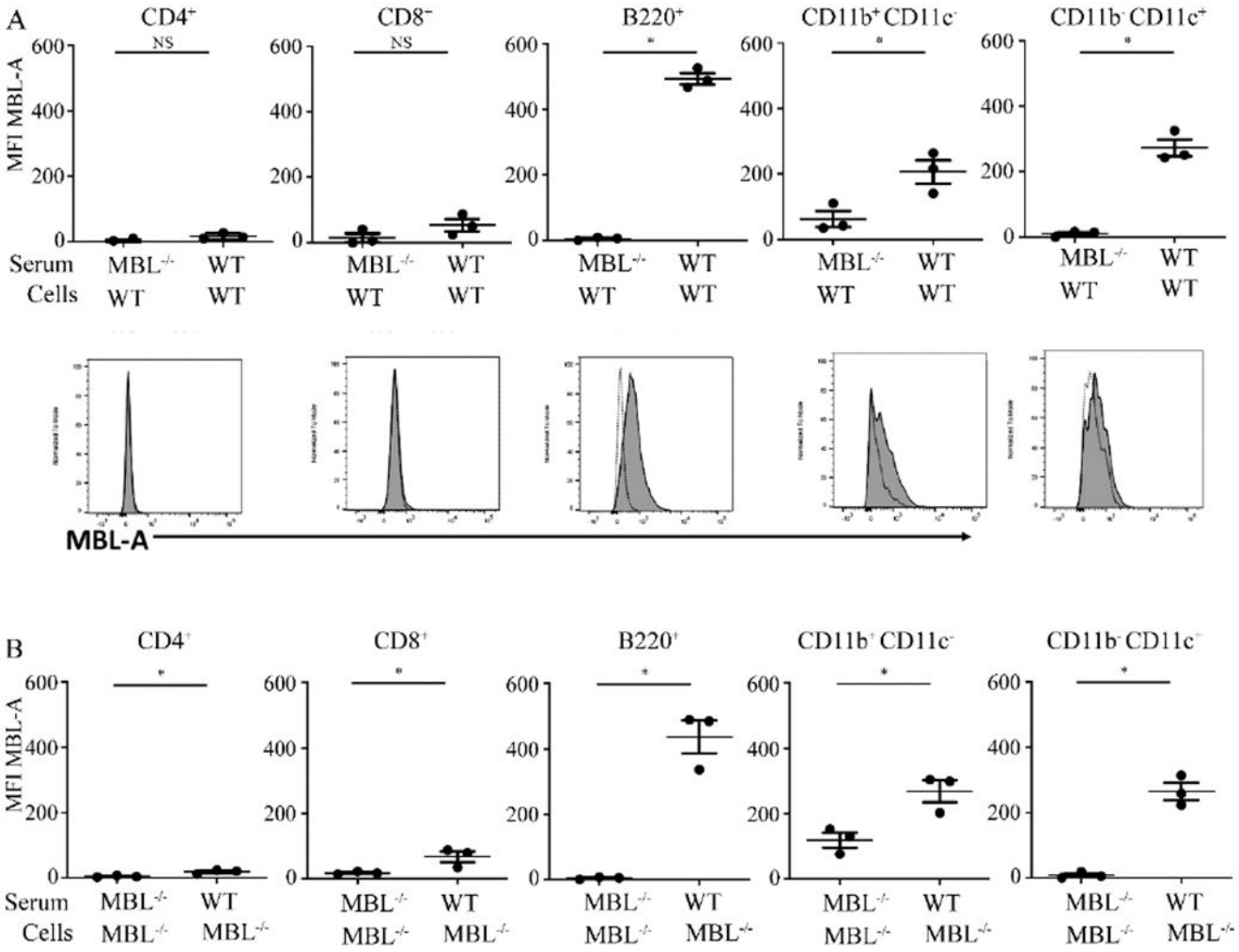

Figure 6. MBL-A is surface expressed on antigen presenting cells.

A. Splenocytes isolated from naïve WT B6 mice, incubated with serum from WT or *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ mice, and analyzed by flow cytometry for MBL-surface expression. Mean fluorescence index (MFI) over isotype control shown in upper panels and representative plots on lower panels. (n=3 mice/grp. *p<0.05). B. Splenocytes from *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ B6 mice incubated with WT or MBL-deficient serum and analyzed by flow cytometry for MBL expression as described above. (n=3 mice/grp. *p<0.05). Statistics calculated using student's t-test with a p-value <0.05 considered statistically significant.

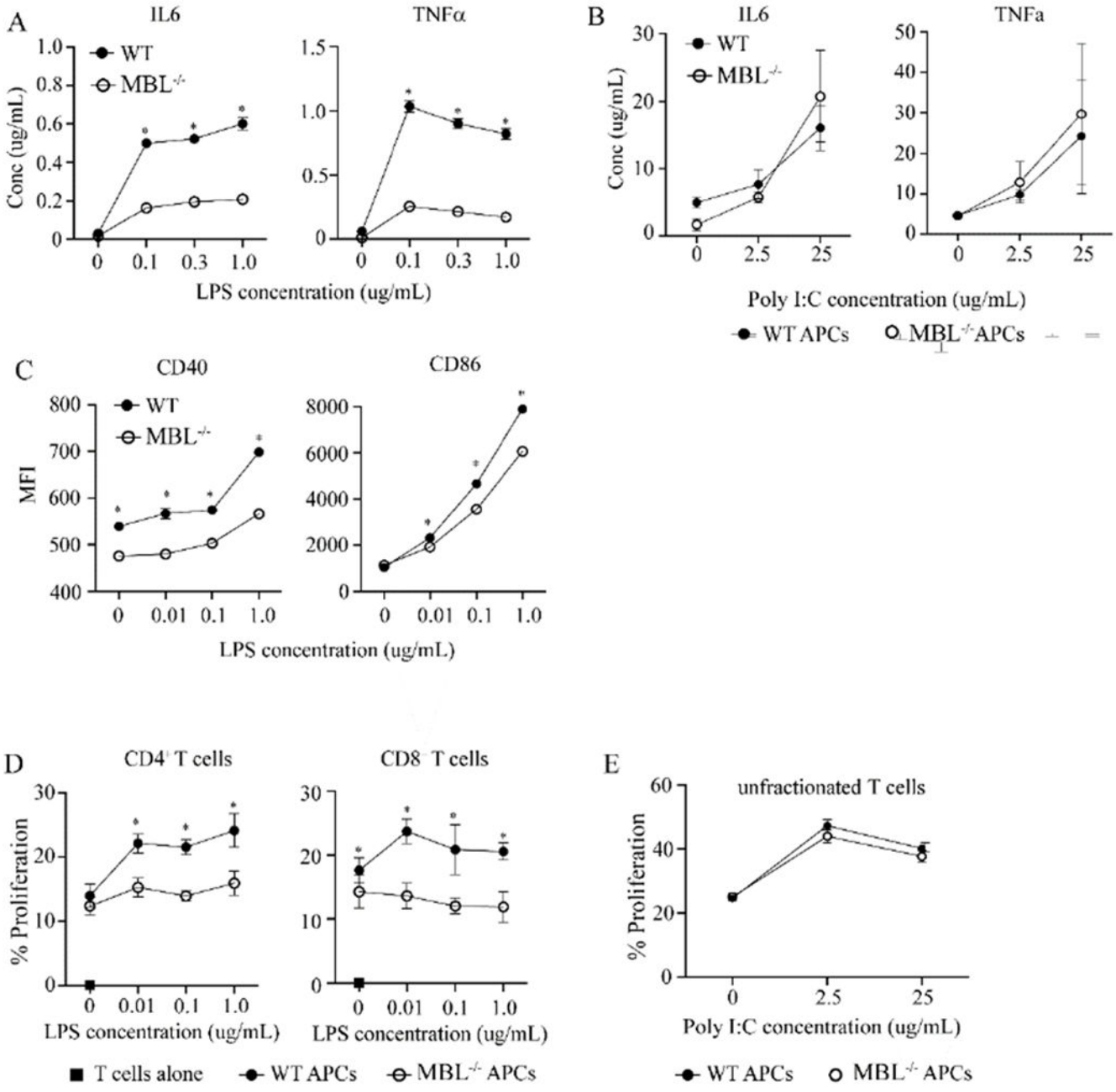

Figure 7. MBL facilitates LPS-induced APC activation and T cell proliferation.

A-C. WT or *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ B6 APCs were co-cultured with allogeneic WT BALB/c T cells for 18 hrs. with varying amounts of LPS (A, C) or Poly I:C (B). A-B. Culture supernatants were analyzed for IL-6 and TNFα by ELISA). C. CD11c+ APCs were analyzed by flow cytometry for expression of activation markers CD40 and CD86 (n=6/grp, *p<0.05). D-E. Proliferation assay (CFSE dilution) of WT BALB/c T cells co-cultured for 3 d with allogeneic WT or *Mbl1*$^{-/-}$*Mbl2*$^{-/-}$ B6 APCs and varying amounts of LPS (D) or Poly

I:C (n=6/grp, *p<0.05). All statistics calculated using student's t-test with a p-value <0.05 considered statistically significant.