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A Critical Role for the TLR4/TRIF Pathway in Allogeneic Hematopoietic Cell Rejection by Innate Immune Cells

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Abstract

We show for the first time that signaling through the TLR4/TRIF pathway plays a critical role in allogeneic bone marrow cell (BMC) rejection. This appears to be unique to BMC as organ allografts are rejected mainly via MyD88 signaling. Using T or T/B cell-deficient mice, we found that BMC allojection occurred early before T cell activation and was T and B cell-independent, suggesting an effector role for innate immune cells in BMC rejection. We further demonstrated the innate immune signaling in BMC allojection by showing superior engraftment in mice deficient in TRIF or TLR4 but not MyD88 or TLR3. The restored cytotoxicity in TRIF deficient recipients transferred with wildtype F4/80⁺ or NK1.1⁺ cells suggests TRIF signaling dependence on macrophages or NK cells in early BMC rejection. Production of the proinflammatory cytokine IL-6 and TRIF relevant chemokine MCP-1 was significantly increased early after bone marrow transplantation. *In vivo* specific depletion of macrophages or NK innate immune cells in combination with anti-CD154/rapamycin resulted in additive-enhanced allogeneic engraftment. The requirement for irradiation was completely eliminated when both macrophages and NK cells were depleted in combination with anti-CD154/rapamycin to target T and B cells, supporting the hypothesis that two barriers involving innate and adaptive immunity exist in mediating rejection of allogeneic BMC. In summary, our results clearly demonstrate a previously unappreciated role for innate immunity in BMC allojection via signaling through a unique MyD88-independent TLR4/TRIF mechanism. These findings may have direct clinical impact on strategies for conditioning recipients for stem cell transplantation.

Keywords

TLR4; TRIF; bone marrow transplantation; innate immunity

Introduction

Hematopoietic stem cell transplantation is curative for red blood cell disorders, inherited disorders of metabolism, numerous autoimmune disorders, and induces donor-specific tolerance to organ transplants. An understanding of the mechanism of bone marrow cell

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Conflict-of-interest disclosure:

S. Ildstad is the founding scientist and director of Regenerex, a biotech start-up company; it has not been capitalized.

L. Bozulic is a research scientist and employee of Regenerex.

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(BMC) rejection has allowed reduced-intensity conditioning approaches to be developed, resulting in significantly less morbidity and mortality (25,50). T cells have been implicated as the primary effector cells in alloreactivity (21). Recent studies in a pre-sensitized model of bone marrow cell rejection demonstrated that humoral immune responses predominate over T cell responses, although both contribute (47). The role of innate immunity in bone marrow transplantation (BMT) has not been fully explored.

The innate immune system acts as a sentinel for the immune system and functions to rapidly respond to infection. The molecular patterns on pathogenic cells are exquisitely distinguished from mammalian cells. Therefore, research in transplantation on rejection, tolerance, and immunosuppressive agents has been directed toward the adaptive immune response. The role of innate immunity in transplantation, with the exception of natural killer (NK) cells, has generally been ignored. However, not all clinical observations are explained by adaptive immune responses, suggesting factors other than recognition of MHC. For example, more immunogenic allografts such as skin, lung, and intestine are in close contact with the environment and commensal organisms (17,26). The overall survival for these grafts is significantly inferior to that of renal transplantation (26). The “danger signal” theory has been proposed to explain immune recognition and reaction to damage signals in the body regardless of their origin (26). In transplantation, the danger signals which may contribute to the initiation of an inflammatory response are considered to be antigen-independent stimuli, and include ischemia-reperfusion, surgical injury, systemic stress, and brain death (22,40).

There is growing evidence that innate immunity plays a role in many aspects of organ transplantation. In recent decades, the role of innate immune responses in the control of adaptive immunity has been revealed with the study of the toll-like receptors (TLR), one important recognition receptor of the pathogen-associated molecular patterns (10,20). Emerging evidence suggests that in addition to responding to pathogen-associated molecular patterns of microorganisms, TLR can be activated by endogenous ligands expressed by mammalian cells (3). TLR activation has been shown to be a barrier to the induction of transplantation tolerance (2,3,21). A critical role for the toll-like-receptor signaling adaptor protein myeloid differentiation factor 88 (MyD88) was recently reported in the rejection of organ allografts (11). Clinical studies suggest that activation of TLR4 signaling in lung transplant recipients contributes to the development of acute rejection (31).

In the present study, we show for the first time that the signaling through the TLR4/toll-interleukin 1 receptor domain-containing adapter inducing interferon- β (TRIF) pathway plays a critical role in allogeneic BMC rejection. The innate immune system acts as the first line barrier that precedes the fully appreciated adaptive immune barrier in BMT. We demonstrate that innate immune cells of NK cells and macrophages both elicit potent cytotoxicity against allogeneic BMC. This effect is independent of T and B cell responses in early allogeneic BMC rejection. These findings may have direct application to novel strategies to condition recipients for bone marrow transplantation.

Materials and Methods

Animals

Male C57BL/6 (B6; H-2^b), BALB/cJ (BALB/c; H-2^d), B10.BR/SgSnJ (B10.BR, H-2^k), B6.129P2-Tcrb^{tm1Mom}Tcrd^{tm1Mom}/J (TCR β / δ ^{-/-}, B6 background), B6.129S7-Rag1^{tm1Mom}/J (Rag^{-/-}, B6 background), B6.B10ScN-Tlr4^{lps-del}/JthJ (TLR4^{-/-}, B6 background), C57BL/10, B6;129S1-Tlr3^{tm1Flv}/J (TLR3^{-/-}), and B6129SF2/J (controls for TLR3^{-/-} mice with same genetic background) were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6J-*Ticam1*^{Lps2/Lps2} (TRIF^{Lps2/Lps2}, B6 background) pairs were purchased from The

Jackson Laboratory and bred at the University of Louisville. TRIF^{Lps2/Lps2} mice express a truncated form of TRIF that is inactive (14). MyD88^{-/-} mice (B6 background) were kind gifts from Shizuo Akira (via Ross Kedl; 3M Corporation, Minneapolis, MN) and bred at the University of Louisville. Animals were housed at the Institute for Cellular Therapeutics under specific pathogen-free conditions, and cared for according to NIH guidelines.

Pre-conditioning and bone marrow transplantation

Recipient B6 mice were conditioned with agents targeting innate immune cells in combination with T cell targeting based strategies and 0–100 cGy total body irradiation (TBI, γ cell 40; Nordion, Ontario, Canada). Agents targeting innate immune cells included anti-mouse Gr-1 (RB68C5, Rat IgG2b, κ , 500 μ g on day -3 and day -2), to deplete neutrophils; anti-mouse NK1.1 (PK136, mouse IgG2a, 1 mg on day -3) to delete NK cells; and clodronate liposomes (CloLip, Vrije Universiteit, Netherlands, 1.2 mg/10 g of animal weight at day -2) to deplete macrophages. With T cell targeting-based strategies, B6 mice were pre-treated i.p. with anti- $\alpha\beta$ -TCR (H57-597: hamster anti-mouse IgG, generated in our laboratory; 30 μ l per mouse at day -3), anti-CD154 (MR-1: hamster anti-mouse IgG3; BioXcell, Lebanon, NH; 0.5 mg per mouse at day 0 and +3), and rapamycin (3 mg/kg, day 0 through +4; LC Laboratory, Woburn, MA). Conditioned mice were transplanted with 15×10^6 untreated donor BMC via lateral tail vein injection 4–6 h after TBI. The intravenous injection of CloLip suspension has been reported to induce the complete depletion of splenic and hepatic macrophages within 24 h (43,42).

Chimerism testing

Recipients were characterized for chimerism using flow cytometry to determine the relative percentages of donor-derived peripheral blood leucocytes (PBL) at 4 wk. Peripheral blood was stained with Abs specific for MHC class-I antigens of donor (FITC-conjugated anti-H-2K^d, 36-7-5, mouse IgG2a) and recipient (PE-conjugated anti-H-2K^b, AF6-88.5, mouse IgG2a) origin. All antibodies were obtained from BD/PharMingen (San Jose, CA). Nonspecific background staining was controlled for using isotype-specific Ab directed against irrelevant Ag conjugated with the same fluorochrome.

Flow cytometric analysis of TCR V β families

BALB/c mice express superantigen I-E, resulting in the clonal deletion of superantigen-specific V β 5.1/2⁺ and V β 11⁺ T cells. As recipient B6 mice do not express I-E, they do not delete these two V β subfamilies. Peripheral blood (8–100 μ l) from mixed chimeras and naive control mice was stained with anti-V β 5.1/2-FITC (MR9-4), V β 6-FITC (RR4-7), V β 8.1/2-FITC (MR-5-2), or V β 11-FITC (RR3-15) vs. anti-host H2K^b-PE, anti-CD8-PerCP, and anti-CD4-APC (all from BD PharMingen). Relative expression indicates the percentage of V β -positive cells within the CD8⁺ or CD4⁺ T cell subsets of the host (H2K^b) lymphoid gate in peripheral blood.

In vivo cytotoxicity assay

Target and internal control splenocytes or BMC (100×10^6 /mL) were incubated with 4.0 μ M or 0.2 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands), respectively, at room temperature for 10 min. Equal volume of fetal bovine serum (Gibco BRL, Grand Island, NY) was added to quench the reaction. After washing, cells were mixed in a 1:1 ratio and resuspended in PBS, and 20×10^6 cells from each were injected intravenously. Peripheral blood was collected from individual mice at selected time points after cell infusion: 0.5 hr, 1 hr, 3 hr, day 1, day 2, day 3, and day 7. After lysis of red blood cells, peripheral-blood lymphocytes were analyzed for CFSE

expression by fluorescence-activated cell sorting (FACS). The percentage of killing was determined by calculating the ratio between target and internal control cells.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

Cytokines are evaluated using ELISA MAX^{TM} Set Deluxe (for IL-1 β and IL-6; Biolegend, San Diego, CA), VeriKine^{TM} Mouse ELISA Kit (for IFN- α and IFN- β ; PBL InterferonSource, Piscataway, NJ), ELISA Ready-SET-Go kit (for monocyte chemotactic protein-1 [MCP-1]; eBioscience, San Diego, CA), and Platinum ELISA 96 tests kit (for interferon-gamma-induced protein 10 [IP-10], eBioscience) as per the manufacturer's directions. Briefly, the specific antibody for each cytokine was pre-coated. One hundred microliters of the serum sample or cytokine standard was incubated for 2 h. After washing, the detection antibody specific for the particular cytokine was added and incubated for 1 h followed by 100 μ l of diluted Avidin-HRP solution for 30 min. The color was developed by adding 100 μ l TMB substrate solution and incubated in the dark for 15–30 min. After adding a stop solution of dilute hydrochloric acid, the optical density of color product was measured at 450 nm using ELISA microplate reader.

Statistical analysis

Data are presented as the average $\pm$ SD. Means of 2 groups were compared by the two-tailed *t* test (two-sample, assuming unequal variances). Means of more than 2 groups were compared by ANOVA. Statistical analysis was carried out using SigmaStat software (Jandel Scientific, San Raphael, CA). The difference between groups was considered significant at $P < 0.05$.

Results

Innate immunity mediates early allorejection of donor BMC

To evaluate the role of innate immunity in BMC rejection, T cell-deficient B6 mice (TCR β / $\delta^{-/-}$) as well as wildtype B6 mice were used as recipients in *in vivo* cytotoxicity assays. 20×10^6 CFSE-labeled allogeneic BALB/c donor (high CFSE fluorescence intensity) and control recipient (low CFSE fluorescence intensity) splenocytes or BMC were injected (Fig. 1A, B). Wildtype B6 mice that received only syngeneic CFSE-labeled cells with high or low intensity also served as a control. We found that the kinetics of cytotoxicity were very similar using either splenocytes or BMC as target cells. Therefore, the results from splenocytes or BMC were summarized together. Donor cells were rapidly eliminated in control wildtype B6 mice and rejection was complete by day 3. The kinetics of donor cell elimination in TCR β / $\delta^{-/-}$ mice resembled wildtype controls. The cytotoxic activity observed in this experiment was not due to the CFSE labeling as the syngeneic controls of B6 cells labeled with CFSE high/low exhibited no cytotoxicity. These results indicate that BMC rejection in wildtype mice was T cell-independent. To rule out any potential involvement of naturally occurring antibodies in the cytotoxicity observed, Rag $^{-/-}$ mice which produce no mature T and B cells were used. A similar kinetic of donor cell killing was observed (Fig. 1C, D). The rapid loss of allogeneic cells from immunocompetent mice is comparable with that in T or T/B cell-deficient mice, indicating that allogeneic BMC are subject to B cell and T cell-independent rejection early after transplantation (< 3 days). Thus, the effectors that mediate BMC rejection would presumably be innate immune cells.

Donor BMC are not sequestered preferentially in tissues

To rule out sequestration of donor BMC in other tissues, the cytotoxic activity in peripheral blood was compared with that for spleen, lymph node, and femur. 20×10^6 CFSE-labeled BALB/c donor (high intensity) and B6 recipient (low intensity) BMC were injected into

naïve B6 mice. Spleens, lymph nodes, femurs, and peripheral blood were collected from each recipient on day 1 after BMC infusion. Significant killing rates were observed in these different tissues tested (Fig. 1E) resembling the results in peripheral blood. These results suggest that the killing rates of allogeneic BMC in peripheral blood reflect the fate of donor cells in other tissues. The allogeneic BMC are not trapped preferentially in specific tissues.

Allogeneic BMC rejection is dependent on the TRIF and not the MyD88 pathway

An effector role for innate immune cells in BMC rejection was suggested in our findings in T-deficient mice that BMC allojection was T cell-independent. Innate immune system initiates defense mechanism with sensing function of the family of toll receptors. The signaling function of TLR depends upon intracellular adaptors. The majority of TLR signal via MyD88 and TRIF. The adaptor MyD88 transmits signals emanating from all TLR, except TLR3, while TRIF specifically mediates TLR3 and TLR4 signaling via type 1 IFN (51,30). To determine which TLR signaling pathway is involved in BMC rejection, MyD88 deficient ($MyD88^{-/-}$) and TRIF deficient ($TRIF^{Lps2/Lps2}$) mice were nonmyeloablatively conditioned with established conditioning strategies targeting T and B cells: anti-CD154/rapamycin/100 cGy TBI and transplanted with 15×10^6 BALB/c BMC (Fig. 2A). A threshold of alloengraftment was observed in our previous study with anti-CD154/rapamycin/100 cGy in wild-type B6 mice that served as controls in our current experiment. As expected, engraftment was achieved in 20.0% of recipients at 1 month after BMT. Surprisingly, engraftment was achieved in only 30% of $MyD88^{-/-}$ recipients at 1 month, resembling that for wild-type B6 mice. In contrast, 100% engraftment was achieved in $TRIF^{Lps2/Lps2}$ mice (Fig. 2B). The level of donor chimerism in $TRIF^{Lps2/Lps2}$ mice was $5.1 \pm 0.1\%$ at one month, which was significantly higher than in $MyD88^{-/-}$ ($1.6 \pm 0.8\%$; $P < 0.05$) or in wild-type B6 mice ($1.3 \pm 0.8\%$; $P < 0.05$) and thereafter throughout all time points tested up to 6 months (Fig. 2C, $P < 0.05$). The levels of donor chimerism increased with time, reaching $17.1 \pm 8.6\%$ in $TRIF^{Lps2/Lps2}$ recipients at six months. These results suggest that allogeneic BMC rejection is independent of the MyD88 pathway but requires TRIF signaling.

Allogeneic BMC rejection is dependent on the TLR4 pathway

TRIF cascade is a MyD88-independent signaling pathway specifically associated with TLR3 and TLR4. We therefore next determined whether the TLR3 or TLR4 pathway was involved in BMC rejection using $TLR3^{-/-}$ and $TLR4^{-/-}$ mice as recipients. $TLR3^{-/-}$ and $TLR4^{-/-}$ mice were conditioned with anti-CD154/rapamycin/100 cGy TBI for BMT (Fig. 2D, E). None of the $TLR3^{-/-}$, 20% of B6129SF2/J mice ($TLR3^{-/-}$ controls), and only one of 10 wild-type B6 mice (controls for $TLR4^{-/-}$) engrafted at 1 month. In striking contrast, 85.7% of $TLR4^{-/-}$ mice engrafted. The donor chimerism was $4.2 \pm 1.8\%$ at 1 month and increased with time, reaching $10.8 \pm 4.7\%$ at 5 months. The levels of donor chimerism were comparable between $TLR4^{-/-}$ and $TRIF^{Lps2/Lps2}$ recipients over the 5 months follow-up ($P > 0.1$). Taken together, these results suggest that rejection of allogeneic BMC is exclusively dependent on the TLR4/TRIF signaling pathway.

Depletion of macrophages, NK cells, or both, but not granulocytes from recipients delays donor cell allojection

The timing of acute rejection of BMC in wildtype B6 was similar with T cell-deficient recipients and occurred within 3 days prior to the time required for T cell activation. Thus the effectors mediated BMC rejection at this period of time would be innate immune cells: macrophages, neutrophils, or NK cells. To differentiate the role for these innate immune cell populations in BMC allojection, *in vivo* cytotoxicity assays were performed in mice from which macrophages, NK cells, or neutrophils had been depleted with CloLip, anti-NK1.1, or

anti-Gr-1, respectively (Fig. 3A, B). The kinetics of elimination of donor cells in mice treated with anti-Gr-1 mAb was similar to saline-treated controls. Donor cells were totally eliminated by day 3. The rejection of donor cells was delayed in recipients treated with CloLip alone, anti-NK1.1 alone, or both, and reached the most significance ($P < 0.003$) at day 1 after infusion compared with saline-treated controls ($88.7 \pm 4.5\%$). At day 1, the killing percentages of CFSE labeled BALB/c cells were $58.0 \pm 2.9\%$, $66.9 \pm 14.0\%$, or $42.6 \pm 7.5\%$ in recipients treated with anti-NK1.1 alone, CloLip alone, or both, respectively. The impaired cytotoxicity remained significant ($P < 0.05$) at day 3. Subsequently, all donor cells were eliminated in all three groups by day 7, most likely representing the initiation of adaptive immunity. The decreased cytotoxicity in recipients treated with CloLip and anti-NK1.1 was additive, indicating that in addition to NK cells, macrophages play an important and non-redundant role in early allogeneic BMC rejection.

Depletion of macrophages and/or NK cells in combination with T cell targeting based strategies eliminates the requirement for irradiation for engraftment

Targeting host-versus-donor reactive T cells in recipients has significantly reduced the minimum TBI dose required for engraftment (48, 49). Because our data suggested that innate immune cells were effectors in early BMC rejection, we hypothesized that by specifically targeting both macrophages and NK cells in addition to adaptive immune cells, engraftment could be achieved without irradiation as a novel approach to conditioning.

To evaluate the efficacy of targeting selected innate immune cells in conditioning for allogeneic engraftment, B6 mice received CloLip, anti-NK1.1 mAb, or anti-Gr-1 mAb. These agents were used in combination with anti- $\alpha\beta$ -TCR/anti-CD154/rapamycin. Conditioned recipients were transplanted with 15×10^6 BALB/c BMC (Fig. 4A). Allogeneic engraftment was achieved in 45.5% of recipients conditioned with anti- $\alpha\beta$ -TCR/anti-CD154/rapamycin plus 100 cGy TBI. The addition of CloLip alone, anti-NK1.1 alone, or both agents to the conditioning resulted in engraftment in 100% of recipients with donor chimerism of $23.5 \pm 8.0\%$, $19.1 \pm 11.4\%$, and $39.4 \pm 3.4\%$, at one month (Fig. 4B, C), respectively. Engraftment remained 100% at 6 months in all three groups (Fig. 4D). When anti-Gr-1 was utilized, only 33.3% of animals engrafted. When TBI was eliminated from preconditioning, none of the control mice engrafted when conditioned with anti- $\alpha\beta$ -TCR/anti-CD154/rapamycin. However, engraftment occurred in 40% or 60% of recipients treated with addition of anti-NK1.1 or CloLip, respectively. When both anti-NK1.1 and CloLip were added, engraftment occurred in 100% of recipients (Fig. 4B). Donor chimerism was $1.9 \pm 0.9\%$ at 1 month and the animals remained chimeric 6 months (Fig. 4C, D). Moreover, specific V β 5.1/2 and V β 11 clonal deletion was detected in host original CD4⁺ T cells in all chimeras ($n = 23$, donor % ranged from 2.5% to 46.7%), suggesting the induction of central tolerance to donor alloantigens.

We next assessed whether chimerism could be successfully achieved without use of T cell depletion. B6 mice were conditioned with CloLip and/or anti-NK1.1 mAb in combination with anti-CD154/rapamycin (Fig. 5A–C). Allogeneic engraftment was achieved in 30% of control recipients conditioned with anti-CD154/rapamycin plus 100 cGy TBI. With the addition of CloLip alone to anti-CD154/rapamycin/100 cGy conditioning, 72.7% ($n = 11$) of recipients engrafted with levels of donor chimerism $2.8 \pm 1.6\%$ 1 month after BMT. The addition of anti-NK1.1 alone or both of anti-NK1.1 and CloLip to anti-CD154/rapamycin/100 cGy conditioning resulted in 100% engraftment, with levels of donor chimerism $8.4 \pm 1.1\%$ or $25.0 \pm 8.0\%$, respectively at 1 month. With anti-NK1.1 and CloLip plus anti-CD154/rapamycin, without irradiation and host T cell depletion, 100% engraftment was also achieved with $1.9 \pm 1.2\%$ donor chimerism at one month and remained durable and stable during 6-month follow up (Fig. 5C).

The early rejection of allogeneic BMC by macrophages or NK cells is TRIF signaling dependent

To study the role of TRIF signaling in BMC rejection by macrophages and NK cells, TRIF^{Lps2/Lps2} mice were used as recipients for *in vivo* cytotoxicity assays after adoptive transfer of wildtype macrophages and NK cells (Fig. 6). Wildtype F4/80⁺ macrophages were sorted from B6 spleens and peritoneal cavities and NK1.1⁺ NK cells from B6 spleens. The doses of transferred NK1.1⁺ and F4/80⁺ cells were 370,000 and 140,000 per recipient, respectively. One day after transfer, 20×10^6 CFSE-labeled BALB/c target (high intensity) and internal control B6 (low intensity) BMC were injected. TRIF^{Lps2/Lps2} mice that did not receive transferred cells and wildtype B6 mice treated with saline served as controls.

As expected, donor cells were rapidly eliminated in control wildtype B6 mice and rejection was complete by day 3. The rejection of donor cells was significantly less in TRIF^{Lps2/Lps2} mice without receiving transferred cells compared with wildtype B6 controls, from marginal significance ($P = 0.04$) at 3 hr to the most significance ($P = 0.0001$) at day 3 after cell infusion. At day 3, the killing rates were $91.4 \pm 1.4\%$ in TRIF^{Lps2/Lps2} mice without transferred cells and $97.6 \pm 1.5\%$ in wildtype B6 controls. The eliminating rates of donor cells were increased in TRIF^{Lps2/Lps2} recipients received either F4/80⁺ or NK1.1⁺ cells and the kinetics of elimination of donor cells was shifted to resemble B6 controls with no significant difference between them at these 3 time points (P values: 0.11 to 0.87). However, the eliminating rates of donor cells were significantly increased in TRIF^{Lps2/Lps2} recipients received either F4/80⁺ or NK1.1⁺ cells when compared with TRIF^{Lps2/Lps2} controls at all time points (P values: 0.038 to 0.002), except the one at 3hr when compared between TRIF^{Lps2/Lps2} recipients received NK1.1⁺ cells and TRIF^{Lps2/Lps2} controls ($P = 0.058$). At day 1, the killing percentages of CFSE labeled BALB/c cells were $71.0 \pm 5.1\%$, $69.1 \pm 3.9\%$, or $61.1 \pm 5.8\%$ in TRIF^{Lps2/Lps2} recipients received either F4/80⁺ cells, NK1.1⁺ cells, or none, respectively. Taken together, these data suggest that TRIF signaling is essential for macrophage- and NK cell-mediated allo-bone marrow cell rejection.

Interleukin-6 and monocyte chemoattractant protein (MCP)-1 production is significantly increased early and transiently after allogeneic BMC infusion

TLR signaling mediates activation of the NF- κ B-dependent promoter to trigger the induction of inflammatory cytokines such as IL-1 β and IL-6 (20,36). IL-6 also promotes Th2 and Th17 type responses to generate cytotoxic effectors and inflammation. Activation of type I IFN, IFN- α and IFN- β , is also involved in the immune response of the adaptor protein TRIF in innate immunity (13). Moreover, IP-10 and MCP-1 have been shown to be TRIF relevant chemokines (18,51). Therefore, the acute phase cytokines of IL-1 β and IL-6, type I IFN (IFN- α and IFN- β), as well as chemokines of IP-10 and MCP-1 in recipients were assessed following allogeneic BMC infusion. IL-6 production was detectable very early at 0.5 and 1 hour after BMT, then peaked at 4 and 8 hours. The serum level of IL-6 decreased rapidly to undetectable levels at 12 hours (Fig. 7A). Notably, MCP-1 levels significantly increased 1 hour after allogeneic BMC infusion ($P = 0.0034$) when compared with the values pre-BMT (Fig. 7B). This peak was significantly higher than values at 4 hours ($P = 0.032$). Other cytokines and chemokines, including IL-1 β , IFN- α , IFN- β , and IP-10, were not detectable (data not shown). These data suggest that IL-6 and MCP-1 are critical cytokine and chemokine that may participate in mediating allo-rejection of BMC.

Discussion

The barrier for rejection of allogeneic BMC has been attributed primarily to adaptive immunity, especially T cell immune responses. With T cell based targeting conditioning strategies, significant progress has been made in developing non-myeloablative conditioning

strategies to achieve mixed chimerism. It has been well known that T cell depletion (48,49) combined with other conditioning strategies, such as immunosuppressive drugs or costimulation-blocking agents (23,37,46) enhances allogeneic BM engraftment. The role of the innate immune system in BMC allojection has not been adequately addressed. However, in addition to T cell targeting-based conditioning, better alloengraftment still requires additional conditioning from nonspecific reagents, such as irradiation and immunosuppressive drugs. The need of other conditioning to improve alloengraftment may suggest the existence of another barrier in addition to T cell barrier in BMT. As the humoral immunity is unlikely a barrier for BMT in unprimed naive recipients, innate immune system is most likely the another barrier in BMT. In the present studies, we have evaluated the role of innate immunity in rejection of BMC and show for the first time that the allojection of BMC is predominantly mediated by TLR4 signaling through the TRIF pathway.

T and T/B cell-deficient mice were used as recipients to study the fate of allogeneic cells very early after transplantation. Donor cell elimination occurred within 3 days in TCR β / δ ^{-/-} and Rag^{-/-} mice, similar to wildtype B6 controls, indicating that the early rejection of allogeneic BMC occurred before T cell-activation and was T and B cell-independent. The T cell-independent mechanism was also demonstrated in previous findings in that allogeneic BMC survived very similarly between recipients with T cell depletion by mAb and naïve recipients (12). The disappearance of allogeneic cells in peripheral blood was the result of cytotoxic activity and not due to sequestering in other tissues or the CFSE labeling as confirmed by the controls. These results implicate an effector role for innate immune cells in early BMC rejection.

The innate immune system represents the first line defense against pathogens. In transplantation, the role of these defenses has not been fully appreciated until recently. Goldstein found that rejection of HY-incompatible skin grafts is MyD88 dependent and not mediated by TLR2 or TLR4 (11). They successfully extended their findings to a MHC fully mismatched model but the co-stimulatory blockade was required (45). MyD88 is an adaptor molecule essential for signaling via most of the TLR to induce translocation of NF- κ B and pro-inflammatory cytokine secretion (24,33). The possible mechanisms for these findings are that activation of innate immunity early during organ transplantation generates a pro-inflammatory environment that makes recipients more resistant to tolerance induction.

Whether and how the innate immune system recognizes or responds to allogeneic BMC remains unknown. To our surprise, our findings in mice deficient in TRIF or MyD88 adaptor indicate that allogeneic BMC rejection occurred exclusively through TRIF signaling but not MyD88. This is in contrast with prior reports demonstrating the critical role of the MyD88 pathway in rejection of transplanted skin (45) and lung (31,35). This may reflect the specificity of BMT as cellular transplants compared to other transplants. Moreover, we found that mice deficient in TLR4 but not TLR3 had superior engraftment of allogeneic BMC compared with wild type controls. These results suggest that allogeneic BMC transplantation triggers TLR4 and subsequently the TRIF pathway, resulting in the activation of innate immune macrophages/NK cells. The innate immune response to allogeneic BMC via TLR4 but not TLR3 may be a result of their biological locations. TLR4 is cell membrane-bound, while TLR3 is associated with the intracellular endosomal membrane (32). Alternatively, it may be due to the fact that bone marrow transplants involve a single cell infusion while organ allografts are transplanted as a primarily vascularized unit.

In transplantation, TLR activation has been demonstrated to generate a pro-inflammatory environment that makes the recipient less susceptible to the tolerance induction (9,11,28). TLR4 activation by LPS at the time of co-stimulation blockade shortens the survival of skin,

cardiac, and islet allografts (5,27,39). Although it has been difficult to absolutely exclude microbial contaminants to activate TLR, non-infectious endogenous inflammatory signals have been implicated to contribute to acute allograft rejection (2,10). The endogenous TLR ligands including heat shock proteins, hyaluronan, and high mobility box group 1 (HMGB1) have been found to activate the innate immune system predominantly via TLR2 and TLR4 (29,38,41). The engagement of hyaluronan with TLR2 and TLR4 has been demonstrated to result in activation of macrophages (16). However, the endogenous activators released after allogeneic BMT remain unknown and need to be further defined.

NK cells have been shown to play a major role in marrow rejection (12,7,15,19). They express inhibitory and activating receptors that are implicated in engraftment. The role of macrophages and neutrophils in rejection of BMC has not been defined, nor has the TLR signaling pathways. We observed that macrophages contributed significantly to the early elimination of transplanted cells. We further found that depletion of macrophages or NK cells combined with T and B cell conditioning-based strategies significantly enhanced alloengraftment. An additive effect was observed when both cellular populations were depleted, suggesting a non-redundant role. Targeting of innate immunity in addition to adaptive immunity completely eliminated the need for TBI to establish chimerism. Alternatively, depleting both NK cells and macrophages plus targeting T cells may provide sufficient “space” in recipient marrow for the donor stem cells to populate. Our data strongly support the hypothesis that two barriers involving innate and adaptive immunity coexist in response to allogeneic BMC. Thus, chimerism could be achieved with minimal regimen-related morbidity by targeting both the innate and adaptive arms of the immune response. This safe approach to condition recipients to establish mixed hematopoietic chimerism could have significant impact in the clinical application of bone marrow transplantation to treat hemoglobinopathies, autoimmune disorders, and induce tolerance to solid organ and cellular transplants. Moreover, the restored cytotoxicity in TRIF^{Lps2/Lps2} recipients received either F4/80⁺ or NK1.1⁺ cells suggests that the early rejection of allogeneic BMC by macrophages or NK cells is TRIF signaling dependent.

Innate immunity serves as an important link between antigen-dependent and antigen-independent responses. The cytokine environment plays a primary role to determine the link (44). When an organ or tissue is transplanted, an inflammatory response is induced through an intricate cascade of events initiated by cytokines. These signals are crucial for the generation of a potent adaptive immune response. We found that IL-6 was up-regulated early within a 12-hour in response to allogeneic BMC infusion. IL-6 promotes Th2 responses and also plays a critical role in the promotion of Th17 differentiation in combination with TGF- β (1,52). Modulation of macrophage function is linked to increased production of IL-17 by CD4 T cells (34). A counter-regulatory relationship between Th17 and regulatory T cells (T_{reg}) has been established. Thus, IL-6-mediated enhancement of Th17 would likely decrease acceptance of transplants. Indeed, IL-17 secreted by Th17 cells has been demonstrated in acute transplant rejection (6,53). In addition, we found that the serum levels of MCP-1 increased in response to allogeneic BMT. MCP-1 gene is one of the IFN-inducible genes that function in a MyD88-independent manner (8,18). Type-I IFN activation is involved in the innate immune response through TRIF (13). The increased MCP-1 may also chemoattract more macrophages and NK cells into the site of inflammation (4), amplifying the pro-inflammatory responses.

In summary, we show that BMC allorejection was critically dependent on TLR4/TRIF signaling. We further demonstrated that innate immune cell populations of macrophages and NK cells mediate early BMC rejection. By targeting both of these innate cell populations as conditioning, allogeneic BMC engraftment was achieved without irradiation. Based on these findings, we propose that the allogeneic BMT may cause release of certain damage-

associated molecular patterns into the extracellular matrix that subsequently trigger the TLR4 signaling pathway of both macrophages and NK cells. The activation of the TLR4 leads to TRIF-dependent NF- κ B activation and subsequently the release of the MCP-1 and IL-6. The increased MCP-1 chemoattracts more NK cells and macrophages into the site of inflammation; thus, activated innate immune cells by TLR4 and subsequent induction of pro-inflammatory cytokines eradicate allo-BMC. The findings of the role of innate immunity in BMC rejection may allow development of reduced intensity conditioning regimens to promote engraftment with reduced toxicity.

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Reference List

- Aggarwal S, Gurney AL. IL-17: prototype member of an emerging cytokine family. *J Leukoc Biol.* 2002; 71:1–8. [PubMed: 11781375]
- Alegre ML, Goldstein DR, Chong AS. Toll-like receptor signaling in transplantation. *Curr Opin Organ Transplant.* 2008; 13:358–365. [PubMed: 18685330]
- Andrade CF, Waddell TK, Keshavjee S, Liu M. Innate immunity and organ transplantation: the potential role of toll-like receptors. *Am J Transplant.* 2005; 5:969–975. [PubMed: 15816875]
- Chen D, Carpenter A, Abrahams J, Chambers RC, Lechler RI, McVey JH, Dorling A. Protease-activated receptor 1 activation is necessary for monocyte chemoattractant protein 1-dependent leukocyte recruitment in vivo. *J Exp Med.* 2008; 205:1739–1746. [PubMed: 18606855]
- Chen L, Wang T, Zhou P, Ma L, Yin D, Shen J, Molinero L, Nozaki T, Phillips T, Uematsu S, Akira S, Wang CR, Fairchild RL, Alegre ML, Chong A. TLR engagement prevents transplantation tolerance. *Am J Transplant.* 2006; 6:2282–2291. [PubMed: 16970798]
- Chen Y, Wood KJ. Interleukin-23 and TH17 cells in transplantation immunity: does 23+17 equal rejection? *Transplantation.* 2007; 84:1071–1074. [PubMed: 17998858]
- Cudkowicz G, Bennett M. Peculiar immunobiology of bone marrow allografts. II. Rejection of parental grafts by resistant F1 hybrid mice. *J Exp Med.* 1971; 134:1513–1528. [PubMed: 4942407]
- Doyle S, Vaidya S, O'Connell R, Dadgostar H, Dempsey P, Wu T, Rao G, Sun R, Haberland M, Modlin R, Cheng G. IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity.* 2002; 17:251–263. [PubMed: 12354379]
- Goldstein DR. Toll like receptors and acute allograft rejection. *Transpl Immunol.* 2006; 17:11–15. [PubMed: 17157206]
- Goldstein DR. The identity of innate immune activators in organ transplantation: origins from within or exterior to the host? *Am J Transplant.* 2007; 7:1692–1694. [PubMed: 17524077]
- Goldstein DR, Tesar BM, Akira S, Lakkis FG. Critical role of the Toll-like receptor signal adaptor protein MyD88 in acute allograft rejection. *J Clin Invest.* 2003; 111:1571–1578. [PubMed: 12750407]
- Hamby K, Trexler A, Pearson TC, Larsen CP, Rigby MR, Kean LS. NK cells rapidly reject allogeneic bone marrow in the spleen through a perforin- and Ly49D-dependent, but NKG2D-independent mechanism. *Am J Transplant.* 2007; 7:1884–1896. [PubMed: 17617852]

13. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. A Toll-like receptor recognizes bacterial DNA. *Nature*. 2000; 408:740–745. [PubMed: 11130078]
14. Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO, Goode J, Lin P, Mann N, Mudd S, Crozat K, Sovath S, Han J, Beutler B. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature*. 2003; 424:743–748. [PubMed: 12872135]
15. Huang Y, Rezzoug F, Xu H, Chilton PM, Schanie CL, Fugier-Vivier I, Ildstad ST. NK cells play a critical role in the regulation of class I-deficient hemopoietic stem cell engraftment: evidence for NK tolerance correlates with receptor editing. *J Immunol*. 2005; 175:3753–3761. [PubMed: 16148121]
16. Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. *Annu Rev Cell Dev Biol*. 2007; 23:435–461. [PubMed: 17506690]
17. Jones ND, Turvey SE, Van Maurik A, Hara M, Kingsley CI, Smith CH, Mellor AL, Morris PJ, Wood KJ. Differential susceptibility of heart, skin, and islet allografts to T cell-mediated rejection. *J Immunol*. 2001; 166:2824–2830. [PubMed: 11160350]
18. Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, Hoshino K, Akira S. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. *J Immunol*. 2001; 167:5887–5894. [PubMed: 11698465]
19. Kean LS, Hamby K, Koehn B, Lee E, Coley S, Stempora L, Adams AB, Heiss E, Pearson TC, Larsen CP. NK cells mediate costimulation blockade-resistant rejection of allogeneic stem cells during nonmyeloablative transplantation. *Am J Transplant*. 2006; 6:292–304. [PubMed: 16426313]
20. Kumar H, Kawai T, Akira S. Toll-like receptors and innate immunity. *Biochem Biophys Res Commun*. 2009; 388:621–625. [PubMed: 19686699]
21. LaRosa DF, Rahman AH, Turka LA. The innate immune system in allograft rejection and tolerance. *J Immunol*. 2007; 178:7503–7509. [PubMed: 17548582]
22. Laskowski IA, Hancock WW, Pratchke J, Wilhelm MJ, Tilney NL. Accelerated graft dysfunction in renal isografts from non-heart-beating donors. *Transplant Proc*. 2001; 33:909–910. [PubMed: 11267125]
23. Luo B, Nanji SA, Schur CD, Pawlick RL, Anderson CC, Shapiro AM. Robust tolerance to fully allogeneic islet transplants achieved by chimerism with minimal conditioning. *Transplantation*. 2005; 80:370–377. [PubMed: 16082333]
24. Luster AD. The role of chemokines in linking innate and adaptive immunity. *Curr Opin Immunol*. 2002; 14:129–135. [PubMed: 11790543]
25. Maris M, Sandmaier BM, Maloney DG, McSweeney PA, Woolfrey A, Chauncey T, Shizuru J, Niederwieser D, Blume KG, Forman S, Storb R. Non-myeloablative hematopoietic stem cell transplantation. *Transfus Clin Biol*. 2001; 8:231–234. [PubMed: 11499966]
26. Matzinger P. The danger model: a renewed sense of self. *Science*. 2002; 296:301–305. [PubMed: 11951032]
27. Miller DM, Thornley TB, Pearson T, Kruger AJ, Yamazaki M, Shultz LD, Welsh RM, Brehm MA, Rossini AA, Greiner DL. TLR agonists prevent the establishment of allogeneic hematopoietic chimerism in mice treated with costimulation blockade. *J Immunol*. 2009; 182:5547–5559. [PubMed: 19380803]
28. Obhrai J, Goldstein DR. The role of toll-like receptors in solid organ transplantation. *Transplantation*. 2006; 81:497–502. [PubMed: 16495791]
29. Ohashi K, Burkart V, Flohe S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. *J Immunol*. 2000; 164:558–561. [PubMed: 10623794]
30. O'Neill LA, Bowie AG. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nat Rev Immunol*. 2007; 7:353–364. [PubMed: 17457343]
31. Palmer SM, Burch LH, Davis RD, Herczyk WF, Howell DN, Reinsmoen NL, Schwartz DA. The role of innate immunity in acute allograft rejection after lung transplantation. *Am J Respir Crit Care Med*. 2003; 168:628–632. [PubMed: 12773319]

32. Sabroe I, Parker LC, Dower SK, Whyte MK. The role of TLR activation in inflammation. *J Pathol.* 2008; 214:126–135. [PubMed: 18161748]
33. Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. *Nat Immunol.* 2001; 2:947–950. [PubMed: 11547333]
34. Siciliano NA, Skinner JA, Yuk MH. Bordetella bronchiseptica modulates macrophage phenotype leading to the inhibition of CD4+ T cell proliferation and the initiation of a Th17 immune response. *J Immunol.* 2006; 177:7131–7138. [PubMed: 17082630]
35. Sugimoto S, Lin X, Okazaki M, Lai J, Tietjens JR, Huang H, Patterson GA, Krupnick AS, Kreisel D, Gelman AE. Monocyte differentiation is controlled by MyD88 after mouse orthotopic lung transplantation. *Transplant Proc.* 2009; 41:388–390. [PubMed: 19249563]
36. Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol.* 2005; 17:1–14. [PubMed: 15585605]
37. Taylor PA, Lees CJ, Wilson JM, Ehrhardt MJ, Campbell MT, Noelle RJ, Blazar BR. Combined effects of calcineurin inhibitors or sirolimus with anti-CD40L mAb on alloengraftment under nonmyeloablative conditions. *Blood.* 2002; 100:3400–3407. [PubMed: 12384443]
38. Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, Miyake K, Freudenberg M, Galanos C, Simon JC. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. *J Exp Med.* 2002; 195:99–111. [PubMed: 11781369]
39. Thornley TB, Phillips NE, Beaudette-Zlatanova BC, Markees TG, Bahl K, Brehm MA, Shultz LD, Kurt-Jones EA, Mordes JP, Welsh RM, Rossini AA, Greinerm DL. Type 1 IFN mediates cross-talk between innate and adaptive immunity that abrogates transplantation tolerance. *J Immunol.* 2007; 179:6620–6629. [PubMed: 17982052]
40. Tilney NL, Paz D, Ames J, Gasser M, Laskowski I, Hancock WW. Ischemia-reperfusion injury. *Transplant Proc.* 2001; 33:843–844. [PubMed: 11267092]
41. Tsung A, Klune JR, Zhang X, Jeyabalan G, Cao Z, Peng X, Stolz DB, Geller DA, Rosengart MR, Billiar TR. HMGB1 release induced by liver ischemia involves Toll-like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling. *J Exp Med.* 2007; 204:2913–2923. [PubMed: 17984303]
42. van Rooijen N, Sanders A, van den Berg TK. Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. *J Immunol Methods.* 1996; 193:93–99. [PubMed: 8690935]
43. van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods.* 1994; 174:83–93. [PubMed: 8083541]
44. Wadia PP, Tambur AR. Yin and yang of cytokine regulation in solid organ graft rejection and tolerance. *Clin Lab Med.* 2008; 28:469–479. [PubMed: 19028264]
45. Walker WE, Nasr IW, Camirand G, Tesar BM, Booth CJ, Goldstein DR. Absence of innate MyD88 signaling promotes inducible allograft acceptance. *J Immunol.* 2006; 177:5307–5316. [PubMed: 17015716]
46. Wekerle T, Kurtz J, Ito H, Ronquillo JV, Dong V, Zhao G, Shaffer J, Sayegh MH, Sykes M. Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. *Nat Med.* 2000; 6:464–469. [PubMed: 10742157]
47. Xu H, Chilton PM, Tanner MK, Huang Y, Schanie CL, Dy-Liacco M, Yan J, Ildstad ST. Humoral immunity is the dominant barrier for allogeneic bone marrow engraftment in sensitized recipients. *Blood.* 2006; 108:3611–3619. [PubMed: 16888094]
48. Xu H, Chilton PM, Huang Y, Schanie CL, Ildstad ST. Production of donor T cells is critical for induction of donor-specific tolerance and maintenance of chimerism. *J Immunol.* 2004; 172:1463–1471. [PubMed: 14734723]
49. Xu H, Chilton PM, Huang Y, Schanie CL, Yan J, Ildstad ST. Addition of cyclophosphamide to T-cell depletion based nonmyeloablative conditioning allows donor T-cell engraftment and clonal deletion of alloreactive host T-cells after bone marrow transplantation. *Transplantation.* 2007; 83:954–963. [PubMed: 17460568]

50. Xu H, Huang Y, Chilton PM, Hussain LR, Tanner MK, Yan J, Ildstad ST. Strategic nonmyeloablative conditioning: CD154:CD40 costimulatory blockade at primary bone marrow transplantation promotes engraftment for secondary bone marrow transplantation after engraftment failure. *J Immunol.* 2008; 181:6616–6624. [PubMed: 18941252]
51. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science.* 2003; 301:640–643. [PubMed: 12855817]
52. Yao Z, Painter SL, Fanslow WC, Ulrich D, Macduff BM, Spriggs MK, Armitage RJ. Human IL-17: a novel cytokine derived from T cells. *J Immunol.* 1995; 155:5483–5486. [PubMed: 7499828]
53. Yoshida S, Haque A, Mizobuchi T, Iwata T, Chiyo M, Webb TJ, Baldrige LA, Heidler KM, Cummings OW, Fujisawa T, Blum JS, Brand DD, Wilkes DS. Anti-type V collagen lymphocytes that express IL-17 and IL-23 induce rejection pathology in fresh and well-healed lung transplants. *Am J Transplant.* 2006; 6:724–735. [PubMed: 16539629]

Figure 1. Innate immunity is responsible for early allorecognition of donor BMC

To determine the role of innate immune cell populations in rejection of donor BMC, T cell-deficient (TCR β / δ ^{-/-}, H-2^b) (A and B) and T plus B cell-deficient mice (Rag^{-/-}, H-2^b) (C and D) were used as recipients *in vivo* cytotoxicity assays. 20 × 10⁶ CFSE-labeled donor BALB/c (H-2^d) splenocytes (as targeting cells, high CFSE fluorescence intensity) and recipient control splenocytes (as internal controls, low CFSE fluorescence intensity) were injected intravenously into T cell-deficient mice and B6 wildtype control mice. Wildtype B6 mice that received only syngeneic CFSE labeled cells with either high intensity or low intensity also served as a control. Donor cell survival was compared over time. Peripheral blood was collected from individual mice at selected time points after cell infusion: 1 h, 3 h, day 1, and day 3. Representative histograms from each group are presented (B and D). Spleens (SP), lymph nodes (LN), femurs for bone marrow (BM), as well as peripheral blood (PB) were collected from each recipient on day 1 after BMC infusion to compare the cytotoxic activity in these different tissues (E). Peripheral blood lymphocytes were analyzed for CFSE expression by a FACSCalibur after lysis of red blood cells. The results of TCR β / δ ^{-/-} mice are the summary of 3 experiments. The results of RAG^{-/-} mice are the summary of 2 experiments.

Figure 2. Allogeneic BMC rejection is dependent on the TLR4/TRIF signaling pathway
 In order to define the innate signaling pathways active in allojection, MyD88^{-/-} and TRIF^{Lps2/Lps2} mice (**B** and **C**) were used as recipients of allogeneic BMT. Then, TLR3^{-/-} and TLR4^{-/-} (**D** and **E**) were recipients in allogeneic BMT. Wildtype mice with the same genetic background served as controls. Recipients were conditioned anti-CD154, rapamycin, and 100 cGy TBI and transplanted with 15×10^6 untreated donor BMC. The schematic treatment of recipient is shown in (A). Donor peripheral blood chimerism was tested monthly. Figures show the frequency of engraftment from 1 to 6 months after BMT (**B**, **D**), level of chimerism in animals that engrafted from 1 to 5 or 6 months (**C**, **E**).

Figure 3. Depletion of innate immune cells enhances allogeneic BMC survival

To determine the effect of innate immune cell populations on rejection of donor BMC, *in vivo* cytotoxicity assays were performed in mice treated with anti-NK1.1, anti-Gr-1, or CloLip. 20×10^6 CFSE labeled donor BALB/c splenocytes (CFSE high) and recipient B6 splenocytes (internal controls, CFSE low) were infused as target cells. Recipients treated with saline served as controls. Peripheral blood was collected at selected time points after cell infusion: 1 h, 3 h, day 1, day 3, and day 7. After lysis of red blood cells, peripheral blood lymphocytes were analyzed for CFSE expression by a FACSCalibur. Representative histograms from a mouse that received CloLip and a control that received PBS treatment at day 1, 3 and 7 are presented (A). The kinetics of killing is the summary of four separate experiments (B).

Figure 4. Depletion of macrophages or/and NK cells promotes alloengraftment

To evaluate the efficacy of targeting different innate immune cell populations in promoting allogeneic engraftment, B6 mice received CloLip, anti-NK1.1 mAb, or anti-Gr-1 mAb. These agents were used in combination with conditioning strategies that target T and B cells: anti- $\alpha\beta$ -TCR + anti-CD154 + rapamycin (Rapa) + irradiation (TBI). The schematic procedures of recipient are shown in (A). Conditioned recipients were transplanted with 15×10^6 untreated BALB/c BMCs via lateral tail vein injection at least 4–6 hr after TBI. Chimerism was followed by flow cytometric analysis monthly with peripheral blood staining of mAbs specific for donor (anti-H-2K^d FITC) and recipient (anti-H2K^b PE) origin (B): percentage of animals with engraftment; (C): level of donor chimerism in engrafted recipients at 1 month; and (D): kinetics of donor chimerism. These results are the summary of 5 experiments.

Figure 5. Depletion of both macrophages and NK cells eliminates the requirement for T cell depletion and irradiation for engraftment

In order to determine whether BMT could be successfully achieved without use of T cell-depletion, B6 mice were conditioned with CloLip and/or anti-NK1.1 mAb in combination with anti-CD154 + rapamycin without anti- $\alpha\beta$ -TCR and transplanted with 15×10^6 untreated donor BMC. Chimerism testing was performed monthly after BMT; (A): engraftment; (B): level of donor chimerism at 1 month; and (C): kinetics of donor chimerism. The results are the summary of 3 experiments.

Figure 6. The role of TRIF signaling in BMC rejection by macrophages and NK cells
 TRIF^{Lps2/Lps2} mice were used as recipients in *in vivo* cytotoxicity assays after adoptive transfer of wildtype macrophages and NK cells. Wildtype macrophages and NK cells were stained with F4/80 PE and NK1.1 FITC, respectively. F4/80⁺ and NK1.1⁺ cells were sorted from spleens of B6 mice and F4/80⁺ cells also sorted from cells collected from peritoneal cavities. One day after transfer, target (BALB/c) and internal control (B6) BMC were incubated with 4.0 μ M or 0.2 μ M CFSE, respectively. 20×10^6 cells from each were injected intravenously. Peripheral blood was collected from individual mouse at different time points after cell infusion: 3 hr, day 1, and day 3. After lysis of red blood cells, peripheral-blood lymphocytes were analyzed for CFSE expression by fluorescence-activated cell sorting (FACS). The percentage of killing was determined by calculating the ratio between target and internal control cells. TRIF^{Lps2/Lps2} mice that did not receive transferred cells and wildtype B6 mice treated with saline served as controls.

Figure 7. Serum levels of cytokines and chemokines

B6 mice were transplanted with 15×10^6 BALB/c allogeneic BMC. Serum was collected at 0.5, 1, 4, 8, and 12 hours, and day 1, 3 and 7 following transplantation. Control serums are from untransplanted B6 mice. The levels of IL-1 β , IL-6, IFN- α , IFN- β , MCP-1, and IP-10 were determined by ELISA. Production of each cytokine is represented in picograms per milliliter. There were 4–5 samples at each time point, and each sample was run in duplicate. Only IL-6 (**A**) and MCP-1 (**B**) were detectable and showed.