Modeling an autism risk factor in mice leads to permanent immune dysregulation

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Increasing evidence highlights a role for the immune system in the pathogenesis of autism spectrum disorder (ASD). As immune dysregulation is observed in the brain, periphery, and gastrointestinal tract of ASD individuals. Furthermore, maternal infection (maternal immune activation, MIA) is a risk factor for ASD. Modeling this risk factor in mice yields offspring with the cardinal behavioral and neuropathological symptoms of human ASD. In this study, we find that offspring of immune-activated mothers display altered immune profiles and function, characterized by a systemic deficit in CD4\(^+\) TCR\(^{\gamma\delta}\) Foxp3\(^+\) CD25\(^+\) regulatory cells, increased IL-6 and IL-17 production by CD4\(^+\) T cells, and elevated levels of peripheral Gr\(^+\)1 cells. In addition, hematopoietic stem cells from MIA offspring exhibit altered myeloid lineage potential and differentiation. Interestingly, repopulating irradiated control mice with bone marrow derived from MIA offspring does not confer MIA-related immunological deficits, implicating the peripheral environmental context in long-term programming of immune dysfunction. Furthermore, behaviorally abnormal MIA offspring that have been irradiated and transplanted with immunologically normal bone marrow from either MIA or control offspring no longer exhibit deficits in stereotyped/repetitive and anxiety-like behaviors, suggesting that immune abnormalities in MIA offspring can contribute to ASD-related behaviors. These studies support a link between cellular immune dysregulation and ASD-related behavioral deficits in a mouse model of an autism risk factor.

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Immune-related susceptibility genes, such as those encoding MET receptor tyrosine kinase, PRKCB1, complement C4B, and specific HLA haplotypes (3, 4). In addition, antibrain antibodies are elevated in some ASD sera and in some mothers of autistic children (5–7). Family members of autistic children, particularly the mothers, show a higher incidence of allergy or autoimmune diseases (8, 9). Consistent with immune involvement are findings that maternal infection is a risk factor for autism (2). After the 1964 rubella pandemic, 8–13% of children born to infected mothers developed features of autism (10). In a recent study surveying all children born in Denmark from 1980 to 2005, a very significant association was found between autism and maternal viral infection during the first trimester of pregnancy (11). Moreover, elevation of IFN-γ, IL-4, or IL-5 in maternal serum is associated with increased risk for ASD in the offspring (12), as is elevation of monocyte chemoattractant protein-1 in the amniotic fluid (13).

Whether the immune abnormalities in ASD actually contribute to its behavioral symptoms or whether they are an epiphene-non of primary neural dysfunction is an outstanding question. The immune system exhibits lifelong reciprocal interactions with the central nervous system, and the fact that the immune status can influence behavioral responses is exemplified by early studies demonstrating that responses to infection and inflammation are related to the brain, resulting in the induction of fever and sickness behavior (14). Moreover, administration of certain cytokines to human subjects frequently causes striking changes in mental state (15). Conversely, emotional and psychological state can influence immune function. Perhaps the strongest evidence to date is the finding that both short- and long-term stress lead to disruption of immune function (16). Immune dysregulation has also been implicated in the etiology of a variety of neurodegenerative, psychiatric, and neurodevelopmental disorders, including Parkinson, Huntington, and Alzheimer’s diseases, multiple sclerosis, major depression, schizophrenia, and addiction (17–20).

Here we ask whether a mouse model exhibiting many features of autism also displays altered immune function. We use the maternal immune activation (MIA) model, which is based on maternal infection as a key environmental risk factor for autism. Pregnant mice are injected with the synthetic, double-stranded RNA, poly(I:C), to initiate a proinflammatory antiviral response. This type of MIA yields offspring with the core behavioral and neuropathological symptoms of autism, including reduced social interaction, abnormal communication, stereotyped/repetitive behavior, and a spatially restricted deficit in Purkinje cells (21, 22).

In this study, we profile peripheral immune subtypes, assess the functional activities of major leukocyte lineages and evaluate the lineage potential of fetal and adult hematopoietic stem cells (HSCs) and progenitors. To explore the potential for prenatal programming of long-term immune dysfunction, we examine whether transferring HSCs from MIA offspring into non-MIA offspring can induce cell-autonomous immune abnormalities. To gain insight into whether immune abnormalities in MIA offspring contribute to the pathogenesis of ASD-related behaviors, we behaviorally assess poly(I:C) offspring reopulated with bone marrow (BM) from saline offspring. Our findings demonstrate that immune challenge during prenatal life leads to persistent immune alterations in the postnatal offspring, which can further impact the development or maintenance of abnormal behavior.

Results

MIA Offspring Exhibit Deficits in Regulatory T Cells and Elevated CD4\(^+\) T-Cell Responses. Many studies of immune dysregulation in human ASD report a bias toward a proinflammatory phenotype (3, 4).

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We therefore investigated regulatory T cells (Tregs) as known suppressors of the innate and adaptive immune responses. Compared with controls, adult offspring of poly(I:C)-injected mothers display a ~50% decrease in splenic CD4⁺ Foxp3⁺ CD25⁺ Tregs (Fig. 1C). This deficit is also reflected in significantly decreased levels of total CD4⁺ Foxp3⁺ cells and possible decreases (P = 0.0846) in CD4⁺ Foxp3⁺ CD25⁺ T cells. Similar deficits in total CD4⁺ Foxp3⁺, Foxp3⁻ CD25⁺, and Foxp3⁻ CD25⁻ cells are also observed in mesenteric lymph node (MLNs) from adult poly(I:C) offspring (Fig. S1A). Despite these differences in Treg levels, there is no difference in the suppression of CD4⁺ CD25⁺ T-cell proliferation between Tregs from saline versus poly(I:C) offspring (Fig. S2). Taken together, these findings indicate a systemic deficit in the abundance of Tregs.

We further tested MIA offspring for levels of IL-17-producing CD4⁺ T (Th17) cells, given their proinflammatory nature and reported reciprocal relationship with Tregs. In assays done in parallel with those for Tregs, we find no significant difference from controls in levels of CD4⁺ TCRβ⁺ IL-17⁺ (Th17) cells, with or without IFN-γ expression (Fig. 1B). There is also no difference in the level of CD4⁺ TCRβ⁻ IL-17⁻ IFN-γ⁺ (Th1) cells. To further examine CD4⁺ T cells, we measured their secretion of IL-6 and IL-17 in response to in vitro stimulation. Compared with controls, CD4⁺ cells from spleens of 15-week-old poly(I:C)-offspring release significantly more IL-6 and IL-17 after in vitro stimulation, with no difference in TNF-α secretion (Fig. 1C). This result is similarly observed with splenic CD4⁺ T cells derived from 3-week-old offspring (Fig. S3A) and 1-year-old offspring (Fig. S3B), suggesting an early onset of persistent immune dysfunction. Interestingly, splenic CD4⁺ T cells from 3-week-old mice produce much lower levels of IL-17 than do such cells from adult offspring (0–10 pg/mL compared with 50–400 pg/mL), reflecting the immunological immaturity reported in young versus adult mice and humans (23). CD4⁺ T cells from MLNs of poly(I:C)-offspring are also hyperresponsive to in vitro stimulation, suggesting that this abnormality is common to secondary lymphoid organs (Fig. S1 C and D). Overall, MIA leads to lower Treg levels and elevated CD4⁺ T-cell responsiveness in spleens and MLNs from MIA offspring. This result can be characterized as a persistent, proinflammatory T-helper-cell phenotype.

**MIA Offspring Display Increased Levels of Gr-1⁺ Cells and Skewed HSC Differentiation.** To determine whether MIA during fetal development alters the profile of other immune subtypes in the offspring, we assessed major leukocyte classes in spleens from poly(I:C) and saline offspring. Compared with controls, adult poly(I:C) offspring exhibit a 1.5-fold higher level of Gr-1⁺ cells and trending increase in CD11b⁺ cells (P = 0.0125) (Fig. 2A). In contrast, there is no difference from controls in the percentages of total B220⁺ B cells, NK1.1⁺ NK cells, CD4⁺ T cells, or CD8⁺ T cells. Moreover, no significant differences are detected for any of the primary leukocyte subtypes in the MLNs (Fig. S4A). Gr-1⁺ cells reflect a heterogeneous group of immune subtypes that includes neutrophils and monocytes, inflammatory cells, and suppressor cells, alike. To determine whether the elevated levels of Gr-1⁺ cells observed in poly(I:C) spleens could be attributed to particular Gr-1⁺ subtypes, we further characterized the splenic Gr-1⁺ population using CD11b, Ly6c, and Ly6G markers. Interestingly, poly(I:C) offspring exhibit mild increases in all three populations of Gr-1⁺ cells resolved: (i) Gr-1⁺ CD11b⁺ Ly6c⁺ Ly6G⁻ SSC(lo) (ii) Gr-1⁺CD11b⁺ Ly6c⁺ Ly6G⁻ SSC(mid) and (iii) Gr-1⁺CD11b⁺Ly6c⁺Ly6G⁻SSC(high). Gr-1⁺ subtypes i and ii are referred to as neutrophils, by histology and in line with their high granularity, and subtype iii is identified as a monocyte population (24). Overall, that poly(I:C) offspring display increases in all identified Gr-1⁺ populations suggests that skewing at the HSC or progenitor level may underlie the elevated Gr-1 phenotype observed in adult poly(I:C) offspring compared with controls.

To evaluate the origin of the increase in splenic Gr-1⁺ cells, we assessed the lineage potential and differentiation of BM cells from adult poly(I:C) versus saline offspring. Using a colony-forming assay to morphologically assess lineage differentiation, we find that HSCs and progenitors from poly(I:C) offspring expressed differentiation into CFU-G (granulocyte) precursors and decreased differentiation into early CFU-GM (granulocyte-macrophage) precursors (Fig. 2B). To the fetus, we find that similar differentiation is observed with fetal liver HSCs and progenitors from poly(I:C) offspring (Fig. 2C). Thus, MIA induces preferential differentiation of fetal as well as adult HSCs and progenitors into granulocyte precursors, which may account for the increased levels of mature Gr-1⁺ cells in spleens of poly(I:C) offspring compared with controls.

**Immune Abnormalities Observed in MIA Offspring Are Not Transferred via BM Transplant.** Our data demonstrate that MIA leads to an altered profile of peripheral immune cells in the offspring, characterized by decreased levels of Tregs, hyperresponsive CD4⁺ T⁺ cells, and elevated levels of Gr-1⁺ cells. To explore whether these immune abnormalities (and the Gr-1 phenotype, in particular) can be attributed to cell-intrinsic developmental programming of HSCs, we transferred BM from the immunologically aberrant poly(I:C) offspring into irradiated saline and MIA offspring. (A) Compared with controls, adult poly(I:C) offspring exhibit decreased levels of CD4⁺ Foxp3⁺ splenocytes and CD4⁺ Foxp3⁻ CD25⁻ Tregs (n = 5, where each sample represents a pool of three spleens). (B) Adult poly(I:C) offspring exhibit decreased levels of splenic CD4⁺ TCRβ⁺ Foxp3⁺ CD25⁺ Tregs, but no significant difference in splenic CD4⁺ TCRβ⁺ IFN-γ⁻ IL-17⁺ Th17⁺ Th1 cells or CD4⁺ TCRβ⁺ IFN-γ⁻ IL-17⁺ Th17 cells (n = 4, where each sample represents a pool of three spleens). (C) CD4⁺ T cells from the spleens of adult offspring secrete elevated levels of IL-6 and IL-17 in response to PMA/ionomycin stimulation. In contrast, CD4⁺ T cells from spleens of adult poly(I:C) and saline offspring do not differ the level of TNF-α secreted (n = 11–16). *P < 0.05, **P < 0.01, ***P < 0.001; n.s., not significant. All panels show one representative experiment of at least two separate trials.
poly(I:C) offspring and assessed whether any MIA-associated immune abnormalities were reestablished.

We find that saline and poly(I:C) offspring that were irradiated and reconstituted with poly(I:C) BM do not develop or retain any of the immune abnormalities exhibited by unmanipulated MIA offspring. All BM-transplanted groups exhibit levels of splenic CD4^+ TCRβ^+ CD25^+ Foxp3^+ Tregs and Gr-1^+ cells that are comparable to those observed in untransplanted saline offspring (Fig. 3 A and B). There is also no difference between saline and BM-transplanted mice in distributions of progenitor subtypes after in vitro differentiation of BM HSCs (Fig. S5). Furthermore, CD4^+ T cells isolated from spleens of any of the types of BM-transplanted mice secrete levels of IL-6 and IL-17 in response to phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation that are similar to those produced by CD4^+ T cells isolated from spleens of untransplanted saline offspring (Fig. 3C). Importantly, the fact that saline mice transplanted with saline BM display equivalent levels of Tregs and Gr-1^+ cells and similar CD4^+ T-cell responsiveness to those observed in unmanipulated saline offspring suggests that the irradiation and BM-transplant procedure itself does not skew immune system profiles under these conditions. Overall, these experiments indicate that MIA-associated immune abnormalities are not transferred via transplant of poly(I:C) BM into irradiated saline or poly(I:C) offspring. This finding suggests either that the HSC microenvironment is important for maintaining altered HSC potential in MIA offspring, or that the particular MIA-associated immune abnormality is not programmed at the stem cell level.

**BM Transplant in MIA Offspring Normalizes Repetitive and Anxiety-Like Behavioral Abnormalities.** To investigate whether the immune abnormalities found in MIA offspring are an independent parallel pathology or if they actually contribute to the development or maintenance of ASD-like behaviors, we assessed behavioral performance in poly(I:C) offspring transplanted with saline BM. Poly(I:C) offspring were first confirmed to exhibit their expected behavioral phenotypes before the procedure. Compared with controls, poly(I:C) offspring display a deficit in prepulse inhibition (PPI) (Fig. S6C). PPI is used to measure sensorimotor gating of the startle reflex, and decreased PPI is frequently observed in autistic individuals (25). Poly(I:C) offspring also exhibit core behavioral symptoms of autism, including increased repetitive behavior as measured by higher levels of stereotypic marble burying and decreased social preference, as assessed by reduced duration spent and entries into a chamber housing a novel mouse versus a familiar mouse (Fig. S6B and C). In addition, MIA offspring exhibit increased anxiety, as reflected by

**Fig. 2.** MIA leads to increased levels of splenic Gr-1^+ cells in adult offspring and preferential differentiation of HSCs into granulocyte precursors in fetal and adult offspring. (A) Spleens from adult poly(I:C) offspring exhibit increased levels of Gr-1^+ cells and no significant differences in other major lineages compared with controls (n = 4, where each sample represents a pool of three spleens). (B) Compared with BM HSCs from control offspring, BM HSCs from adult poly(I:C) offspring display increased differentiation into CFU-G precursors and decreased differentiation into early CFU-GM precursors (n = 4). Compared with controls, fetal liver HSCs from embryonic day (E) 13.5 (C, Left) and E15.5 (C, Right) poly(I:C) offspring also display increased differentiation into CFU-G and decreased differentiation into CFU-GM, in addition to decreased percentages of CFU-GEMM with E15.5 HSCs and increased percentages of CFU-E with E15.5 HSCs (n = 4, where each sample represents a pool of cells from six fetal livers from a single litter). *P < 0.05, **P < 0.01, ***P < 0.001; n.s., not significant. All panels represent one representative experiment of at least two separate trials.

**Fig. 3.** Immune abnormalities observed in MIA offspring are not transferred by BM transplant into irradiated mice. (A) There is no difference in levels of splenic CD4^+ TCRβ^+ CD25^+ Foxp3^+ Tregs between saline or poly(I:C) offspring transplanted with BM from saline or poly(I:C) offspring. Levels of splenic Tregs between BM transplant groups are comparable to those observed in untransplanted saline offspring. BM-transplanted mice do not display MIA-associated increases in splenic Gr-1^+ cells. Percentages of Gr-1^+ cells are comparable between BM-transplanted groups and untransplanted saline offspring (n = 4–5), but untransplanted poly(I:C) offspring exhibit statistically elevated levels of splenic Gr-1^+ cells. (C) CD4^+ T cells from BM-transplanted mice exhibit statistically equivalent levels of IL-6 (Left) and IL-17 (Right) in response to PMA/ionomycin stimulation in vitro. There are no differences between concentrations of IL-6 and IL-17 secreted by CD4^+ cells from BM-transplanted groups and from untransplanted saline offspring, despite significant hyperresponsiveness of CD4^+ T cells from poly(I:C) offspring (n = 11–16 for saline and poly(I:C) groups, 4–5 for BM transplant groups). *P < 0.05, **P < 0.01. BM transplant data were acquired from one large experiment.
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is typically observed after acute inflammation (32, 33) and is also a feature of a number of chronic diseases (34–36). There have been no reports of altered neutrophil levels in ASD, however. In one study, children with ASD exhibited increased plasma monocyte counts, but no significant abnormalities in major granulocyte subtypes (37). In light of the present results, it will be of interest to extend these immunophenotypic studies to behavioral or symptomatic subpopulations of ASD individuals.

It is intriguing to consider that MIA effects on developing HSCs may underlie some of the persistent peripheral immune changes found in the offspring. We find that both adult and fetal HSCs and progenitor cells display preferential differentiation into CFU-G colonies, which provides an explanation for how increases in such short-lived cells could be maintained over the lifespan of MIA offspring. Maternal poly(I:C) injection is known to induce dramatic increases in proinflammatory cytokines in the placenta, a principal HSC niche during midgestation (38). Because HSCs and progenitors respond to inflammatory signals, such as cytokines and Toll-like receptor ligands, it will be interesting to explore whether MIA-induced changes in the HSC environment can skew hematopoietic lineage decisions and fate.

Despite the fact that HSCs and progenitors isolated from poly(I:C) offspring are developmentally skewed toward granulocyte precursors, transplantation of BM from poly(I:C) offspring into irradiated recipient mice results in significantly lower irradiation after poly(I:C) offspring BM transplantation does not transfer the Gr-1 phenotype. This finding suggests that developmental programming of HSCs to skew lineage differentiation in MIA offspring is not governed solely by stably encoded cell-intrinsic factors, such as epigenetic modification. Rather, it is likely that an MIA-induced peripheral environment is necessary to supply the cues that guide preferential myeloid development. Changes in G-CSF, IL-5, and IL-3 levels, for example, are implicated in fine-tuning the transcription factor activity that governs lineage choice for granulocyte/macrophage progenitors (39). Indeed, we have found that MIA offspring exhibit similar hematopoietic changes in peripheral blood mononuclear cells as adult MIA offspring, including alterations in splenic cytokine profiles (40). It will be of interest to assess whether the BM microenvironment differs in poly(I:C) and saline offspring. Overall, it is intriguing that the Gr-1 phenotype observed in MIA offspring is not transferred via BM transplant, suggesting a lack of sufficient cell-intrinsic epigenetic programming and a key role for environmental factors in promoting this lineage choice.

We demonstrate that subjecting MIA offspring that have validated peripheral deficits to irradiation and reconstitution with immunologically normal BM alters their peripheral phenotype. Namely, these MIA offspring no longer exhibit their previous abnormalities in repetitive marble burying and open-field exploration. These findings imply that correcting immune function can correct some autism-related behavioral abnormalities. However, this experiment has a significant limitation. In the absence of a positive control (MIA offspring that retain behavioral deficits after irradiation and BM transplantation), we are unable to distinguish between the potential restorative effects of the BM itself (that is, the effects of restoring immune phenotype) versus the effect of confounding factors associated with the transplant procedure. The most obvious of these is the effect of irradiation on various aspects of recipient homeostasis, including metabolic function, gastrointestinal microbial composition, and oxidative stress, all of which may indirectly influence behavioral outcome. We are, however, inspired by recent work demonstrating that stereotyped grooming behavior in Hox8 mutant mice is, in fact, transferrable via BM transplant (41). This finding shows, at least in that experimental model, that mice can reconstitute an expected behavioral impairment via BM transplant despite prior irradiation. It will be important to explore other experimental approaches to treat immune dysfunction in MIA offspring and to further assess whether these can alleviate behavioral outcomes.

Nonetheless, our finding that behavioral abnormalities in the MIA offspring can be corrected by the irradiation-BM transplant procedure contributes to a growing number of studies reporting the efficacy of BM transplant on ameliorating symptoms of neurological disorders (41–43). Furthermore, several experiments using RAG1 KO, SCID, and athymic mice demonstrate that primary immune dysfunction can lead to behavioral impairment (44–47). Interestingly, work with germ-free mice links the absence of microbiota and associated alterations in the immune system to abnormal behavioral performance (48). Some immune alterations have been described in the maternal valproic acid model and the BTBR mouse strain that display behavioral features of autism (49, 50). In contrast, much remains to be learned about potential immune changes in mouse models of ASD candidate genes. One such gene is particularly attractive in this regard, MET, which encodes a tyrosine kinase receptor and is known to play a role in immune regulation (51).

Altered peripheral immune profiles and cellular activity are also associated with core behavioral impairments in human ASD. Increased plasma IL-4 levels correlate with greater deficits in communication, and increased plasma IL-8, IL-12 p40, IL-6, and IL-1β are linked to stereotypy, hyperactivity, and lethargy scores from ASD children (52). Altered levels of other immune factors, including TGF, macrophage migration inhibitory factor (MIF), and CD31, have also been associated with the severity of ASD-related behaviors or pathophysiology (4). In addition, reduced levels of plasma IgG and IgM are associated with behavioral severity in ASD children (53).

It is striking that in a mouse model of an autism environmental risk factor that exhibits the cardinal behavioral and neuropathological symptoms of autism, there is also permanent peripheral immune dysregulation. This finding provides the opportunity to explore molecular mechanisms underlying the relationship between brain dysfunction and altered immunity in the manifestation of abnormal behavior. Furthermore, this finding provides a platform for investigating how prenatal challenges can program long-term postnatal immunity, health, and disease. Maternal in utero-mediated epigenetic modification in HSC and progenitor cells is one possible mechanism for how effects may be established by transient environmental changes yet persist permanently into adulthood. However, the BM transplant results suggest that the peripheral environment of the MIA offspring is also critical for maintaining a permanently modified immune state.

Methods

Detailed methods are provided in SI Methods.

MIA. Pregnant C57BL/6N mice were injected on E12.5 with saline or poly(I:C). For poly(I:C) injections, poly(I:C) potassium salt (Sigma Aldrich) was dissolved in saline at 4 mg/mL and administered intraperitoneally at 20 mg/kg [based on the weight of the poly(I:C) itself, not including the total weight of the potassium salt]. Control mice were injected with saline alone at 0.5 μL/g body weight.

CD4+ T-Cell In Vitro Stimulation. 106 CD4+ T cells were cultured in complete RPMI with PMA (50 ng/mL) and ionomycin (750 ng/mL) for 3 d at 37 °C with 5% (vol/vol) CO2. Each day, 0.5 mL supernatant was collected. ELISA to detect IL-6, IL-17, and TNF-α were performed according to the manufacturer’s instructions (eBioscience).

Flow Cytometry. For subtyping of Gr-1+ splenocytes, cells were stained with Gr-1-APC, CD11b-PE, Ly6C-FITC, Ly6G-APC, and CD19-PerCP-Cy5.5 (Biolégend). For detection of TH17 cells and Treg cells, splenocytes were stained for 4 h with PMA/ionomycin in the presence of GolgiPLUG (BD Biosciences). Suspensions were blocked for Fc receptors and labeled with CD4-FITC, TCRB-PerCP-Cy5.5, and CD25-PE before labeling with IFN-γ-PE and Foxp3-APC (eBioscience). Samples were processed using the FACScalibur cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar).

Methylcellulose Colony-Forming Assay. 2 x 104 fetal liver or BM cells were added to 3 mL of thawed methylcellulose supplemented with SCF, IL-3, IL-6, and Epo (StemCell). Total colony number was counted on day 5 and confirmed to be equivalent across groups. CFU-G, -M, -E (erythroid), -GM, and -GEMM colonies were blindly scored on day 12 according to standard protocols.
BM Transplantation. Behaviorally tested, 9-wk-old mice were injected intraperitoneally with high-dose ketamine/xylazine (5.6 µl/g per mouse) and lethally irradiated (1,000 rads) with heads shielded by 4-mm lead. The effectiveness of the head shielding was confirmed by selective grazing of brain color in the irradiated area. Recovered mice were anesthetized with isoflurane and injected retro-orbitally with 5 × 10^6 donor BM cells. Mice were retested in behavioral paradigms at 17–19 wk of age and killed for immunological assays at 19–20 wk of age.

Behavioral Testing. At 6–8 wk of age, mice were behaviorally tested for PPI, open-field exploration, repetitive marble burying, and social preference (22, 38, 54, 55). Eight weeks after transplant, mice were similarly tested in all paradigms but PPI because PPI performance is highly sensitive to handling and prior testing experience (56).

Statistical Analysis. Statistical analysis was performed with Prism software (Graphpad). Differences between two treatment groups were assessed using two-tailed t test, and differences among multiple groups were assessed using one-way ANOVA and Bonferroni post hoc tests. Two-way ANOVA and Bonferroni post hoc test was used for PPI, CD4+ T-cell stimulation and methylcellulose assay data.

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