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Myeloid-Derived Suppresser Cells as a Potential Therapy for Experimental Autoimmune Myasthenia Gravis

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We recently demonstrated that hepatic stellate cells induce the differentiation of myeloid-derived suppressor cells (MDSCs) from myeloid progenitors. In this study, we found that adoptive transfer of these MDSCs effectively reversed disease progression in experimental autoimmune myasthenia gravis (EAMG), a T cell–dependent and B cell–mediated model for myasthenia gravis. In addition to ameliorated disease severity, MDSC-treated EAMG mice showed suppressed acetylcholine receptor (AChR)–specific T cell responses, decreased levels of serum anti-AChR IgGs, and reduced complement activation at the neuromuscular junctions. Incubating MDSCs with B cells activated by anti-IgM or anti-CD40 Abs inhibited the proliferation of these in vitro–activated B cells. Administering MDSCs into mice immunized with a T cell–independent Ag inhibited the Ag-specific Ab production in vivo. MDSCs directly inhibit B cells through multiple mechanisms, including PGE2, inducible NO synthase, and arginase. Interestingly, MDSC treatment in EAMG mice does not appear to significantly inhibit their immune response to a nonrelevant Ag, OVA. These results demonstrated that hepatic stellate cell–induced MDSCs concurrently suppress both T and B cell autoimmunity, leading to effective treatment of established EAMG, and that the MDSCs inhibit AChR-specific immune responses at least partially in an Ag-specific manner. These data suggest that MDSCs could be further developed as a novel approach to treating myasthenia gravis and, even more broadly, other diseases in which T and B cells are involved in pathogenesis.


Myasthenia gravis (MG) is an autoimmune neuromuscular transmission disorder. In most patients, pathology is caused by acetylcholine receptor (AChR) autoantibodies (1, 2). After binding to AChR at the endplates, these autoantibodies damage the neuromuscular junctions, primarily by activating complement, leading to severe muscle weakness (2). Antigenic modulation also contributes to the loss of AChR from the postsynaptic membrane. Because AChR is a T cell–dependent Ag, T cells are also integrally involved in the production of anti-AChR autoantibodies in MG, in addition to the Ab-producing B cells. Experimental autoimmune MG (EAMG) can be induced in mice by immunization with purified Torpedo AChR with adjuvant, and this model has been widely used to understand the pathogenesis of MG and to test novel therapies for this disease (3). Despite MG’s status as an orphan disease, its importance lies in being one of the few disorders that fulfills the strict criteria of autoimmunity, and any therapeutics found to be effective for MG are likely to translate well to other autoimmune disorders.

Myeloid-derived suppressor cells (MDSCs), originally identified in tumors (4, 5), have been found to inhibit host innate immunity and adaptive immunity, especially T cell responses against tumors, thereby permitting tumor survival (6). Existing evidence suggests that MDSC-mediated immunosuppression in peripheral lymphoid organs is mainly Ag specific, because T cells in the peripheral lymphoid organs of tumor-bearing mice and in the peripheral blood of cancer patients can still respond to stimuli other than tumor–associated Ags (7–9). Because of their potent and potentially Ag-specific T cell inhibitory activities, MDSCs hold promise as a novel therapy for autoimmune disease (7). However, because of the impracticality of isolating large numbers of syngeneic MDSCs from tumors for treatment purposes, the development of MDSCs as a new approach to treating autoimmune diseases has been greatly hampered. We recently developed a unique method for generating large numbers of MDSCs from bone marrow progenitors and demonstrated that these MDSCs potently inhibit T cell responses both in vitro and in vivo (10, 11). In this study, we evaluated the efficacy of these MDSCs in treating ongoing EAMG in mice and explored their direct B cell inhibitory activity in addition to their T cell–suppressive activities.

Materials and Methods

MDSC induction and characterization

Hepatic stellate cells (HSCs) and HSC–induced MDSCs were prepared following protocols described in detail previously (10, 11). In brief, HSCs were isolated from B6 mouse liver and cultured in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 20% FBS in 5% CO2 in air at 37°C for 7–14 d. Cell viability was >90% as determined by trypan blue exclusion. The purity of HSCs was >95%, as determined by their staining positive for α-smooth muscle actin (immune staining) and negative for CD45 (flow), as previously described (10). For MDSC induction, bone marrow cells from C57BL/6 mice were cultured in RPMI 1640 medium supplemented with 20% FBS, 5% CO2 in air at 37°C for 7–14 d. Cell viability was >90% as determined by trypan blue exclusion. The purity of MDSCs was >95%, as determined by their staining positive for CD11b (flow), as previously described (10). For MDSC induction, bone marrow cells from C57BL/6 mice were cultured in RPMI 1640 medium supplemented with 20% FBS, 5% CO2 in air at 37°C for 7–14 d. Cell viability was >90% as determined by trypan blue exclusion.
(15-PDGβH) knockout (KO) mice (B6 background; 2 × 10^6 cells/well) were cultured with HSCs (80%) in RPMI 1640 medium containing 10% FBS in the presence of either mouse rGM-CSF alone (8 ng/ml) or GM-CSF (8 ng/ml) plus IL-4 (1000 U/ml; both from Schering-Plough, Kenilworth, NJ) for 5 d. The floating cells (MDCs) were harvested, washed, and resuspended in RPMI 1640 medium. These MDCs comprised ~80% CD11b^+CD11c^−/hi and 20% CD11b^+CD11c^lo/b with monocyte-like morphology (10).

**EAMG induction and treatment**

EAMG was induced in mice following protocols described before with minor modifications (3, 12). In brief, C57BL/6 mice (female, 8–12 wk old; Jackson Laboratory) were immunized at the tail base and in both thighs with 25 μg purified Torpedo AChR protein in complete Freund's adjuvant supplemented with 4 mg/ml Mycobacterium tuberculosis strain H37RA extract (Becton Dickinson, Franklin Lakes, NJ). In 2 wk, the mice were immunized again following the same protocol. The development of EAMG was determined by muscle strength evaluation and serum AChR-specific IgG ELISA 1 wk after the boost immunization. After the development of EAMG was confirmed, mice were randomly divided into two groups (n=11 in each group). For the treatment group, 1.5 × 10^6 of the MDCs were adoptively transferred by tail-vein i.v. injection into each of the mice, and for the control group, the same volume of PBS was injected. All the animal work was approved by the Institutional Animal Care and Use Committee and carried out according to the guidelines of the National Institutes of Health and our institution for the humane care and use of research animals.

**Muscle strength evaluation**

Muscle strength of each mouse was evaluated by grid-hanging time as described previously, with minor modifications (13, 14). Mice were first exercised by gently dragging the tail base across a cage-top grid repeatedly (30 times) as they attempted to grip the grid; following this step, they were placed on the grid, which was then inverted. Hanging time was recorded as the time it took for the mouse to fall from the grid. Hanging time for each mouse was measured at least twice, and the average value was recorded.

**Serum AChR-specific IgG level measurement**

To measure AChR-specific IgG total levels in the muscle serum, we collected samples from the tail vein and incubated them in wells of a 96-well plate coated with 5 μg/ml purified Torpedo AChR protein. After washing, HRP-conjugated rabbit anti-mouse IgG (1:4000; Southern Biotech, Birmingham, AL) was added into each well, and the titers of AChR-specific IgGs in the sera were measured by measuring OD450 after the color development using the 3,3',5,5'-tetramethylebenzidine substrate (Thermo Scientific [Pierce], Rockford, IL). To determine the titers of different AChR-specific isotypes before and after MDCS treatment, we used a similar ELISA protocol with Abs against mouse IgA, IgM, IgG1, IgG2a, IgG2b, and IgG3 from a mouse Ab isotyping kit (Sigma, St. Louis, MO). All samples were run in duplicate.

**AChR-specific T cell recall assays**

AChR-specific Th1 and Th17 responses in the treated versus control mice were assessed by culturing 4 × 10^5 splenocytes with 5 μg/ml purified AChR protein for 3 d, then measuring the levels of IFN-γ and IL-17 in the culture supernatants by standard ELISA. In brief, ELISA plates were coated with 4 μg/ml anti-mouse IFN-γ IgG (BD Biosciences, San Jose, CA) or 4 μg/ml anti-mouse IL-17 IgG (BD Biosciences) overnight and then blocked with 1 mg/ml BSA. After this, culture supernatants were added into each well and incubated for 2 h. After washing, 1 μg/ml biotin anti-mouse IFN-γ or IL-17 IgGs was added into each well and incubated for another 1 h. The plates were then developed after incubation with alkaline phosphatase–labeled goat anti-biotin IgG (1:2000; Vector Laboratories, Burlingame, CA) and para-nitrophenylphosphate substrate (Thermo Scientific [Pierce], Rockford, IL). The concentrations of IFN-γ and IL-17 in the supernatants were calculated by measuring OD450. All samples were run in duplicate.

**Immunoﬂuorescence staining and analysis of complement activation products**

For analysis of complement activation at neuromuscular junctions, cryosections of leg skeletal muscles from the MDCS-treated and control mice were prepared and stained with Alexa Fluor 594–labeled α-bungarotoxin (BTX; Life Technologies, Carlsbad, CA) to locate the endplate. The same sections were also stained with FITC-labeled goat anti-mouse C3 IgG (MI Biocmathens, Solon, OH) or rabbit polyclonal anti-complement C9b-9 (1:1000 dilution; Calbiochem, San Diego, CA) followed by staining with Alexa 488–labeled goat anti-rabbit IgG (Life Technologies, Eugene, OR). Sections were examined with a Nikon Diaphot fluorescence microscope (Nikon Instruments, Melville, NY). For analysis of the deposition of membrane attack complexes (MACs) at the neuromuscular junctions, the stained sections were viewed with an Olympus BX50 fluorescence microscope (Olympus, Center Valley, PA). Digital images captured with a SPOT digital microscope camera (Diagnostic Instruments, Sterling Heights, MI) and analyzed with Image Pro software (Media Cybernetics, Silver Spring, MD). BTX-stained junctions were identified and outlined in Image Pro. The corresponding area was assessed for MAC deposition based on mean pixel density of the area outlined. Four animals from each group (MDCS treated and control) were analyzed. A total of 135 neuromuscular junctions of the MDCS-treated group and 65 of the control group were analyzed. The percent of endplates with specific pixel density was expressed in a histogram.

**Evaluation of MDCS treatment specificity**

Ten wild-type (WT) C57BL/6 mice were immunized with 25 μg AChR protein in CFA following the protocol described earlier for EAMG induction. In 1 wk, after confirming that anti-AChR IgGs developed in these mice by analyzing serum samples using ELISA, mice were randomly divided into two groups; one group was treated with 1.5 × 10^6 MDCSs and the other with the same volume of PBS by i.v. injection. In another week, all the mice were immunized again with a nonrelevant Ag (OVA protein, 100 μg/mouse) in CFA, and sera samples were collected on a weekly basis for another 3 wk. Serum titers of AChR or OVA-specific IgGs were measured by respective ELISAs.

**In vitro B cell inhibitory assays**

The direct impact of MDCSs on B cells was assessed by direct observation of the size and number of cell clusters resulting from proliferating B cells, by flow cytometry analysis of CFSE dilution in proliferating B cells, or both. In brief, splenocytes from naive C57BL/6 mice were first labeled with 10 μM CFSE, then incubated with either 5 μg/ml anti-CD40 IgG plus 100 U/ml IL-4 or 10 μg/ml anti-IgM F(ab')2; (Jackson Immunoresearch, West Grove, PA) plus 100 U/ml IL-4. After 72 h of incubation, the sizes and numbers of clusters from proliferating cells were recorded under a microscope, and CFSE dilution of the proliferating CD19^+ B cells was analyzed by a flow cytometer (BD LSR II; BD Biosciences, San Jose, CA). In experiments to study the role of arginase, inducible NO synthase (iNOS), and PGE2 in the process of MDCS-mediated B cell inhibition, anti-IgM F(ab')2-activated B cells were labeled with CFSE, then mixed with MDCSs at a ratio of 10:1 in the presence of different concentrations of the arginase inhibitor N^ω-lysine monomethyl arginine acetate (l-NMMA), or PGE2, production inhibitor NS398 (Cayman Chemical, Ann Arbor, MI) followed by the same readout assays. Concentrations of PGE2 in the culture supernatants were measured by a PGE2 ELISA kit (Cayman Chemical), following manufacturer-provided protocols.

**In vivo B cell inhibitory assays**

To determine the direct impact of MDCSs on preactivated B cells in vivo, we immunized 8-wk-old female C57BL/6 mice (n=6) with 20 μg of the T cell–independent Ag 4-hydroxy-3-nitrophénylacetyl (NP)-Ficoll (Biosearch Technology, Petaluma, CA) by i.p. injection following protocols described previously (15). When anti-NP IgGs were detectable in the sera as assessed by ELISA (14 d later), 1.5 × 10^6 of the MDCSs were given to half the mice by tail vein i.v. injection. The anti-NP IgG levels in the sera were monitored for another 3 wk by the same ELISA.

**Anti-NP IgG ELISA**

Sera samples were 1:50 diluted in PBS and added into wells of a 96-well plate coated with 5 μg/ml NP-BSA (Biosearch Technologies, Petaluma, CA). After 2 h of incubation, HRP-conjugated rabbit anti-IgM IgG (1:4000) was added and incubated for another hour. The titers of NP-specific IgGs in the sera were assessed by measuring OD450 after the development using tetramethylbenzidine (Thermo Scientific, Rockford, IL).

**Results**

Adaptive transfer of HSC-induced MDCS reverses disease progression in established EAMG

Following previously published protocols (13, 16, 17), we induced EAMG in WT C57BL/6 mice by repeated immunization with AChR in CFA. One week after the boost, we evaluated muscle strength of the immunized mice using a grid-hanging method and measured sera AChR-specific IgG levels by ELISA to confirm the...
establishment of EAMG. After this, we randomly divided the mice into two groups. One group received $1.5 \times 10^6$ MDSCs per mouse through tail-vein i.v. injection, and the other group received the same volume of PBS as controls. We monitored the mice for another 3 wk, assessing their muscle strength and sera anti-AChR IgG levels every week. In addition, we evaluated complement activation levels at the endplates by immunofluorescence staining and analyzed AChR-specific T cell responses by Th1/Th17 recall assays at the end of the experiments. As shown in Fig. 1, the MDSC-treated mice showed improved muscle strength (Fig. 1A) and reduced sera AChR-specific IgG levels (Fig. 1B) starting 1 wk after the treatment, whereas the control mice continued to deteriorate. To further determine the impact of MDSC treatment on different AChR-specific immunoglobulin isotypes, we measured levels of AChR-specific IgA, IgM, IgG1, IgG2a, IgG2b, and IgG3 in sera collected immediately before and 3 wk after treatment by ELISA. These assays showed that, compared with continually increasing levels of all anti-AChR Ig subclasses (except for IgA) in the mock-treated mice (Fig. 2A), levels of anti-AChR IgG2a, IgG2b, and IgM decreased in the MDSC-treated EAMG mice (Fig. 2B).

Complement C3 deposition at the endplates was intense in the control mice, whereas little complement C3 deposition was detected in the treated mice (Fig. 3A). The BTX-identified endplates were also analyzed for MAC formation by density scan analysis. As shown in Fig. 3B, consistent with the C3 deposition staining assays, density scan analysis confirmed significant differences in MAC formation on endplates between MDSC-treated and mock-treated mice in EAMG. The MDSC-treated mice with EAMG had a significantly lower percentage of high-intensity MAC staining ($p < 0.001$, two-tailed test) than the mock-treated mice with EAMG.

Consistent with the reported T cell inhibitory activity of MDSCs, treated mice showed significantly suppressed AChR-specific Th1 and Th17 responses compared with control mice, as measured by IFN-γ and IL-17 production in the recall assays (Fig. 3C). These results indicate that adoptive transfer of the MDSCs after disease onset was effective in reversing EAMG disease progression in association with suppressed AChR-specific T and B cell responses.

MDSC treatment in EAMG mice does not significantly inhibit their immune responses to a nonrelevant Ag

Previous studies have suggested that MDSCs suppress immune response in an Ag-specific manner (7–9). We therefore tested whether treating EAMG mice with MDSCs would suppress their immune responses to OVA, a nonrelevant Ag. We reimmunized MDSC-treated and control EAMG mice with OVA in CFA 1 wk after MDSC administration, collected sera weekly for another 3 wk, and assessed both AChR- and OVA-specific IgG titers in the sera by respective ELISAs. Consistent with what we have observed before (Fig. 1B), we found that AChR-specific IgG titers decreased in MDSC-treated mice but kept increasing in control mice, demonstrating that treating mice with MDSCs even after they had developed anti-AChR IgGs effectively reversed the development of these autoantibodies (Fig. 4A). In contrast, OVA-specific Ab titers in both MDSC-treated and control mice kept rising over the same time course (Fig. 4B). However, when we examined the data more closely, we found that OVA-specific IgG titers in MDSC-treated mice were slightly lower than those in control mice (Fig. 4D), whereas AChR-specific IgG titers were significantly lower in the same MDSC-treated than those in the control mice at the same time points (Fig. 4C). Taken together, these results support the hypothesis that MDSCs can at least partially suppress immune responses in an Ag-specific manner.

HSC-induced MDSCs directly inhibit B cell proliferation in vitro

The suppressed AChR-specific B cell responses (reduced serum anti-AChR IgG titers) observed in the earlier experiments could come from suppressed T cell responses because AChR is a T cell–dependent Ag. However, it is also possible that in addition to inhibiting T cells, MDSCs may directly inhibit B cell responses. To test this hypothesis, we cocultured CFSE-labeled, anti-CD40 mAb/IL-4–activated B cells with different numbers of MDSCs for 4 d, then assessed B cell inhibition by analyzing sizes and numbers of cell clusters formed from proliferating B cells and by measuring CD19+ B cell proliferation (CFSE dilution) using flow-cytometry analysis. These assays (Fig. 5A) showed that when mixed with B cells at a ratio of 1:5, MDSCs almost completely inhibited B cell proliferation (reduced from 85.5 to 6.9%), and that at a ratio of 1:10, MDSCs still significantly inhibited B cell proliferation (from 85.5 to 13.7%).

Because signaling through BCRs is a more physiologically relevant form of B cell activation than cross-linking CD40, we next incubated different numbers of MDSCs with B cells exposed to anti-IgM F(ab′)2 plus IL-4, then assessed B cell proliferation after 3 d of incubation using the same method as described earlier. These assays showed that, similar to the results obtained with the anti-CD40 mAb–activated B cells, HSC-induced MDSCs inhibited the proliferation of the activated B cells in a dose-dependent fashion (Fig. 5B), indicating that MDSCs directly inhibit B cells.

To test whether MDSCs could inhibit the proliferation of preactivated B cells, we activated B cells again by signaling through BCRs using anti-IgM F(ab′)2 plus IL-4, and after 24 h of incubation, we added different numbers of MDSCs into the culture and measured B cell proliferation by direct imaging and by flow cytometry following the same protocol. These experiments showed that MDSCs inhibited the proliferation of preactivated B cells in a dose-dependent manner (Fig. 5C).

**FIGURE 1.** Adoptive transfer of HSC-induced MDSCs is effective in treating established EAMG. Mice were immunized to induce EAMG following the protocol described. After the second boost, the development of EAMG was confirmed using the grid-hanging method and measurement of serum anti-AChR IgG levels; the mice were then randomly divided into two groups: one group ($n = 11$) received $1.5 \times 10^6$ MDSCs by tail-vein injection and the other ($n = 11$) received the same volume of PBS as controls. Mice were then monitored for another 3 wk, with muscle strength (A) and total sera anti-AChR IgG levels (B) assessed every week. Error bars are SD.
HSC-induced MDSCs directly inhibit preactivated B cells in vivo

To verify the earlier results in vivo, we treated WT C57BL/6 mice immunized with the T cell–independent Ag NP-Ficoll (18), in which the production of anti-NP IgGs does not involve T cells. After verifying the presence of anti-NP IgGs in the mouse sera by ELISA 2 wk after immunization, we treated half the mice with MDSCs (1.5 \( \times \) 10^6 cells/mouse), the other half with the same volume of PBS, and then monitored serum anti-NP IgG levels by ELISA every week for another 3 wk. These studies showed that anti-NP IgG levels in treated mice started to decrease 1 wk after MDSC treatment, whereas serum NP-specific IgG levels continued to increase in the mock-treated mice, demonstrating that MDSCs directly inhibited the preactivated B cells in vivo (Fig. 6).

PGE2 is integrally involved in MDSC-mediated B cell inhibition

We next tried to explore possible mechanisms by which MDSCs inhibit activated B cell proliferation. Previous isolated reports have demonstrated that MDSCs produce PGE2 (9, 19) and that PGE2 inhibits B cells (20). In light of these reports, we set up B cell inhibition assays again in which the anti-IgM F(ab')2-activated B cells were incubated with MDSCs in the presence of 0, 2, or 5 \( \mu \)M of the PGE2-production inhibitor NS398 (21). After 72 h of incubation, we assessed B cell proliferation by microscopy/flow cytometry and measured PGE2 concentrations in the culture supernatants by ELISA. These assays showed that, consistent with the earlier described results, MDSCs inhibited the proliferation of activated B cells, and that NS398 significantly enhanced B cell proliferation in the presence of MDSCs in a dose-dependent manner (Fig. 7A, 7B), indicating that production of PGE2 is an important mechanism by which MDSCs inhibit B cells. ELISA analysis of the culture supernatants confirmed that MDSC-derived PGE2 was significantly decreased in the presence of NS398 (Fig. 7C).

To further verify the role of PGE2 in MDSC-mediated direct B cell inhibition, in addition to the earlier described experiments using PGE2 production inhibitors, we also compared the relative efficacies of MDSCs derived from WT or 15-PDGH KO mice in inhibiting the proliferation of activated B cells. It is known that 15-PDGH inactivates PGE2 (22), and consequently a deficiency of 15-PDGH leads to significantly increased levels of PGE2 (23). These experiments showed that MDSCs derived from 15-PDGH KO mice were more potent in inhibiting the proliferation of activated B cells than those from WT mice (Fig. 7D), which also indicated an important role of PGE2 in the MDSC-mediated direct B cell inhibition.

Other mechanisms underlying MDSC-mediated B cell inhibition

It has been demonstrated that iNOS and arginase are two mechanisms by which MDSCs inhibit T cell responses (7). To determine whether they are also important in MDSC-mediated B cell inhibition in ad-
dition to PGE2, we cocultured activated B cells with MDSCs in the absence or presence of different concentrations of respective iNOS and arginase inhibitors, l-NMMA (24) and N^\text{G}-hydroxy-nor-arginine (25), then compared B cell proliferation in 72 h. These experiments showed that inhibition of iNOS or arginase reduced the B cell inhibitory activity of MDSCs in a dose-dependent manner (Fig. 8), suggesting that in addition to PGE2, iNOS and arginase are also important for MDSCs to directly inhibit B cells.

**Discussion**

In this study, we demonstrated that adoptive transfer of HSC-induced MDSCs reversed EAMG disease progression when the

**FIGURE 4.** MDSC treatment does not significantly suppress host immune responses to a nonrelevant Ag. (A) AchR-specific IgG titers in MDSC-treated EAMG mice and control mice. Sera were collected at different time points and serum anti-AChR IgG titers were measured by ELISA. (B) OVA-specific IgG titers in MDSC-treated EAMG mice and control mice after OVA immunization. (C) AchR-specific IgG titers in MDSC-treated EAMG mice and control mice at different time points; (D) OVA-specific IgG titers in MDSC-treated EAMG mice and control mice after OVA immunization as measured over the same time course. Error bars are SD. ^{*}p < 0.05.

**FIGURE 5.** HSC-induced MDSCs directly inhibit B cells in vitro. (A) Splenocytes from C57BL/6 mice (4 × 10^5 cells) were labeled with CFSE, activated by 1 μg/ml rat anti-CD40 IgG and 100 U/ml IL-4, and then immediately cocultured with different numbers of MDSCs. The proliferation of the activated CD19^+^ B cells was assessed on day 4 by flow cytometry. (B) Similar experiments were done except that B cells were activated by 10 μg/ml goat anti-mouse IgM F(ab')2 and 100 U/ml IL-4. Cells were analyzed on day 3. Representative results of more than three experiments. (C) B cells were activated by anti-IgM F(ab')2/IL-4 and MDSCs were added 24 h later, showing that MDSCs inhibit the proliferation of preactivated B cells. Upper panel, Images of cell clumps (arrows) formed after B cell proliferation; lower panel, B cell proliferation assessed by CFSE dilution assays. Representative results of five experiments.
cells were given after disease onset. In conjunction with significantly improved muscle strength, MDSC-treated EAMG mice had suppressed AChR-specific T cell responses, reduced AChR-specific humoral responses, and decreased neuromuscular junction complement activation. MDSC treatment did not significantly inhibit host immune responses to a nonrelevant Ag. In addition to the established T cell inhibitory activities of MDSCs, we found that these cells directly inhibit B cells, which could contribute to their potency in treating EAMG. Using respective inhibitors and MDSCs derived from 15-PGDH KO mice, we found that MDSCs directly inhibit B cells through multiple mechanisms including PGE2, iNOS, and arginase.

Because AChR-specific autoantibodies cause pathology in most MG patients by activating complement and/or directly blocking AChR function, targeting B cells to reduce AChR-specific Ab production is a valid treatment option. In fact, rituximab, a B cell-depleting mAb, has been successfully used to treat MG (26–28). Because AChR is a T cell–dependent Ag, inhibiting T cell responses should also be effective. Indeed, previous studies using reagents primarily targeting T cells have shown effectiveness in treating active immunization-induced EAMG (29, 30); in the clinical context, thymectomy (surgical removal of the thymus, where T cells develop) and administration of the immunosuppressive agent mycophenolate, which primarily inhibits T cell replication, are options for treating MG (31). It is reasonable to hypothesize that a new treatment simultaneously suppressing both autoreactive T and B cell responses would work synergistically and be highly effective in treating MG. Our data suggest that HSC-induced MDSCs fit these criteria, because they concurrently inhibit both AChR-specific T and B cell responses, and these MDSCs indeed were highly effective in reversing disease progress in EAMG.

MDSCs have been shown to inhibit T cells by producing NO through iNOS (32) and by expressing IDO to locally reduce tryptophan levels and to produce bioactive tryptophan metabolites (kynurenine) (33). They also appear to be able to induce Foxp3+ regulatory T cell differentiation to regulate T cell responses (34). Although it is well established that MDSCs inhibit T cells through multiple mechanisms (7), whether MDSCs have any direct effect on B cells and what the underlying mechanism is have been understudied. A recent report showed that MDSCs can be induced by infection in a murine model of retrovirus-induced AIDS (35). This study also found that the infection-induced MDSCs directly suppressed the proliferation of B cells that had been activated in vitro by LPSs or anti-CD40 mAb. This suppression occurred through the generation of reactive nitrogen species, as shown by the fact that the NO synthetase inhibitor L-NMMA significantly reduced the ability of MDSCs to inhibit B cells (35). We have found, as demonstrated in this report, that HSC-induced MDSCs also directly suppress the proliferation of B cells that had been activated in vitro by anti-CD40 or anti-IgM mAbs, and that

**FIGURE 6.** HSC-induced MDSCs directly inhibit B cells in vivo. WT B6 mice (n = 6) were immunized with 20 μg/mouse of the T cell–independent Ag NP-Ficoll by i.p. injection, and the development of anti-NP IgG in the sera was determined by ELISA in 2 wk. After this, half the mice were treated with MDSCs (1.5 × 10^6 cells/mouse) by tail-vein i.v. injection, and the other half received the same volume of PBS. The sera titers of anti-NP IgG were monitored by ELISA every week for another 3 wk. Error bars are SD. *p < 0.01.

**FIGURE 7.** HSC-induced MDSCs inhibit B cells through PGE2. Splenocytes from C57BL/6 mice (4 × 10^5 cells) were labeled with CFSE, activated by 10 μg/ml goat anti-mouse IgM F(ab')2 and 100 U/ml IL-4. These cells were cocultured without or with MDSCs (10:1 ratio) in the presence of 0, 2, and 5 μM NS398. Cell images (A) were taken on day 2 to show the formed clusters (arrows) as a result of B cell proliferation; then CFSE dilution assays on CD19+ B cells (B) were performed the next day (day 3), and PGE2 concentrations in the culture supernatants were measured by ELISA (C). In parallel experiments, efficacies of MDSCs derived from WT or 15-PGDH KO mice inhibiting B cells were compared using the same CFSE-based B cell proliferation assay (D). Representative results of two or three experiments. Error bars are SD. *p < 0.05.
showed that HSC-induced MDSCs produce large amounts of PGE2, and that B cell proliferation was significantly increased in the presence of different concentrations of inhibitors. The proliferation of the activated CD19+ B cells was assessed on day 4 by flow cytometry.

Recent studies also indicate that MDSCs suppress T cell responses and diseases because of their known potent T cell inhibitory activity. Parallel studies using MDSCs derived from 15-PGDH KO mice have been isolated reports showing that MDSCs produce PGE2 (19, 40). The role of PGE2 in immunoregulation is controversial. Some studies have found that PGE2 promotes immune inflammation through enhancing Th1 cell differentiation and Th17 cell expansion by interacting with its receptors on T cells and dendritic cells (38); other studies have shown that PGE2 suppresses T and B cell responses (20, 39). Although there have been isolated reports showing that MDSCs produce PGE2 (19, 40), their potential impact on B cells was unclear. Our ELISA assays showed that the 15-PGDH–deficient MDSCs were more efficient at decreasing PGE2 production as expected, as suggested by the studies using the PGE2 production inhibitors.

Multiple mechanisms including PGE2, iNOS, and arginase are integrally involved in the MDSC-mediated B cell inhibition. This finding is consistent with the previous results observed on the retroviral infection-induced MDSCs, suggesting that the direct B cell inhibitory potential could apply to MDSCs induced by means of several different stimuli. More importantly, in vivo studies using the T cell–independent Ag NP-Ficoll, we found that MDSCs inhibited the production of the anti-NP-Ficoll IgGs when given after these Abs were already present in the sera, demonstrating that MDSCs are effective in directly inhibiting preactivated B cells in vivo. The potent B cell inhibitory activity of MDSCs, together with their established T cell inhibitory activity, could explain the significant effect that MDSCs had in reversing disease progression in established EAMG.

PGE2 is a primary product of arachidonic metabolism and is synthesized via the cyclooxygenase and PG syntheses pathways. Produced PGE2 is inactivated by 15-PGDH, which metabolizes PGE2 by oxidizing the 15(S)-hydroxyl group into a keto group, resulting in the inactivated 15-keto PGE2 (36). Not surprisingly, genetic deletion of 15-PGDH leads to increased tissue levels of PGE2 (37). The role of PGE2 in immunoregulation is controversial. Some studies have found that PGE2 promotes immune inflammation through enhancing Th1 cell differentiation and Th17 cell expansion by interacting with its receptors on T cells and dendritic cells (38); other studies have shown that PGE2 suppresses T and B cell responses (20, 39). Although there have been isolated reports showing that MDSCs produce PGE2 (19, 40), their potential impact on B cells was unclear. Our ELISA assays showed that HSC-induced MDSCs produce large amounts of PGE2, as reported in studies using MDSCs from other sources (19, 40). By adding cyclooxygenase-2 inhibitors NS398 in the B cell/MDSC cocultures, we found that PGE2 production decreased as expected and that B cell proliferation was significantly increased in the presence of MDSCs in association with decreased levels of PGE2, suggesting that PGE2 is important for MDSCs to directly inhibit B cells. Parallel studies using MDSCs derived from 15-PGDH KO mice showed that the 15-PGDH–deficient MDSCs were more efficient at inhibiting B cells than WT MDSCs, which further confirmed the importance of PGE2 in the MDSC-mediated direct B cell suppression, as suggested by the studies using the PGE2 production inhibitors.

MDSCs hold promise as a new strategy for treating autoimmune diseases because of their known potent T cell inhibitory activity. Recent studies also indicate that MDSCs suppress T cell responses in an Ag-specific manner (7), a great advantage over the use of other nonspecific immunosuppressive reagents such as prednisone and cyclosporine, which often leads to unwanted negative side effects in patients. We found that anti-AChR IgG titers decreased in MDSC-treated mice but kept increasing in control mice, and over the same time course, anti-OVA IgG titers in both the treated and control mice kept increasing, suggesting that the administered MDSCs markedly suppressed AChR-specific immune responses but did not significantly inhibit the anti-OVA IgG development. Although anti-OVA IgG titers in MDSC-treated EAMG mice were slightly lower than those in control EAMG mice after OVA reimmunization, comparing with significantly decreased anti-AChR IgG titers in MDSC-treated EAMG mice, these results suggest that MDSCs can work at least partially in an Ag-specific manner as suggested by previous reports (7–9). Another possible explanation for these results is that by the time when we reimmunized the mice with OVA Ag (1 wk after MDSC administration), MDSCs had “hit and run” and had been cleared out, whereas their inhibitory effects on AChR-specific immune reactions persisted. Nevertheless, these results suggest that our MDSC treatment does not have a significant impact on the immune responses against nonrelevent Ags, which is an advantage over other existing pan-immunosuppressive drugs.

The development of MDSCs for treating autoimmune diseases has been greatly hampered because of the lack of good methods to efficiently generate potent syngeneic MDSCs in large numbers (7). To address this obstacle, we recently developed a protocol to induce MDSC differentiation from bone marrow progenitors by using HSCs (10). The induction of MDSCs by HSCs is not MHC restricted, because HSCs isolated from C57BL/6 mice induce MDSC differentiation from bone marrow progenitors from BALB/C as efficiently as from C57BL/6 mice (data not shown), suggesting that this approach could be further developed in clinic for the generation of syngeneic MDSCs for treatment purposes. In these cases, only bone marrow cells or PBMCs containing hematopoietic progenitors from the same patients are required to differentiate into active MDSCs after their incubation with HSCs from other donors.

In summary, we have found that adoptive transfer of HSC-induced MDSCs was highly effective in reversing EAMG disease progression in association with suppressed AChR-specific T cell responses, reduced serum anti-AChR IgG levels, and decreased endplate complement activation. The MDSC treatment did not significantly inhibit the host responses to a nonrelevant Ag, which was introduced 1 wk later. In addition to their potent T cell inhibitory activities, these MDSCs directly inhibited preactivated B cells in which multiple mechanisms including PGE2, iNOS, and arginases are integrally involved in the underlying mechanism. The concurrent inhibition of both T and B cells by these MDSCs, the potential Ag-specific immune suppression, as well as the relative convenience in preparing large numbers of syngeneic MDSCs, should make the HSC-induced MDSCs attractive for further clinical development for patients diagnosed with MG, or even more broadly, for patients diagnosed with other diseases in which T and B cells are involved in the pathogenesis.

Disclosures

The authors have no financial conflicts of interest.

References
