

Myeloid Suppressor Cells Induced by Retinal Pigment Epithelial Cells Inhibit Autoreactive T-Cell Responses That Lead to Experimental Autoimmune Uveitis

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PURPOSE. To test whether retinal pigment epithelial (RPE) cells are able to induce myeloid-derived suppressor cell (MDSC) differentiation from bone marrow (BM) progenitors.

METHODS. BM cells were cocultured with or without RPE cells in the presence of GM-CSF and IL-4. Numbers of resultant MDSCs were assessed by flow cytometry after 6 days of incubation. The ability of the RPE cell-induced MDSCs to inhibit T cells was evaluated by a CFSE-based T-cell proliferation assay. To explore the mechanism by which RPE cells induce MDSC differentiation, PD-L1-deficient RPE cells and blocking antibodies against TGF- β , CTLA-2 α , and IL-6 were used. RPE cell-induced MDSCs were adoptively transferred into mice immunized with interphotoreceptor retinoid-binding protein in complete Freund's adjuvant to test their efficacy in suppressing autoreactive T-cell responses in experimental autoimmune uveitis (EAU).

RESULTS. RPE cells induced the differentiation of MDSCs. These RPE cell-induced MDSCs significantly inhibited T-cell proliferation in a dose-dependent manner. PD-L1-deficient RPE cells induced MDSC differentiation as efficiently as wild-type RPE cells, and neutralizing TGF- β or CTLA-2 α did not alter the numbers of induced MDSCs. However, blocking IL-6 reduced the efficacy of RPE cell-induced MDSC differentiation. Finally, adoptive transfer of RPE cell-induced MDSCs suppressed IRBP-specific T-cell responses that led to EAU.

CONCLUSIONS. RPE cells induce the differentiation of MDSCs from bone marrow progenitors. Both cell surface molecules and soluble factors are important in inducing MDSC differentiation. PD-L1, TGF- β , and CTLA-2 α were not measurably involved in RPE cell-induced MDSC differentiation, whereas IL-6 was important in the process. The induction of MDSCs could be another mechanism by which RPE cells control immune reactions in the retina, and RPE cell-induced MDSCs should be further investigated as a potential approach to therapy for

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Myeloid-derived suppressor cells (MDSCs) were originally identified in patients and in mice with cancer.¹⁻³ MDSCs potently suppress host T-cell responses to permit tumor survival. In mice, MDSCs are characterized as CD11b⁺Gr-1⁺ cells that are immunosuppressive.⁴ Because of their potent T-cell inhibitory activities, MDSCs have potential as a novel therapy for T-cell-mediated autoimmune diseases^{5,6} and for the prevention of transplanted allograft rejection.⁶ However, because it is impractical to isolate syngeneic MDSCs from tumors for treatment purposes, the lack of a reliable, syngeneic source of large numbers of MDSCs has greatly hampered the development of MDSCs as a new therapeutic approach. Therefore, understanding the mechanisms that underlie MDSC differentiation and developing new methods to generate large numbers of MDSC in vitro are of clinical relevance.

In addition to tumors, MDSCs have been identified in infections^{7,8} and autoimmune diseases, including experimental autoimmune uveitis (EAU),⁹ a murine model of autoimmune posterior uveitis in which retina-specific T cells cause local inflammation, resulting in breakdown of the blood-retina barrier, leukocyte infiltration, retinal granulomas, retinal folding, and retinal detachment.¹⁰ It is possible that the MDSCs identified in EAU are induced, at least in part, by myeloid progenitors in the blood that enter the eye during uveitis by local retinal cells such as retinal pigment epithelial (RPE) cells.

Previous studies have demonstrated that RPE cells directly inhibit T and B cells in the retina by expressing PD-L1 and TGF- β .¹¹⁻¹³ They can also induce foxp3⁺ T regulatory (Treg) cell differentiation by producing CTLA-2 α , a cathepsin L inhibitor.¹⁴ However, whether there are other mechanisms that RPE cells use to control the immune reactions are unclear. In this report, we found that RPE cells inhibited dendritic cell (DC) propagation and induced MDSC differentiation from myeloid progenitor cells in bone marrow (BM) cells. Similar to the MDSCs identified in tumors, the RPE cell-induced MDSCs were CD11b⁺Gr-1⁺ and had profound T-cell inhibitory activities. The lack of PD-L1 on RPE did not alter the numbers of RPE cell-induced MDSCs, nor did blocking the activities of TGF- β or CTLA-2 α . However, blocking IL-6 in the RPE-BM cell cocultures significantly inhibited MDSC differentiation, suggesting that IL-6 is important for RPE cells to induce MDSCs. Finally, the adoptive transfer of RPE cell-induced MDSCs significantly inhibited autoreactive T-cell responses that lead to retinal injury in EAU. These results demonstrated a novel mechanism by which RPE cells regulate immune responses and could lead to new methods to generate large numbers of syngeneic MDSCs for potential therapeutic applications.

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METHODS AND REAGENTS

Mice

C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) 8 to 12 weeks old were used in all studies. *PD-L1*^{-/-} mice¹⁵ (C57BL/6 background) were generously provided by Lieping Chen (Johns Hopkins University, Baltimore, MD). All animal studies were carried out using an approved institutional animal protocol in the animal resource center of Case Western Reserve University and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Mouse RPE Isolation and Characterization

Primary RPE from wild-type (WT) and respective knockout mice were isolated according to methods described by Sun et al.,¹⁶ with minor modifications. In brief, the cornea, lens, and neural retina were first removed from the eyes, then posterior eyecups were immersed in 0.25% trypsin/EDTA and incubated for 1 hour at 37°C. RPE cells were harvested from eyecups by gentle pipetting. The cells were spun down at 1000 rpm for 5 minutes, then resuspended in complete DMEM with 10% of FBS and cultured in a six-well plate. The purity of the isolated cells was >90%, as assessed by staining with an anti-RPE65 antibody (Millipore, Billerica, MA). Cells with passage numbers 3 to 6 were used in all experiments.

Induction of MDSCs by RPE

An established protocol for DC generation was followed to test the efficacy of RPE in inducing MDSC differentiation. In brief, primary RPE cells were cocultured with BM cells at a ratio of 1:20 in the presence of 8 ng/mL GM-CSF and 100 U/mL IL-4. BM cells cultured in the presence of cytokines but not RPE cells were included as controls. After incubation (6 days), differentiated MDSCs (CD11b⁺Gr-1⁺ cells) in nonadherent cells were assessed by flow cytometry using an LSRI flow cytometer (BD Biosciences, Franklin Lakes, NJ). In transwell experiments, culture conditions remained the same except that RPE cells were cultured in an insert (0.4 μm pore size; BD Biosciences), permeable to soluble factors but not allowing physical cell-cell contact. For TGF-β and IL-6 blocking experiments, 5 μg/mL anti-TGF-β mAb (clone TW7-20B9, sodium azide free; BioLegend, San Diego, CA), anti-IL-6 mAb (clone MP5-20F3, sodium azide free; BioLegend), or isotype control was added into the RPE-BM cell cocultures. For CTLA-2α blocking experiments, 10 μg/mL rabbit anti-mouse CTLA-2α IgG¹⁴ and purified rabbit IgG (Southern Biotechnology Associates, Birmingham, AL) were used.

T-Cell Proliferation Assay

The efficacy of the RPE cell-induced MDSCs in inhibiting T-cell responses was assessed by a CFSE-based T-cell proliferation assay.¹⁷ For these assays, naive C57BL/6 mouse spleen cells were first labeled by incubating them with 0.3 μM CFSE (Invitrogen, Carlsbad, CA) at 37°C for 8 minutes. After washing, 1 μg/mL anti-CD3 mAb (BD Biosciences) was added to the CFSE-labeled spleen cells to activate T cells. The CFSE-labeled, anti-CD3 mAb activated cells were then aliquotted into wells of a 96-well plate at a concentration of 0.4 × 10⁶ cells/well and were incubated with different numbers of the RPE cell-induced MDSCs (T cell/MDSC ratios: 0, 1:10, 1:20, 1:40, and 1:80) in triplicate. After 2 days of incubation, T-cell inhibitory activity was assessed by checking proliferating T cell-formed clusters under a microscope and by measuring CFSE dilution on CD4⁺ T cells using flow cytometry.

EAU Induction and MDSC-Based Treatments

EAU was induced in female C57L/6 mice by IRBP₁₋₂₀ peptide immunization (200 μg/mouse) in CFA together with pertussis toxin using protocols described previously.⁹ To test the efficacy of the RPE cell-induced MDSCs in suppressing T-cell responses in vivo, 2 × 10⁶ MDSCs suspended in 0.5 mL PBS were injected intravenously through the tail vein in half the mice after immunization. The same volume of

PBS was given to the other half of immunized mice as controls. In 3 weeks, all mice were euthanized. Spleen cells were collected to assess IRBP-specific Th1 and Th17 responses by IFN-γ and IL-17 ELISA, and the eyes were examined to evaluate the severity of EAU.

EAU Scoring

Mouse eyes were fixed in 10% formalin overnight. After this, paraffin-embedded ocular sections were made and stained with hematoxylin and eosin. The EAU histopathologic severity of each eye was evaluated in a blinded fashion on a scale of 0 to 4 using established criteria¹⁰: 0, no change; 0.5 (trace), few (one to two) very small, peripheral focal lesions, minimal vasculitis/vitritis; 1, mild vasculitis, fewer than five small focal lesions and one linear lesion; 2, multiple (more than five) chorioretinal lesions and/or infiltrations, severe vasculitis (large size, thick wall, infiltrations), few linear lesions (fewer than five); 3, pattern of linear lesions, large confluent lesions, subretinal neovascularization, retinal hemorrhages, and papilledema; 4, large retinal detachment and retinal atrophy.

T-Cell Response Recall Assays

Spleen cells (2 × 10⁶) from the MDSC-treated and mock-treated mice were incubated with 5 and 20 μg/mL of IRBP peptide for 3 days. Then IFN-γ and IL-17 levels in the supernatants were measured using conventional ELISA kits (BD Biosciences).

Statistical Analysis

All experiments were performed at least twice with similar results. Data were analyzed using an independent *t*-test, except for the EAU histopathological score, which were analyzed by an ANOVA test treating each mouse (average of both eyes) as one statistical event. *P* ≤ 0.05 was considered significant.

RESULTS

RPE Cells Induce CD11b⁺Gr-1⁺ Cell Differentiation

To test whether RPE cells are capable of inducing MDSC differentiation from BM cells, we followed a well-established protocol for the generation of DCs from BM progenitors. We cocultured BM cells with and without RPE cells in the presence of GM-CSF and IL-4. After 6 days of incubation, we stained the nonadherent cells for CD11b, Gr-1, and CD11c, followed by flow cytometry analysis. These experiments (Fig. 1) showed that, consistent with previous reports, BM cells differentiated into CD11b⁺CD11c⁺ DCs in the presence of GM-CSF and IL-4. However, in cultures with RPE cells, the generation of CD11b⁺CD11c⁺ DCs was significantly inhibited by 2.5-fold (Fig. 1A), whereas the generation of CD11b⁺Gr-1⁺ cells was increased by 3-fold (Fig. 1B). Although the CD11b⁺CD11c⁺ cells had a typical DC morphology (Fig. 1C), the CD11b⁺Gr-1⁺ cells appeared to have a mononuclear cell-like morphology (Fig. 1D). These results indicated that RPE cells inhibit DC propagation and skew the differentiation of the BM progenitors into cells with phenotypic features of MDSCs.

RPE Cell-Induced CD11b⁺Gr-1⁺ Cells Inhibit T-Cell Responses

In addition to the CD11b⁺Gr-1⁺ cell surface marker expression profile, an important feature of MDSCs is their ability to inhibit T-cell responses.¹⁷ To determine whether the CD11b⁺Gr-1⁺ cells induced by RPE are indeed functional MDSCs, we examined their T-cell inhibitory activity using a CFSE-based T-cell proliferation assay. These assays (Fig. 2) showed that the RPE cell-induced CD11b⁺Gr-1⁺ cells potently inhibited T-cell responses in a dose-dependent manner. At a ratio of 1:5, the RPE cell-induced CD11b⁺Gr-1⁺ cells inhibited T-cell proliferation

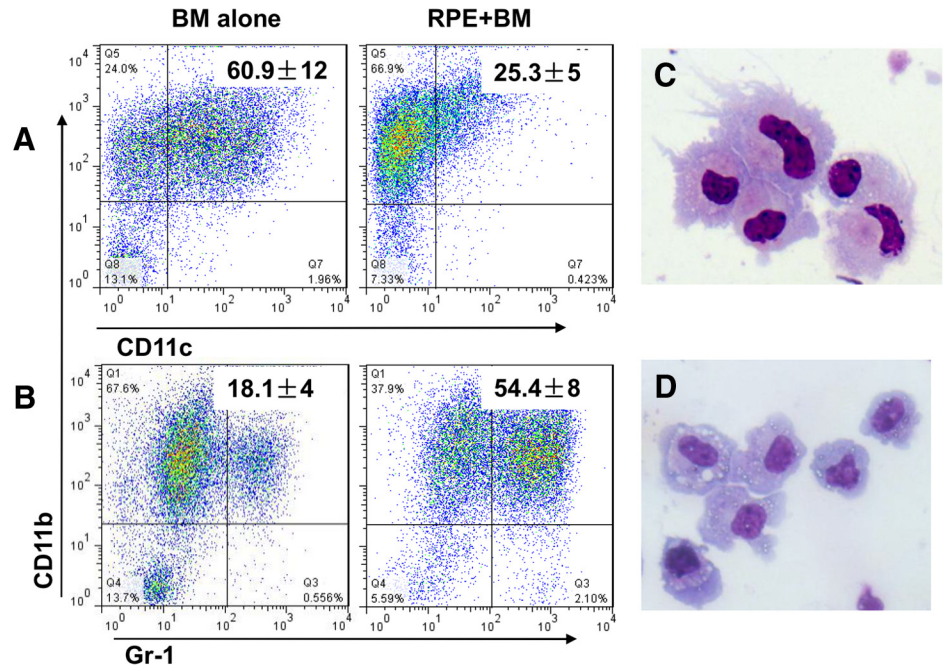


FIGURE 1. RPE cells inhibited DC propagation and induced MDSC differentiation. BM cells were cultured without and with RPE (at a ratio of 20:1) in the presence of GM-CSF and IL-4. After incubation, nonadherent cells were analyzed for markers of DCs (CD11b⁺CD11c⁺) (A) and MDSCs (CD11b⁺Gr-1⁺) (B). Photographs of the resultant DCs (C) and MDSC (D) were taken after conventional Giemsa staining. Results are representative of more than individual experiments. Data are mean ± SD.

by approximately 80%, whereas the inhibitory effect waned at the ratio of 1:40 (Fig. 2B). Consistent to the CFSE dilution assays, directly assessing cell clusters formed by the proliferating T cells under a microscope showed the same pattern (Fig. 2A, upper panel). In addition, to determine whether the RPE cell-induced CD11b⁺Gr-1⁺ cells inhibit inflammatory cytokine production from activated T cells, we repeated the experiments, collected culture supernatants, and measured levels of IFN- γ by ELISA. These assays showed that in association with inhibited T-cell proliferation, IFN- γ production was significantly reduced in a dose-dependent manner by the RPE cell-

induced CD11b⁺Gr-1⁺ cells (Fig. 2C). All these data indicate that the RPE cell-induced CD11b⁺Gr-1⁺ cells are indeed MDSCs with potent T-cell inhibitory activities.

Requirement of Both Direct Cell-Cell Contact and Soluble Factors for RPE to Induce MDSCs

We next began to explore the underlying mechanism by which RPE cells induce MDSCs. To distinguish whether cell surface proteins on RPE cells or soluble factors produced by RPE cells are involved in the induction of MDSCs, we repeated the

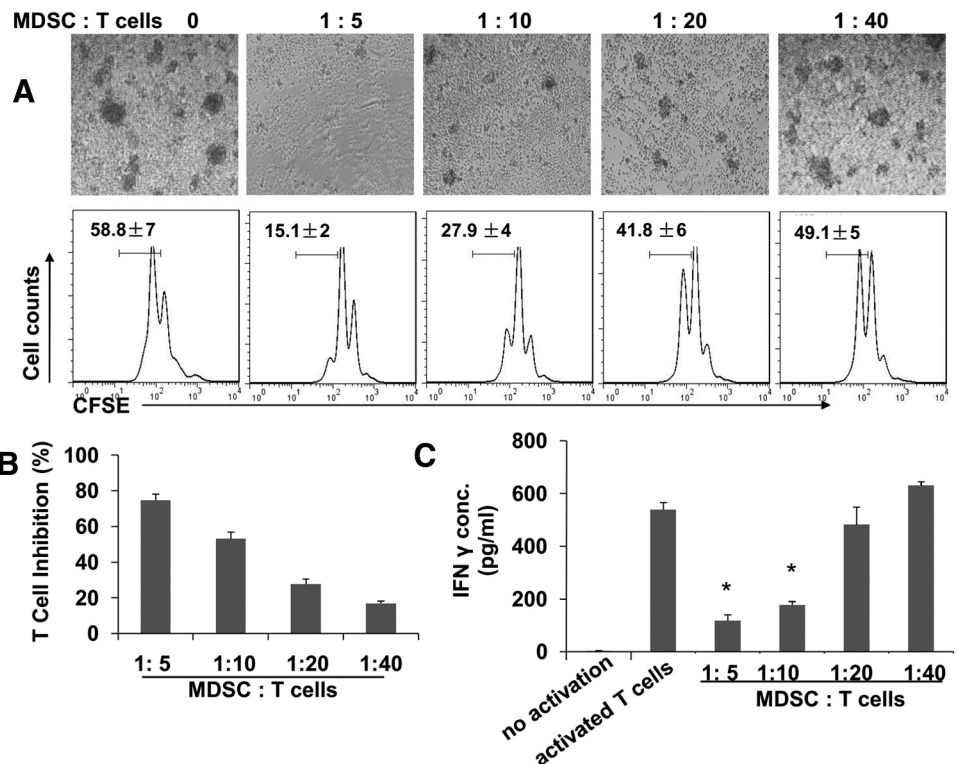


FIGURE 2. RPE cell-induced MDSCs inhibited T-cell responses. Spleen cells (5×10^5) from naive C57BL/6 mice were labeled with CFSE and activated by anti-CD3 mAb, then cocultured with different numbers of the RPE cell-induced MDSCs. In 2 days, the inhibition of T-cell responses was assessed by evaluating proliferating T cell-formed clusters directly under a microscope (A, top) and by measuring CFSE dilution using flow cytometry, gating on the CD4⁺ T cells (A, bottom). The inhibition of T-cell responses (B) was calculated using the following formula: inhibition (%) = $1 - [(b - a)/a]$, where *a* is the number of proliferating T cells without MDSCs and *b* is the number of proliferating T cells with MDSCs. IFN- γ levels in the supernatants were measured by standard ELISA (C).

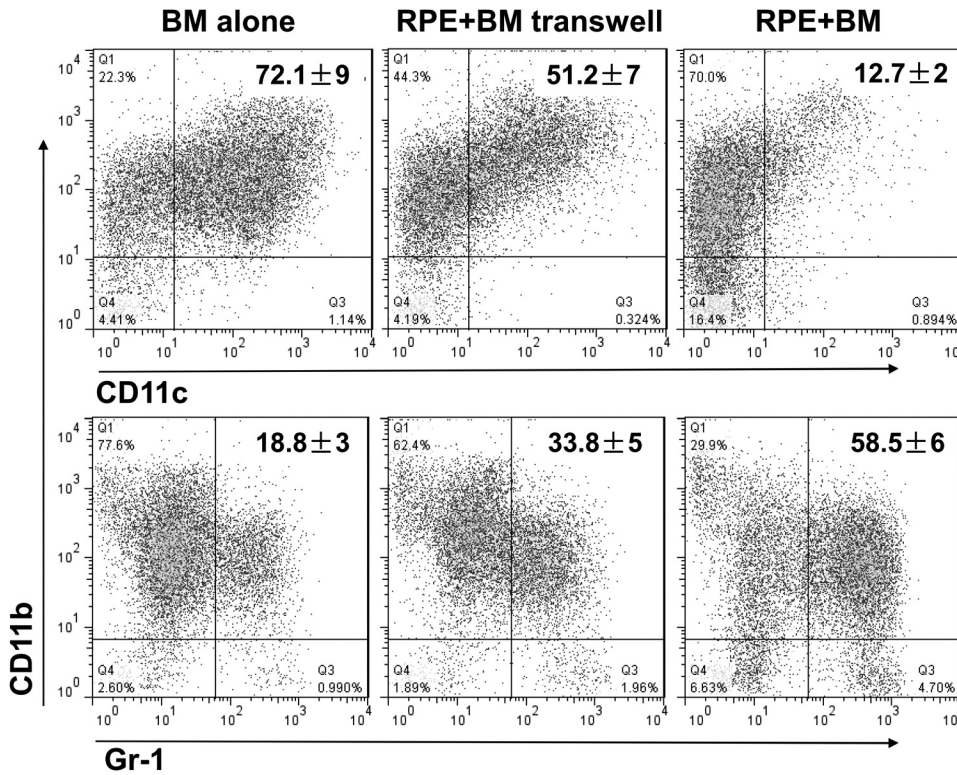


FIGURE 3. Both RPE cell surface molecules and RPE cell-derived soluble factors were involved in suppressing DC propagation and inducing MDSC differentiation. BM cells were cultured without and with RPE (at a ratio of 20:1) in direct contact or in culture inserts together with GM-CSF and IL-4. After incubation, non-adherent cells were analyzed for markers of DCs (CD11b⁺CD11c⁺) and MDSCs (CD11b⁺Gr-1⁺). Results are representative of two individual experiments. Data are mean ± SD.

cocultures with a transwell system in which soluble factors could be exchanged between the RPE in the culture inserts and the BM cells in the underneath plate wells, but direct cell-cell contact was not allowed. These experiments (Fig. 3) showed that transwell separation of BM cells from RPE cells increased the generation of CD11b⁺CD11c⁺ DCs from 12.7% ± 2% to 51.2% ± 7% compared with BM alone generation of DCs at

72.1% ± 9%. In contrast, the generation of CD11b⁺Gr-1⁺ MDSCs was increased from 18.8% ± 3% in BM cultures alone to 33.8% ± 5% but less than the 58.5% ± 6% generation of MDSCs in cocultured RPE plus BM cells. Thus, these transwell experiments showed that the effects of RPE cells on MDSC induction and DC inhibition were not completely abolished when RPE and BM cells were physically separated, suggesting that both

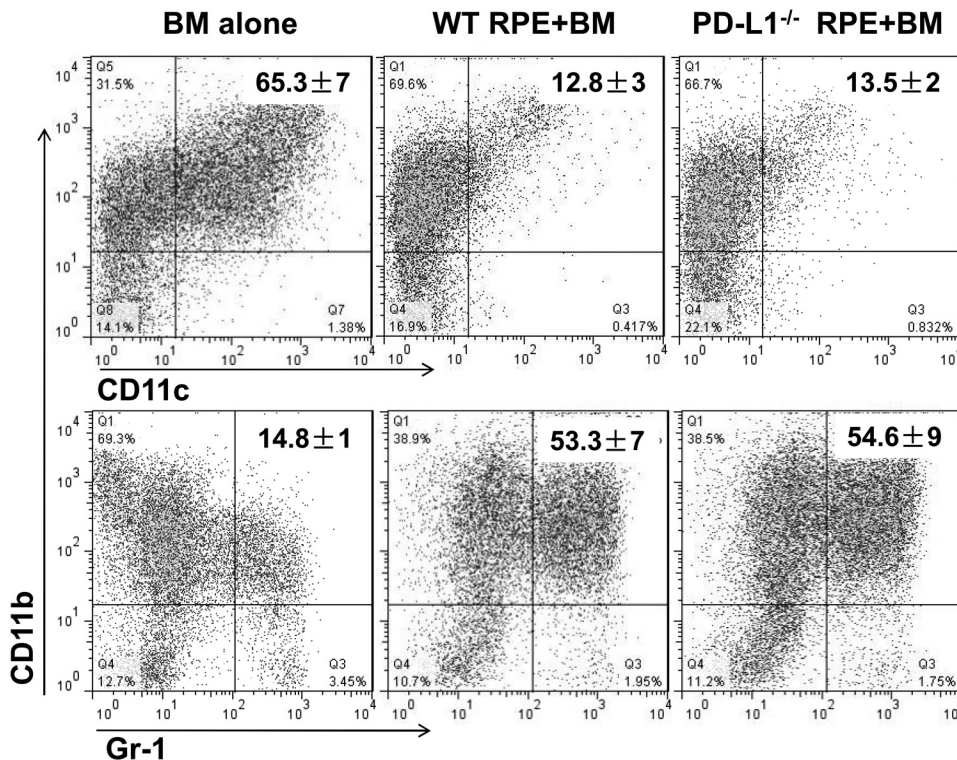


FIGURE 4. The cell surface molecule PD-L1 was not involved in RPE cell-induced MDSC differentiation. BM cells were cultured without and with WT or PD-L1^{-/-} RPE (at a ratio of 20:1), together with GM-CSF and IL-4. After incubation, nonadherent cells were analyzed for markers of DCs (CD11b⁺CD11c⁺) and MDSCs (CD11b⁺Gr-1⁺). Results are representative of two individual experiments. Data are mean ± SD.

cell surface proteins on RPE cells and soluble factors produced by RPE cells are important in the process of RPE cell-induced MDSC differentiation and DC inhibition.

Exploration of Candidate Molecules Involved in RPE Cell-Induced MDSC Differentiation

Previous studies^{12,18} demonstrated that PD-L1 is present on the RPE cell surface and that it is critical for RPE cells to directly inhibit T-cell responses. We hypothesized that PD-L1 could be one of the cell surface molecules important for RPE cell-induced MDSC differentiation. To test this hypothesis, we isolated primary RPE cells from *PD-L1*^{-/-} mice and compared the efficacy of MDSC induction by both WT and *PD-L1*^{-/-} RPE cells using the same protocol described. As shown in Figure 4, consistent with previously described experiments, BM cells cocultured with WT RPE cells efficiently inhibited DC propagation and induced MDSC differentiation. However, the same number of BM cells cocultured with the same number of *PD-L1*^{-/-} RPE cells resulted in similar numbers of DCs and MDSCs, indicating that PD-L1 is not important in the induction of MDSCs by RPE.

We next examined other factors that could be integrally involved in RPE cell-induced MDSC differentiation. It has been reported that RPE cells produce TGF- β ¹⁹ and that TGF- β induces MDSC differentiation in tumors,²⁰ so we first tested the role of TGF- β by using its neutralizing mAbs. However, blocking TGF- β did not significantly change the numbers of MDSCs induced by RPE cells (data not shown). Because CTLA-2 α has been recently identified to be critical for RPE cells to induce foxp3⁺ Treg cells,¹⁴ we also examined its role in RPE cell-induced MDSC differentiation by using a function blocking rabbit anti-CTLA-2 α IgG.¹⁴ These experiments found that neither the control rabbit IgG nor the rabbit anti-CTLA-2 α IgG reduced the numbers of the resultant CD11b⁺Gr-1⁺ MDSCs (data not shown). Finally, in light of the isolated reports that RPE cells produce IL-6²¹⁻²³ and that IL-6 stimulates MDSC differentiation,^{24,25} we measured IL-6 levels in the cocultures by ELISA. These assays (Fig. 5A) showed that consistent with previous reports, RPE cells in our experimental system produce IL-6. To explore the role of IL-6 in the process, we repeated the MDSC induction experiments using WT RPE cells together with an anti-IL-6 mAb to neutralize the IL-6 activity. These experiments (Fig. 5B) showed that blocking IL-6 in the cocultures reduced the resultant CD11b⁺Gr-1⁺ MDSC generation from 53.8% \pm 5% to 38.2% \pm 4%, indicating that IL-6 is one of the important soluble factors that are integrally involved in RPE cell-induced MDSC differentiation.

RPE Cell-Induced MDSCs Inhibit Autoreactive T-Cell Responses That Lead to EAU

We next examined whether RPE cell-induced MDSCs can inhibit in vivo autoreactive T-cell responses that cause retinal injury in EAU.²⁶ We induced EAU in C57BL/6 mice by immunizing them with IRBP₁₋₂₀ peptide in CFA, together with pertussis toxin, as previously described.²⁷ We randomly divided the immunized mice into two groups. We treated one group with 2×10^6 of the RPE cell-induced MDSCs through tail vein intravenous injection. We gave the other group the same volume of PBS as controls. In 3 weeks, we assessed the severity of EAU disease by retinal histopathologic analysis. We also compared IRBP-specific T-cell responses in the treated and control mice by ELISA using isolated spleen cells. These experiments showed that adoptive transfer of the RPE cell-induced MDSCs markedly reduced EAU disease severity (Fig. 6A) with decreased retinal leukocyte infiltration and photoreceptor/RPE damage (Fig. 6B). In accordance with the ameliorated disease severity, IRBP-specific T-cell responses in the RPE cell-induced

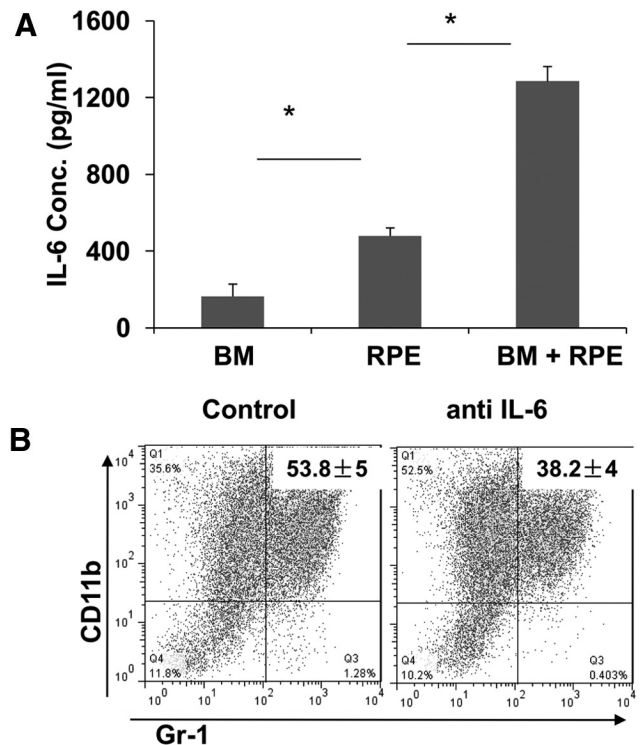


FIGURE 5. The soluble factor IL-6 is important for RPE cell-induced MDSC differentiation. BM cells were cultured without and with RPE (at a ratio of 20:1), together with GM-CSF and IL-4 for 3 days. Then supernatants were collected, and concentrations of IL-6 were measured by ELISA (A). The same experiments were repeated except that in half the wells, 5 μ g/mL anti-IL-6 IgG was added. In the other half, the same amount of isotype control was added. After incubation, nonadherent cells were analyzed for markers of MDSC (CD11b⁺Gr-1⁺). Results are representative of two individual experiments. Data are mean \pm SD. **P* < 0.05.

MDSC-treated mice were also reduced compared with spleen cells from the mock-treated controls, as assessed by the measurements of IFN- γ and IL-17 produced by the respective spleen cells ex vivo (Fig. 6C).

DISCUSSION

In this study, we demonstrated that RPE cells inhibited DC propagation from myeloid progenitors and induced the differentiation of CD11b⁺Gr-1⁺ cells that match the cell surface markers of MDSCs identified in tumors. We found that these RPE cell-induced MDSCs potently inhibited T-cell proliferation and inflammatory cytokine production and that systemic delivery of these cells inhibited in vivo autoreactive T-cell responses that led to retinal injury in EAU. Using *PD-L1*^{-/-} RPE cells, we found that PD-L1 was not essential for the RPE cell-induced MDSC differentiation, and using blocking mAbs we found that neither TGF- β nor CTLA-2 α was important for RPE cells to induce MDSCs, whereas IL-6 was integrally involved in the process.

MDSCs are studied extensively in tumors.²⁸ These cells suppress T-cell responses against tumors, which become a major obstacle for developing effective tumor-targeted immunotherapies. Many studies in tumors are focused on how to inhibit MDSC differentiation and how to inhibit the existing MDSC activities to improve the efficacy of tumor vaccine and other tumor-targeted immunotherapies. On the other hand, because of their profound T-cell inhibitory activity, MDSCs

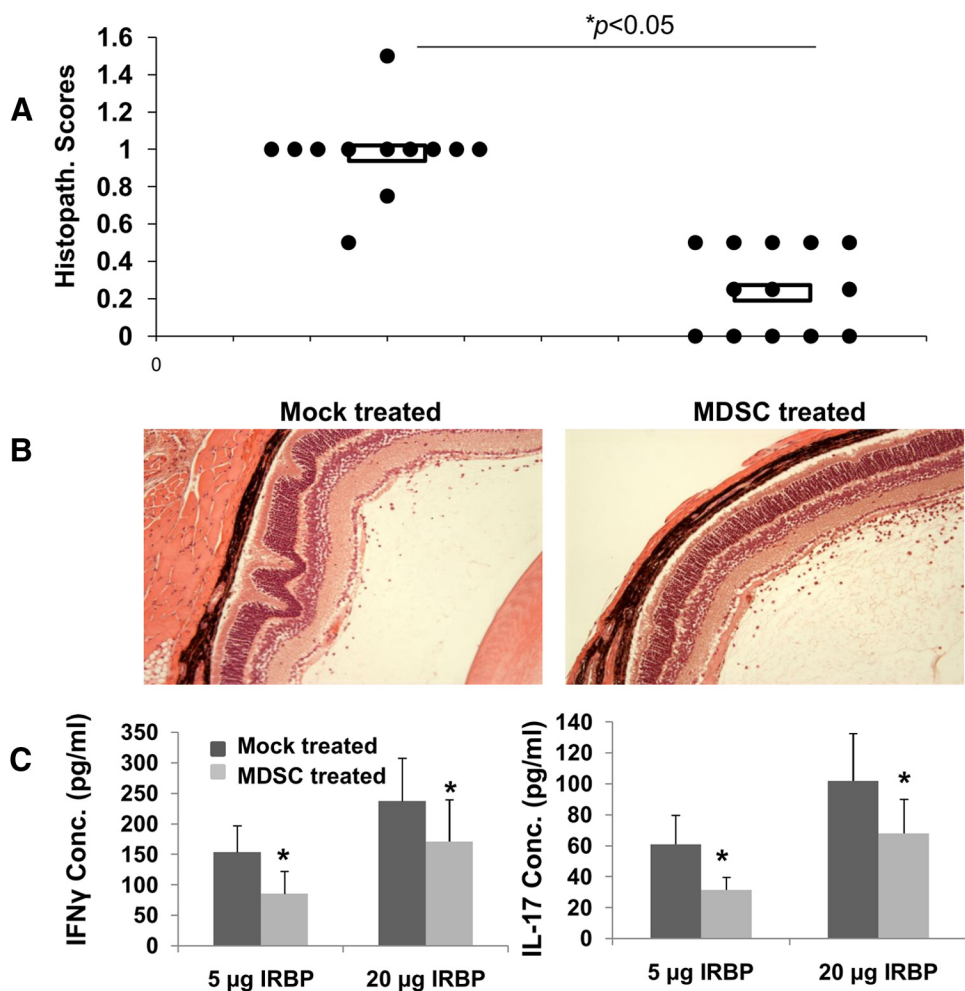


FIGURE 6. RPE cell-induced MDSCs suppress in vivo autoreactive T-cell responses that lead to retinal injury in EAU. RPE cell-induced MDSCs (2×10^6) in 0.5 mL PBS were adoptively transferred into each of the six female C57BL/6 mice after EAU induction by tail vein intravenous injection. The same volume of PBS was administered into each of the six control mice. In 21 days, EAU disease severity was evaluated by histopathologic scoring of the ocular sections in a masked fashion. Each dot represents one eye (A). Shown are representative images of the retina from the mock-treated and MDSC-treated mice (B). IRBP-specific Th1 and Th17 cell responses in the experimental mice were assessed by IFN- γ and IL-17 ELISA using culture supernatants collected from splenocytes restimulated with different concentrations of the IRBP₁₋₂₀ peptide (C).

could represent a novel therapeutic approach to treating patients with autoimmune diseases caused by autoreactive T cells. Because it is impractical to isolate syngeneic MDSCs from cancer patients to treat autoimmune diseases, it has been a challenge to develop MDSCs as a new therapy. Given that human RPE cells can be easily isolated and expanded in vitro from donor eyes²⁹ and syngeneic BM cells or peripheral blood mononuclear cells containing myeloid progenitors can be collected from individual patients, our discovery that RPE cells induce MDSC differentiation from myeloid progenitors suggests a new approach to generate large numbers of syngeneic MDSCs for “personalized” autoimmune disease treatments.

EAU in mice is an established animal model for human autoimmune posterior uveitis, which helps in the understanding of pathologic mechanisms underlying the human disease and in the development of novel therapies.²⁶ In EAU, retinal T cells are primed in the periphery and migrate to the retina to cause local inflammation. The results that systemic delivery of the RPE cell-induced MDSCs effectively protected mice from retinal injury in EAU and that both IRBP-specific Th1 and Th17 responses were suppressed in the lymph organs suggest that the adoptively transferred MDSCs inhibited T-cell autoimmunity in the periphery. These studies provided proof-of-concept that RPE cell-induced MDSCs could be developed as a novel therapy for the treatment of autoimmune posterior uveitis and, even more broadly, for the treatment of other similar autoimmune diseases in which pathologic T-cell activities are involved.

Our studies using a transwell system suggest that both the cell surface molecules and the soluble factors produced by RPE

are required for efficient MDSC induction. Although the cell surface molecule PD-L1 has been found important in RPE cell direct inhibitory activity on T- and B-cell responses,^{12,18} it did not appear to be important in the RPE cell-induced MDSC differentiation in our studies using *PD-L1*^{-/-} RPE. More work is needed to identify the RPE cell surface molecules important for RPE cell-induced MDSC differentiation.

Previous studies^{21,23} have shown that RPE cells produce IL-6. Our ELISA results with the isolated RPE cells are consistent with these findings. Using blocking mAbs, we found that neutralization of IL-6 reduces the number of MDSCs that RPE cells can induce, suggesting that IL-6 is a soluble factor that plays an important role in the induction of MDSC differentiation. This result is consistent with other reports in humans and mice indicating that in tumors, IL-6 induces MDSC differentiation both in vitro and in vivo.^{24,30} Although the difference between samples with and without IL-6 neutralization was statistically significant in our experiments, the changes were moderate (MDSCs down from $53.8\% \pm 5\%$ to $38.2\% \pm 4\%$), suggesting that there are other redundant factors involved in the RPE cell-induced MDSC differentiation. Despite the findings that TGF- β is an important immunosuppressive cytokine produced by RPE, it apparently is not one of the soluble factors for RPE cells to induce MDSCs because blocking TGF- β did not change the efficacy of RPE cell-induced MDSC differentiation. Consistent with previous reports,¹⁴ our isolated RPE cells produced high levels of CTLA-2 α (Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8377/-/DCSupplemental>), a cathepsin L inhibitor important for RPE cell immunosuppressive activities, by inducing FoxP3⁺ Treg cells. However, as with TGF- β , blocking

CTLA-2 α did not reduce the numbers of MDSCs induced by RPE, indicating that CTLA-2 α is also not detectably involved in the process.

Many organs use multiple mechanisms to control local immune responses to maintain their functional homeostasis. In patients or animals with cancer, the induction of MDSCs is a major mechanism by which tumors control host immune responses to permit tumor growth.¹⁷ In the eye, in addition to the physical blood-retina barrier, many ocular cells have been identified as able to suppress immune responses to preserve vision.³¹ RPE cells are an important type of retinal cells that regulate immune reactions in the eye. RPE cells directly inhibit T- and B-cell responses by secreting TGF- β and by expressing PD-L1 on their surfaces. RPE cells also induce foxp3+ Treg cells by producing CTLA-2 α . Although neither of these mechanisms appeared to be involved in the induction of MDSCs by RPE, our results that RPE cells induce MDSC differentiation suggest that this could represent a novel mechanism by which RPE cells control local inflammation in the eye. We hypothesize that during retinal inflammation, myeloid progenitors circulating in the blood could enter the retina along with other inflammatory cells. RPE cells could then induce MDSC differentiation in situ and thereby help to control immune reactions in the eye.

Similar to the eye, the liver is another organ with many mechanisms that tightly control local immune reactions. Under normal conditions, the liver encounters numerous pathogens and inflammation initiators; hence, local immune responses must be dampened to allow the liver to function properly. Indeed, liver allografts are spontaneously accepted in mice. We recently demonstrated that hepatic stellate cells (HSCs), a type of nonparenchymal cells in the liver, induce MDSC differentiation and that cotransplantation of these HSC-induced MDSCs protects islet allografts from T-cell-mediated rejection,³² suggesting that the induction of MDSCs by local liver cells is integrally involved in the immunosuppressive status in the liver. Results described in this report that RPE cells can induce MDSC differentiation suggest that the induction of MDSCs by local cells could be a common mechanism used by immunologically privileged organs such as the liver and the eye to control local immune reactions and to maintain their functional homeostasis.

In summary, we demonstrated that RPE cells inhibited DC propagation and induced MDSC differentiation. Both cell surface molecules on RPE cells and soluble factors produced by RPE cells are required for efficient MDSC induction. PD-L1, TGF- β , and CTLA-2 α did not appear to be essential for RPE cells to induce MDSCs, whereas IL-6 was important. The RPE cell-induced MDSCs were effective in inhibiting autoreactive T-cell responses that led to retinal injury in EAU. These results provide insight into the development of new methods to generate large numbers of syngeneic MDSCs for the treatment of autoimmune posterior uveitis and, more broadly, similar autoimmune diseases. These data also suggest that the inhibition of DC activation and the induction of MDSC differentiation could be another mechanism underlying the immunoregulatory activity of RPE cells, which play important roles in controlling immune reactions in the retina.

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