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Crucial Role of Granulocytic Myeloid-Derived Suppressor Cells in the Regulation of Central Nervous System Autoimmune Disease

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There is a need in autoimmune diseases to uncover the mechanisms involved in the natural resolution of inflammation. In this article, we demonstrate that granulocytic myeloid-derived suppressor cells (G-MDSCs) abundantly accumulate within the peripheral lymphoid compartments and target organs of mice with experimental autoimmune encephalomyelitis prior to disease remission. In vivo transfer of G-MDSCs ameliorated experimental autoimmune encephalomyelitis, significantly decreased demyelination, and delayed disease onset through inhibition of encephalitogenic Th1 and Th17 immune responses. Exposure of G-MDSCs to the autoimmune milieu led to up-regulation of the programmed death 1 ligand that was required for the G-MDSC-mediated suppressive function both in vitro and in vivo. Importantly, myeloid-derived suppressor cells were enriched in the periphery of subjects with active multiple sclerosis and suppressed the activation and proliferation of autologous CD4+ T cells ex vivo. Collectively, this study revealed a pivotal role for myeloid-derived suppressor cells in the regulation of multiple sclerosis, which could be exploited for therapeutic purposes. The Journal of Immunology, 2012, 188: 1136–1146.

R e-establishment of immune homeostasis and self-tolerance remain unresolved issues in autoimmune inflammatory diseases. Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the CNS of unknown etiology (1). Experimental autoimmune encephalomyelitis (EAE) is a well-established animal model that resembles many facets of MS (2) and is used as a tool to study pathogenic, as well as regulatory, processes of the disease. EAE is characterized by periods of exacerbation followed by remission, which mirrors disease course in the majority of MS patients. Disease pathogenesis, progression, and subsequent relapses have been attributed to myelin-reactive Th1 and Th17 cells and their products (3–5). In contrast, several mechanisms involving immune cells, such as CD4+ regulatory T cells (6) and type II monocytes (7) or neutrophils (8), as well as immunosuppressive mediators, like IL-10 (9, 10), have been proposed to regulate EAE both in vivo and in vitro. However, it is poorly understood how inflammation is resolved, disease remits, and, in particular, which immune cells are important for naturally terminating the relapsing phase. Therefore, there is a need to delineate such mechanisms to facilitate the design of more effective protocols for the re-establishment of tolerance and prevention of autoimmune diseases of the CNS.

In recent years, myeloid-derived suppressor cells (MDSCs) have received considerable attention because they potently perturb both innate and adaptive immune responses (11). MDSCs consist of a heterogeneous population of myeloid precursors of macrophages, dendritic cells, and granulocytes and are characterized by the coexpression of Gr-1 and CD11b. They can be divided into cells with monocytic or granulocytic morphology, defined as CD11b+Ly6G− or CD11b+Ly6G−, respectively (12–14). Extensive studies established a prominent role for MDSCs in the regulation of immune responses in mice during cancer (11, 15, 16), infections (15, 17), and transplantation (18–20); in humans, MDSC accumulation at tumor sites downregulates antitumor immunity, promoting tumor surveillance and growth (21, 22). A variety of mechanisms has been attributed to the MDSC-mediated suppression of immune responses, ranging from cell-to-cell contact to soluble mediators (23, 24). However, it is now appreciated that cells of the myeloid lineage are characterized by increased plasticity, and their functional polarization depends on the local microenvironment (25).

Although the importance of MDSCs in antitumor immunity is well defined, their role in the regulation of autoimmune pathology is just emerging. MDSCs were shown to prevent murine type 1 diabetes (26) and to suppress inflammatory responses in the gut (27), retina (28), and skin (29). The role of MDSCs remains controversial in EAE. Circulating Ly6C+ myeloid precursors were shown to perpetuate disease upon migration to the CNS (30, 31),...
whereas other reports demonstrated a regulatory role for MDSCs during EAE (32, 33). Although the increased immunosuppressive properties of MDSCs could make them potential targets for therapeutic intervention of autoimmunity, several questions remain to be addressed. For example, the subset of MDSCs, as well as the precise mechanism exploited by these cells in the modulation of an autoimmune response, is unclear. In addition, the specialized microenvironment that might favor the MDSCs’ function needs to be determined. Finally, it remains elusive whether MDSCs have any role in human autoimmune diseases.

In this study, we demonstrate a significant accumulation of CD11bLy6G+Ly6C− granulocytic MDSCs (G-MDSCs) in the spleen of mice with EAE prior to disease resolution. Adoptive transfer of G-MDSCs, isolated from the autoimmune environment, potently suppress the development of EAE and inhibit the priming of autoantigen-specific Th1 and Th17 cells. In addition, G-MDSCs from myelin oligodendrocyte glycoprotein (MOG)-immunized mice express high levels of the co-inhibitory molecule programmed death ligand (PD-L)1, which was required to suppress the activation and proliferation of autologous T cells in vitro. Together, our data reveal a pivotal role for G-MDSCs in the regulation of CNS autoimmune inflammation.

Materials and Methods

Mice

Female C57BL/6 (B6) mice (6–10 wk) were obtained from the specific pathogen-free facility of the Institute of Molecular Biology and Biotechnology (Heraklion Crete, Greece).

PD-1−/− mice bred on a B6 background were a kind gift of Dr. Zhang (Department of Orthopedic Surgery, University of Chicago, Chicago, IL). PD-L1−/− mice bred on a B6 background (34) were provided by Prof. A. Sharpe (Department of Pathology, Harvard Medical School, Brigham & Women’s Hospital). All procedures were in accordance to institutional guidelines and were approved by the Greek Federal Veterinary Office.

Reagents

The following fluorescent-conjugated mAbs were used for analysis of mouse cells. Ly6C (1G7,1G10) was from Miltenyi Biotec, and Gr-1 (RB6-8C5), CD80 (16-10A1), CD86 (PO3.1), CD40 (1C10), CD273 (PD-L2, 8C5), CD80 (16-10A1), CD86 (PO3.1), CD40 (1C10), CD273 (PD-L2, 8C5), all from BD Biosciences (San Jose, CA). CD4, Ly6G (1A8), CD44 (H1/68.12), CD11b (M1/70), CD3e (145-2C11), CD19 (1D3), CD274 (PD-L1, M1H5), CD45R/B220 (RA3-6B2), CD4 (RM4-5), Ly6G (1A8), CD44 (Pgp-1, Ly24), I-Ab (AF6-120.1), and CD25 (PC61) were from BD Pharmingen.

The following mAbs were used for human cell phenotypes. CD15 (8OH5), CD33 (D3HL60.251), CD84 (R05.1), CD45R/B220 (RA3-6B2), CD4 (RM4-5), Ly6G (1A8), CD44 (Pgp-1, Ly24), I-Ab (AF6-120.1), and CD25 (PC61) were from BD PharMingen.

Flow cytometry and cell sorting

Cells were stained for extracellular markers for 20 min at 4°C in PBS/0.5% FCS. Intracellular Foxp3 staining was performed using the anti-mouse Foxp3 staining set, according to the manufacturer’s protocol (eBioscience). Dead cells were identified and excluded from all analyses by 7AAD (BD Pharmingen). For tetrimer staining, lymph node (LN) cells (2 × 106 cells) were incubated for 5 min with 10 μg/mouse and rat sera (Jackson ImmunoResearch Laboratories), followed by a 45-min staining with 10 μg/ml tetramer at room temperature. mAbs and viability dyes were subsequently added for 20 min on ice. Cells were acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star). Cell sorting was performed using the high-speed cell sorter MoFlo (Dako).

T cell-proliferation assays and cytokine assessment

Draining inguinal LNs were harvested 9–10 d after immunization and were cultured (6 × 105 cells/well) in the presence or absence of 10 ng/ml MOG peptide for 72 h. T cells were then pulsed with 1 μCi [3H]thymidine (TRK120; Amersham Biosciences) for 18 h, and incorporated radioactivity was measured using a Beckman Coulter 1450 tricarb scintillation counter. Results are expressed as stimulation index, which is defined as cpm in the presence of Ag/cpm in the absence of Ag. Cytokines were assessed in culture supernatants collected after 48 h of stimulation. Detection of IL-2, IFN-γ (BD OptEIA; BD Biosciences), and IL-17 (R&D Systems) was performed by ELISA, following the manufacturer’s recommendations. Light absorption at 450 nm was measured using a Vmax plate reader (Bio-Rad). In other experiments, intracellular LNs were dissected and analyzed by flow cytometry, as indicated in the figure legends.

MS subjects

MS patients were recruited through the Neurology Department, University Hospital of Heraklion (Crete, Greece). The disease’s diagnosis and classification were established by the clinical and magnetic resonance imaging characteristics of the International Panel on MS. Patients with MS, acute or subacute immune-mediated or infectious diseases of the CNS were sought out and excluded by appropriate clinical and diagnostic evaluations. MS subjects with active disease were those with acute or subacute neurologic symptoms either due to the initial MS episode (disease onset) or to a subsequent relapse, followed by improvement. In contrast, patients with relapsing-remitting MS who were clinically stable and who had experienced no clinical exacerbation for >2 mo prior to the time of the study were considered to be in remission. The Clinical Research Ethics Board at the University Hospital of Crete (Heraklion, Crete) approved this study. Informed consent was obtained from all patients prior to sample collection.
Human cell isolation from peripheral blood

Heparinized blood was collected from healthy subjects and MS patients, and PBMCs were isolated on Histopaque-1077 (Sigma) density gradient. MDSCs and CD4+ T cells were analyzed by flow cytometry and sorted as described. The Clinical Research Ethics Board at the University Hospital of Crete (Heraklion, Crete) approved this study.

In vitro suppression assay

Naïve mouse CD4+CD25− T cells (from B6 or PD1−/− mice) were sorted (purity > 99%) and were stimulated with 10 μg/ml plate-bound anti-CD3 (145-2C11; BD Pharmingen) and 1 μg/ml anti-CD28 (37.51; BD Pharmingen). Purified CD11b+Ly6G- MDSCs (purity > 95%) were activated with recombinant mouse IFN-γ for 24 h and then added to the culture at a 1:1 ratio. Proliferation was assessed by [3H]thymidine uptake.

Human CD4+CD25− T cells were sorted (purity > 99%) from PBMCs, labeled with CFSE (1 μM for 10 min at 37 °C in labeling buffer-PBS/0.1% BSA), and cocultured (2 × 10^6 cells/well) with sorted MDSCs (purity > 95%), at a 1:1 ratio, in the presence of 2 μg/ml plate-bound anti-CD3 (OKT3; e-Bioscience) and 1 μg/ml anti-CD28 (CD28.2; e-Bioscience). Proliferation of T cells was determined based on CFSE dilution by flow cytometry. The levels of IL-2 in culture supernatants were measured after 48 h using a human cytokine ELISA kit (eBioscience).

Phenotypic analysis

Sorted G-MDSCs or Ly6G-CD11b+ (7AAD-CD3-CD19−) cells were cultured (1.5 × 10^6 cells/ml) in the presence of recombinant mouse IFN-γ (20 ng/ml), LPS (1 μg/ml), or IL-4 (20 ng/ml) for 18–20 h. Cell surface markers were assessed by flow cytometry. Nitrite quantification was assessed using the Greiss reagent system (Promega), following the manufacturer’s instructions. Bone marrow-derived macrophages generated in the presence of L929 cell-conditioned medium containing M-CSF and activated with LPS (1 μg/ml) for 12 h were used as a positive control. Cultured supernatants were assessed for production of IL-10 and IL-12 by ELISA (BD OptEIA; BD Biosciences). Arginase I production was determined by fluorescent analysis of spinal cords demonstrated specific localization of CD11b+Ly6G+ MDSCs among the inflammatory infiltrates (Fig. 1G). Collectively, our data showed an increased accumulation of G-MDSCs in CNS and enhanced recruitment of this subset at lymphoid organs peaking prior to resolution of EAE. Together, these findings raised the possibility of an MDSC-mediated role in the resolution of the autoimmune response.

Adaptive transfer of G-MDSCs ameliorates MOG35–55-induced EAE

To evaluate the ability of G-MDSCs to mediate disease remission, we adoptively transferred purified G-MDSCs isolated from spleens of MOG/CF clandestined mice into recipient mice during the course of EAE (Fig. 2A), as well as the draining cervical LNs (Supplemental Fig. 1). Consistent with the flow cytometry data, immunofluorescent analysis of spinal cords demonstrated specific localization of CD11b+Ly6G+ MDSCs among the inflammatory infiltrates (Fig. 1G). Collectively, our data showed an increased accumulation of G-MDSCs in CNS and enhanced recruitment of this subset at lymphoid organs peaking prior to resolution of EAE. Together, these findings raised the possibility of an MDSC-mediated role in the resolution of the autoimmune response.

G-MDSCs suppress the priming of MOG35–55-specific Th1 and Th17 cells in vivo

Because EAE is initiated and perpetuated by autoreactive Th1 and Th17 cells, we further assessed whether in vivo transfer of G-MDSCs could influence the priming of MOG-specific T cells in the peripheral LNs. To address this, mice were adoptively transferred with G-MDSCs as described in Fig 2A; 9 d after the antigenic challenge, inguinal draining LNs (dLNs) were assessed for MOG35–55-specific T cell responses. We noted that G-MDSC...
transfer resulted in decreased frequency (Fig. 3A) and significantly reduced numbers (Fig. 3B) of MOG38–49/IAb+CD3+CD4+ T cells compared with control untreated mice, indicating that G-MDSCs suppressed the expansion of autoreactive T cells. This was confirmed upon ex vivo stimulation of dLNs with MOG peptide; LNs from G-MDSC–treated mice showed markedly reduced cell proliferation and significant suppression of Th1- and Th-17–secreting cytokines compared with the untreated control group (Fig. 3C). Suppression of MOG-specific Th1 and Th17 responses was accompanied by increased accumulation of G-MDSCs in the dLNs of G-MDSC–injected mice compared with the control group (Fig. 3D). In contrast, no significant difference in the frequency of CD4+Foxp3+ regulatory T cells was observed (Fig. 3E). Collectively, these results demonstrated the suppressive potential of G-MDSCs against MOG-specific autoreactive T cells in vivo.

**Phenotypic characterization of G-MDSCs in MOG/CFA-immunized mice**

Several mechanisms of suppression have been assigned to MDSCs during cancer and infection (11, 35). However, it remains to be determined how G-MDSCs exert their function in an autoimmune setting. To address this, G-MDSCs were sorted from MOG/CFA-immunized mice and treated for 24 h with LPS or IFN-γ, two well-known proinflammatory stimuli. Assessment of cytokines in culture supernatants showed increased secretion of IL-10 upon PMA or LPS/IFN-γ stimulation (Fig. 4A), whereas IL-12 was not detected (Fig. 4B). In addition, neither NO (Fig. 4C) nor Arg-1 (Fig. 4D) could be detected in stimulated G-MDSCs, whereas intracellular ROS accumulation was only observed upon PMA treatment (Fig. 4E). In contrast, detailed cell surface phenotypic analysis of untreated G-MDSCs revealed an increased expression of the inhibitory molecule PD-L1 (Fig. 4F). Interestingly, treatment with LPS caused a significant up-regulation of PD-L1 expression, which was even more robust in IFN-γ–treated G-MDSCs (Fig. 4F). Enhanced expression of PD-L1 on G-MDSCs upon treatment with IFN-γ was specific, because expression of other costimulatory/inhibitory markers, such as CD80, CD86, CD40, and PD-L2, was not altered (Fig. 4F).

**PD-L1 is required for G-MDSC–mediated suppression of EAE**

PD-L1/PD-1 interactions were reported to deliver co-inhibitory signals leading to attenuation of T cell responses both in vitro and in vivo (34, 36). To test the functional significance of the increased PD-L1 expression observed in G-MDSCs, we first compared the activation and proliferation of naive CD4+CD25– T cells isolated from wild-type (WT) versus PD-1 knockout (PD-1−/−) mice in the presence or absence of IFN-γ–treated G-MDSCs (Fig. 5A). To this end, the presence of G-MDSCs significantly reduced the frequency of CD4+CD25+ activated WT T cells, whereas the activation of PD-1−/− T cells was not affected (Fig. 5B). Moreover, G-MDSCs significantly inhibited WT T cell proliferation, whereas PD-1−/− T cells were resistant to G-MDSC–mediated suppression (Fig. 5C). Overall, these data indicated that IFN-γ–exposed G-MDSCs suppressed T cell responses via the PD-1/PD-L1 inhibitory pathway in vitro. To examine whether PD-L1 expression by G-MDSCs confers a dominant mechanism of EAE suppression, we adoptively transferred G-MDSCs isolated from MOG/CFA-immunized PD-L1−/− mice into syngeneic recipients over, G-MDSCs significantly inhibited WT T cell proliferation, whereas PD-1−/− T cells were resistant to G-MDSC–mediated suppression (Fig. 5C). Overall, these data indicated that IFN-γ–exposed G-MDSCs suppressed T cell responses via the PD-1/PD-L1 inhibitory pathway in vitro. To examine whether PD-L1 expression by G-MDSCs confers a dominant mechanism of EAE suppression, we adoptively transferred G-MDSCs isolated from MOG/CFA-immunized PD-L1−/− mice into syngeneic recipients during the course of EAE, as described in Fig. 2A. Deficiency of PD-L1 on G-MDSCs abrogated their suppressive ability, because the disease onset and severity were not significantly different between treated and control mice (Fig. 5D). This finding correlated with immunohistological analysis of the spinal cords of the two groups of mice, for which a comparable degree of demyelination was observed (Fig. 5E). Moreover, analysis of the dLNs of PD-L1−/− G-MDSC–treated mice showed a comparable frequency of MOG38–49/IAb+CD3+CD4+ effector T cells as in untreated mice (Fig. 5F). Collectively, these data provided direct...

Human G-MDSCs from MS patients potently suppress the activation and proliferation of autologous T cells in vitro

We next examined the presence of MDSCs in MS subjects in the active phase of disease or during remission. Human MDSCs have mainly been studied in cancer patients and are characterized as HLA-DR \(^{low}\)/CD14\(^{low}\)/CD15\(^{+}\)/CD33\(^{+}\) (37, 38). Flow cytometry analysis revealed significantly increased frequency (Fig. 6A) and numbers (Fig. 6B) of HLA-DR \(^{low}\)/CD14\(^{low}\)/CD33\(^{+}\)/CD15\(^{+}\) MDSCs in the peripheral blood of patients with active MS compared with patients in remission or healthy controls. A significant decrease in MDSC numbers was also observed upon longitudinal analysis of CD33\(^{+}\)/CD15\(^{+}\) cells in seven active MS patients who achieved remission (Fig. 6C). Similar to mouse G-MDSCs, morphologic analysis of sorted HLA-DR \(^{low}\)/CD14\(^{low}\)/CD33\(^{+}\)/CD15\(^{+}\) MDSCs in the peripheral blood of patients with active MS compared with patients in remission or healthy controls. A significant decrease in MDSC numbers was observed. Importantly, although responder T cells underwent at least three or four cell divisions (1st, 24.4%; 2nd, 7.52%; 3rd, 3.06), as traced by the dilution of CFSE, the presence of MDSCs in the culture caused a proliferation arrest of responder T cells to only one or two cell divisions (1st, 15.4%; 2nd, 5.82%) after 5 d of culture (Fig. 7B). MDSC-mediated suppression of T cell proliferation was significant, as extrapolated by enumeration of the CD4\(^{+}\) T cells following the 5-d coculture (Fig. 7C). This was further supported by a significant reduction in IL-2 secretion in supernatants of MDSCs/CD4\(^{+}\)CD25\(^{+}\) cocultures compared with control cultures (Fig. 7D). Reduced CD25 expression and T cell proliferation was not due to unspecific blocking effects of the CD33\(^{+}\)/CD15\(^{+}\) cells, because culture of T cells with autologous CD14\(^{+}\) monocytes increased T cell proliferation and CD25 expression (Supplemental Fig. 2). To examine whether PD-L1 was expressed by the CD33\(^{+}\)/CD15\(^{+}\) MDSCs, we performed flow cytometry analysis on freshly purified PBMCs. Our data demonstrated an increased expression of PD-L1 in CD33\(^{+}\)/CD15\(^{+}\) cells from MS patients compared with cells from healthy individuals (Fig. 7E, left panel). Moreover, treatment of PBMCs from active MS patients for 24 h resulted in up-regulation of PD-L1 (Fig. 7E, right panel), suggesting that PD-L1 might be involved in MDSC-mediated suppression. To address this, we set up coculture experiments, as described above, in the presence or absence of blocking anti-human PD-L1 mAb. A shown in Fig. 7F, blocking of PD-L1 was able to partially block the inhibitory potential of CD33\(^{+}\)/CD15\(^{+}\) cells but not completely restore the proliferative ability.
CD4+CD25+ autologous T cells. These results demonstrated that PD-L1 expression by human CD33+CD15+ MDSCs might be involved in MDSC-mediated suppression and indicated that other mediators have to participate, because blocking of PD-L1 does not fully restore human T cell proliferation. Overall, our results demonstrated a significant enrichment of G-MDSCs in active MS patients with a potent ability to suppress the activation and expansion of autologous T cells.

Discussion

Resolution of inflammation during the course of an autoimmune disease is governed by diverse and complex mechanisms likely acting in concert. A comprehensive understanding of the cellular and soluble mediators should facilitate the design of more specialized therapeutic protocols. In this article, we provide compelling evidence for a pivotal role for granulocytic CD11bhiLy6G+ MDSCs (G-MDSCs) in the regulation of CNS autoimmune inflammation. Thus, in vivo transfer of highly purified G-MDSCs ameliorated EAE, significantly reduced the expansion of autoreactive T cells in the dLNs, and constrained pathogenic Th1 and Th17 immune responses in a PD-L1/IFN-γ-dependent fashion. Importantly, our results demonstrate for the first time, to our knowledge, an important role for G-MDSCs in patients with MS, because this subset was significantly increased in the periphery during active disease and potently suppressed autologous T cell proliferation in vitro. Together, these data highlight the potential of G-MDSCs to serve as a novel target for pharmacologic intervention in autoimmune inflammatory diseases.

It is not clear whether the G-MDSCs described in this article represent a subset of neutrophils or are undifferentiated myeloid suppressor cells. G-MDSCs isolated from MOG/CFA-immunized mice had a high side scatter profile and phenotypically displayed features of neutrophils, such as multilobed nucleus and expression of the typical marker Ly6G. Mounting evidence suggests that, similar to macrophages, polymorphonuclear cells (PMNs) are versatile cells and could acquire diverse functions depending upon the microenvironment. Thus, in the malignancy setting, tumor-associated neutrophils were polarized into either an antitumorigenic (N1) or a protumorigenic (N2) population, depending upon the tumor milieu (39). Moreover, in a systemic inflammatory model, two operationally different PMN populations were characterized: PMN-I and PMN-II, which produced high levels of IL-12 and IL-10, respectively (40). Similarly, in an infectious disease setting, mycobacteria-exposed neutrophils secreted high levels of IL-10, thus possessing anti-inflammatory properties (41). We found that, in the autoimmune environment generated by the in-
Injection of a self-Ag in adjuvant, G-MDSCs secreted elevated levels of IL-10 but not IL-12, a profile consistent with the PMN-II regulatory cells characterized above. Irrespective of the differentiation status of G-MDSCs in the autoimmune setting, our data undoubtedly established a regulatory role for granulocytic “neutrophil”-like myeloid cells in the resolution of autoimmune inflammation. Further phenotypic characterization of the regulatory granulocytic cells and development of genetic approaches for the specific depletion of the polarized PMN subsets are needed to reassess their potential in the regulation of immune responses.

Our work provides evidence for a G-MDSC regulation of EAE at the target tissue. Thus, upon adoptive transfer of G-MDSCs in MOG/CFA-immunized mice, we observed reduced inflammation and demyelination in the spinal cord. This, in turn, correlated with delayed disease onset and amelioration of clinical symptoms. Importantly, we also observed an increased accumulation of G-MDSCs at the meningeal lesions of spinal cord, suggesting that G-MDSCs at the meningeal lesions of spinal cord, suggesting that G-MDSCs could exert their function at the peripheral lymphoid organs, as well as at the target tissue. This is in agreement with recent data demonstrating that MDSCs suppress preferentially at the inflammatory site in a mouse model of prostate cancer (42). It undoubtedly established a regulatory role for granulocytic “neutrophil”-like myeloid cells in the resolution of autoimmune inflammation. Further phenotypic characterization of the regulatory granulocytic cells and development of genetic approaches for the specific depletion of the polarized PMN subsets are needed to reassess their potential in the regulation of immune responses.

Although the function of MDSCs in a malignant disease environment has been addressed (24), the mechanisms underlying their suppressive activity in an autoimmune setting have not been explored. In our experiments, G-MDSCs isolated from MOG/CFA-immunized mice and exposed to IFN-γ in vitro failed to express NO, Arginase I, or ROS, which have been closely linked to MDSC-mediated suppression of antitumor immunity. These findings suggested that the autoimmune environment might induce a novel regulatory signature on G-MDSCs. Indeed, further characterization revealed a significant IFN-γ-mediated up-regulation of the inhibitory molecule PD-L1. Thus, adoptive transfer of PD-L1-deficient G-MDSCs during the course of EAE failed to suppress disease pathology and to limit the expansion of encephalitogenic T cells in the dLNs. Published data established a pivotal role for the PD-L1/PD-L1 pathway in the regulation of an autoimmune response (47). Thus, PD-L1-deficient mice develop greatly exacerbated EAE compared with control littermates, which was associated with enhanced autoantigen-specific T cell responses (34, 48). Our data extend those findings, because they
point to a PD-L1–dependent G-MDSC–mediated regulation of EAE. Furthermore, we did not detect expression of the other B7 family inhibitory molecule PD-L2 by G-MDSCs, indicating that this receptor is not involved in the G-MDSC–mediated inhibition of disease. This finding could explain the increased susceptibility of PD-L1<sup>−/−</sup> mice in MOG-induced EAE (34, 49). In line with our G-MDSC phenotypic data, another study demonstrated increased expression of PD-L1 in MDSCs isolated from tumor-bearing mice; however, in this report, MDSC-mediated suppression was independent of PD-L1 (14). The disparity in these results could be explained by the increased plasticity of the MDSC population and is consistent with the concept that MDSC phenotype and function would greatly depend on the microenvironmental milieu. It should be noted that, apart from...

FIGURE 6. G-MDSCs are enriched in the periphery of active MS patients. Frequency (A) and relative numbers (B) of CD33<sup>+</sup>CD15<sup>+</sup> MDSCs in the peripheral blood of MS patients with active disease (n = 14) or in remission (n = 17), as well as healthy individuals (n = 26). Gates were set on HLA-DR<sup>low</sup>CD14<sup>−</sup>CD33<sup>+</sup>CD15<sup>+</sup> cells. In the active MS group, ■ represents patients with first episode, whereas ● represents those in relapse. C, Longitudinal course of CD33<sup>+</sup>CD15<sup>+</sup> MDSCs in MS patients during active disease and 6 mo after the last relapse (remission). Each line represents an individual patient; p value was calculated using the Wilcoxon signed-rank test. D, May–Grunwald–Giemsa staining of sorted CD33<sup>+</sup>CD15<sup>+</sup> MDSCs. Original magnification ×60.

**p = 0.003, t test.
PD-L1, G-MDSCs from MOG/CFA-immunized mice secreted significant amounts of the immunosuppressive cytokine IL-10, indicating that several nonmutually exclusive mechanisms might contribute to the G-MDSC–mediated resolution of autoimmunity. Our findings regarding the IFN-γ-dependent up-regulation of PD-L1 are of interest. Although IFN-γ levels are abundant both in the periphery and the target organ during the course of EAE and in MS patients, its role in the clinical outcome of the disease in both human and mice remains controversial (50–52). Our results support a regulatory role for IFN-γ in the effector phase of disease, because up-regulation of PD-L1 expression by G-MDSCs was greatly dependent on IFN-γ. This is in agreement with studies demonstrating that IFN-γ enhanced the MDSC suppressive function (53–55) and that blocking of IFN-γ totally reversed the inhibitory activity of G-MDSCs in a tumor mouse model (53). Our data reconcile findings that demonstrated exacerbation of EAE in IFN-γ−/− and IFN-γR−/− mice or during neutralization of this cytokine (56–60). In humans, a clinical trial using IFN-γ to treat MS patients led to disease exacerbations (61); however, it was later demonstrated that IFN-γ could induce apoptosis in human oligodendrocytes, thus precipitating the autoimmune response (62, 63). In this context and based on our findings, it is worth postulating that exposure of G-MDSCs to IFN-γ during the course of the disease leads to up-regulation of PD-L1 expression, which might subsequently serve as a regulatory mechanism in controlling pathology and facilitating disease remission.

Promoting and establishing immune modulation could be a beneficial therapeutic strategy in patients with MS. Our data demonstrated that granulocytic CD33⁺CD15⁺ MDSCs were significantly enriched in the periphery of MS patients with active disease, and, importantly, they inhibited the activation and proliferation of autologous T cell in vitro. Consistent with the mouse data, PD-L1 expression by human CD33⁺CD15⁺ MDSCs was up-regulated in the presence of IFN-γ; however, blocking of PD-L1 expression partially restored the proliferation of autologous T cells. Further analysis of human MDSCs is required to delineate the molecules that secrete or express under autoimmune inflammatory conditions and to examine whether these molecules are involved in the MDSC potent suppressive activity. Although our study involved a relatively limited sample of MS patients, the results obtained suggested that MDSC mobilization in the peripheral blood is particularly robust at disease onset, raising the possibility...
that such mobilization may contribute to clinical recovery. Of note, disease remission is more often complete after the initial attack of the disease (MS onset) than after subsequent clinical events (relapses). Because available treatments in MS are only partially effective, development of new therapies that specifically target the inflammatory autoimmune response is mandated (64). Overall, our findings establish a critical role for G-MDSCs in the regulation of MS and provide novel insights into the mechanisms that limit inflammation during autoimmune diseases. Understanding the mechanisms that are involved in disease recovery may provide important insights into aberrant pathways that account for the chronic and progressive form of MS. The tolerogenic and immunosuppressive properties of G-MDSCs demonstrated in this study could be exploited for the development of more cell-specific–based therapies in patients with autoimmune inflammatory diseases.

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Disclosures

The authors have no financial conflicts of interest.

References


Corrections


In Fig. 1B, there is no legend for the x-axis on the FACS plots. The legend for the x-axis is CD11b.

In Fig. 4F, the titles for the second and third FACS plots were incorrectly attributed. The second FACS plot represents CD86 and the third FACS plot represents CD80.