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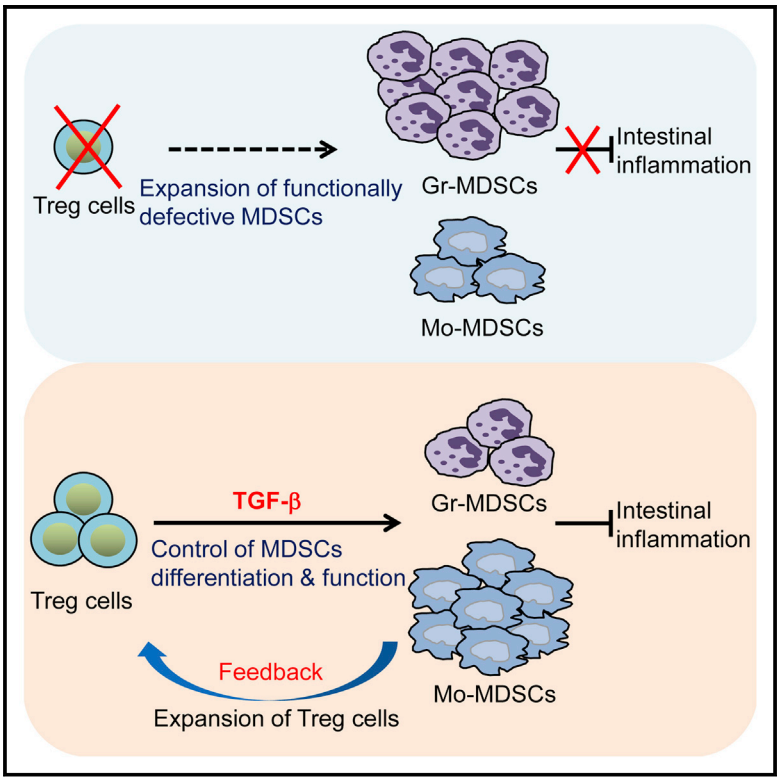
Cell Reports

Myeloid-Derived Suppressor Cells Are Controlled by Regulatory T Cells via TGF-β during Murine Colitis

Graphical Abstract



Authors

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In Brief

Lee et al. find that, under pro-inflammatory conditions, Treg cells and MDSCs establish a positive feedback loop whereby MDSCs support the expansion of Treg cells while Treg cells modulate MDSC differentiation and function. This property of Treg cells is dependent upon Treg cell-derived TGF-β.

Highlights

- Demonstration of MDSC regulation by Treg cells during inflammation
- Treg cells regulate the proliferation and activity of MDSCs
- Treg cells and MDSCs can establish a positive feedback loop during murine colitis

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Myeloid-Derived Suppressor Cells Are Controlled by Regulatory T Cells via TGF-β during Murine Colitis

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SUMMARY

Myeloid-derived suppressor cells (MDSCs) are well known regulators of regulatory T cells (Treg cells); however, the direct regulation of MDSCs by Treg cells has not been well characterized. We find that colitis caused by functional deficiency of Treg cells leads to altered expansion and reduced function of MDSCs. During differentiation of MDSCs in vitro from bone marrow cells, Treg cells enhanced MDSC function and controlled their differentiation through a mechanism involving transforming growth factor-β (TGF-β). TGF-β-deficient Treg cells were not able to regulate MDSC function in an experimentally induced model of colitis. Finally, we evaluated the therapeutic effect of TGF-β-mediated in-vitro-differentiated MDSCs on colitis. Adoptive transfer of MDSCs that differentiated with TGF-β led to better colitis prevention than the transfer of MDSCs that differentiated without TGF-β. Our results demonstrate an interaction between Treg cells and MDSCs that contributes to the regulation of MDSC proliferation and the acquisition of immunosuppressive functions.

INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells composed of immature myeloid cells, including precursors of macrophages, granulocytes, dendritic cells, and myeloid cells at early stages of differentiation (Serafini et al., 2006). In mice, MDSCs are defined as $CD11b^+Gr1^+$ cells and are normally present in low numbers in the bone marrow and spleen of healthy mice (Marigo et al., 2008). However, in response to inflammatory diseases, MDSCs accumulate in greater numbers in the spleen, blood, and lymph nodes (Gabrilovich and Nagaraj, 2009), where they suppress immune responses (Delano et al., 2007; Haile et al., 2010; Marhaba et al., 2007). MDSCs in mice can be further characterized by two epitopes recognized by the anti-Gr1 antibodies Ly6G and Ly6C. These epitopes allow for the classification of MDSCs into two distinct subsets with distinct tissue distribution and function: granulocytic MDSCs (Gr-MDSCs, $CD11b^+Ly6G^{high}Ly6C^{low}$) and monocytic MDSCs (Mo-MDSCs, $CD11b^+Ly6G^{dim}Ly6C^{hi}$) (Bronte, 2009; Haile et al., 2010). The granulocytic and monocytic subsets of MDSCs use different mechanisms to suppress T cell proliferation. Gr-MDSCs express high levels of arginase 1 (ARG1) and produce reactive oxygen species (ROSs), whereas low levels of ARG1 and high levels of nitric oxide (NO) production by inducible nitric oxide synthase (iNOS) characterize the monocytic subset (Gabrilovich and Nagaraj, 2009; Youn et al., 2008).

Human inflammatory bowel disease (IBD) encompasses several diseases, including Crohn's disease and ulcerative colitis (Baumgart and Sandborn, 2007). Studies with murine models of IBD, which mimic many of the features found in human IBD, have shown that the disease results from an imbalance of effector and regulatory T cells (Treg cells) (Maloy and Kullberg, 2008). MDSC levels are elevated in T cell-dependent colitis, and this serves to protect mice from developing intestinal inflammation (Haile et al., 2008). Treg cells play an important role in maintaining self-tolerance and preventing autoimmunity by suppressing self-reactive cells (Sakaguchi et al., 2006). Moreover, Treg cells have been shown to play an important role in intestinal homeostasis (Saurer and Mueller, 2009). Treg cells express cell surface markers, particularly CD4 and CD25, along with the lineage-specific transcription factor Foxp3 (Fontenot et al., 2003; Hori et al., 2003). In addition, Mo-MDSCs can suppress tumor-specific T cell responses directly by secreting NO, transforming growth factor-β (TGF-β), and interleukin-10 (IL-10) and indirectly by converting peripherally derived Treg cells (Huang et al., 2006). However, the effect of Treg cells on MDSCs has not been determined.

We report that Treg cell-derived TGF-β is a crucial controller of MDSC proliferation and function in murine colitis. We have also identified a role for Treg cells in regulating MDSCs and also show a functional interaction between Treg cells and MDSCs in colitis.

RESULTS

MDSCs from *Pdk1*$^{flox/flox}$;*CD4-Cre* Mice Have Impaired Immune-Suppressive Functions Compared with MDSCs from *Pdk1*$^{flox/+}$;*CD4-Cre* Mice

Deletion of the phosphoinositide-dependent protein kinase 1 (*Pdk1*) gene by *CD4-Cre* (T cell-specific deletion) impairs the function of Treg cells, thereby leading to spontaneous colitis in *Pdk1*$^{flox/flox}$;*CD4-Cre* mice because functionally defective Treg cells cannot regulate the activation of TCR$\gamma\delta^+$ T cells, whose *Pdk1* gene remains intact (Park et al., 2010). The mice that develop colitis (*Pdk1*$^{flox/flox}$;*CD4-Cre*) show a high incidence of rectal prolapse, unlike wild-type littermate control mice (*Pdk1*$^{flox/+}$;*CD4-Cre*), which were bred in the same cages. Interestingly, the CD11b$^+$Gr1$^+$ MDSC population and the severity of intestinal inflammation increased with age. CD11b$^+$Gr1$^+$ cells were isolated using a Ficoll density gradient, effectively removing granulocytes, including inflammatory neutrophils (Condamine et al., 2014), and the remaining cells were assumed to be MDSCs. In young mice (6–8 weeks old), *Pdk1*$^{flox/flox}$;*CD4-Cre* mice showed a similar percentage of MDSCs in the spleen and lymph nodes compared with *Pdk1*$^{flox/+}$;*CD4-Cre* mice (Figures 1A and 1B). However, in older mice (more than 12 weeks old), the population of MDSCs in the spleen and lymph nodes was much higher in *Pdk1*$^{flox/flox}$;*CD4-Cre* mice compared with *Pdk1*$^{flox/+}$;*CD4-Cre* mice (Figures 1A and 1B). In these mice, consistent with previous observations (Park et al., 2010), PDK1-deficient Treg cells secreted far less TGF-β and IL-10 (Figure 1C). Interestingly, other immune-regulatory cells, such as MDSCs, showed reduced functions in *Pdk1*$^{flox/flox}$;*CD4-Cre* mice compared with MDSCs from control (*Pdk1*$^{flox/+}$;*CD4-Cre*) mice. For example, expression of iNOS in Mo-MDSCs and ARG1 in both Mo-MDSCs and Gr-MDSCs decreased in *Pdk1*$^{flox/flox}$;*CD4-Cre* mice compared with *Pdk1*$^{flox/+}$;*CD4-Cre* mice (Figure 1D). PD-L1 expression was also lower in both Mo-MDSCs and Gr-MDSCs in *Pdk1*$^{flox/flox}$;*CD4-Cre* mice compared with *Pdk1*$^{flox/+}$;*CD4-Cre* mice (Figure 1E). In addition, suppression of CD4$^+$ T cell proliferation by Mo-MDSCs and Gr-MDSCs from *Pdk1*$^{flox/flox}$;*CD4-Cre* mice was weak compared with MDSCs from *Pdk1*$^{flox/+}$;*CD4-Cre* mice (Figure 1F). Because MDSCs regulate Treg cell expansion (Huang et al., 2006), the ability of MDSCs to induce Treg cell differentiation in vitro was also evaluated under suboptimal conditions with a reduced amount of TGF-β. Our results suggest that induction of induced Treg cell (iTreg cell) differentiation in vitro by MDSCs from *Pdk1*$^{flox/flox}$;*CD4-Cre* mice was less efficient than induction of iTreg cell differentiation in vitro by MDSCs from *Pdk1*$^{flox/+}$;*CD4-Cre* mice (Figure 1G; Figure S1). In addition, increasing iTreg differentiation by MDSCs from *Pdk1*$^{flox/+}$;*CD4-Cre* mice was considerably affected by anti-PD-L1 antibody, whereas this was modestly affected by anti-IL-10 antibody (Figure 1G; Figure S1).

Adoptive Transfer of Wild-Type Treg Cells Restored the Immune-Modulatory Functions of MDSCs in *Pdk1*$^{flox/flox}$;*CD4-Cre* Mice

We next evaluated whether MDSC function could also be affected by Treg cells because the function of MDSCs was significantly reduced in mice deficient in Treg cell function (Figure 1F). For this purpose, we used a mouse model for colitis where inflammation of the intestines is induced by dextran sodium sulfate (DSS) (Figure S2A). Our result showed that reduced body weight in mice with DSS-induced colitis was not significantly reversed by adoptive transfer of MDSCs from *Pdk1*$^{flox/flox}$;*CD4-Cre* mice (Figure 2A). However, when MDSCs from wild-type Treg cell-injected *Pdk1*$^{flox/flox}$;*CD4-Cre* mice were transferred into mice with DSS-induced colitis, body weight improved dramatically (Figure 2A). Colon length is a proven and useful indicator of the severity of colitis (Araki et al., 2005). After 11 days of DSS treatment, mice were sacrificed, and colon length was measured. Our results show that DSS-treated mice had significantly shorter colons compared with unexposed mice, whereas adoptive transfer of MDSCs from *Pdk1*$^{flox/flox}$;*CD4-Cre* mice did not affect colon length in DSS-treated mice (Figure 2B; Figure S2B). However, transfer of MDSCs from wild-type Treg cell-injected *Pdk1*$^{flox/flox}$;*CD4-Cre* mice restored colon length to normal in DSS-treated mice, and this was consistent with weight gain (Figures 2A and 2B). Histological examination of colon sections prepared from normal and DSS-treated mice also showed that recovery from intestinal inflammation and injury could be improved by transfer of MDSCs from wild-type Treg cell-injected *Pdk1*$^{flox/flox}$;*CD4-Cre* mice (Figures 2C and 2D). However, transfer of MDSCs from *Pdk1*$^{flox/flox}$;*CD4-Cre* mice had no appreciable effect on reducing intestinal inflammation and/or tissue injury (Figures 2C and 2D). Finally, it is important to note that the transfer of MDSCs from wild-type Treg cell-injected *Pdk1*$^{flox/flox}$;*CD4-Cre* mice, but not MDSCs from *Pdk1*$^{flox/flox}$;*CD4-Cre* mice, caused a decline in production of cytokines such as IL-10, IL-17A, and interferon γ (IFN-γ) from cultured intestinal tissue (Figure 2E).

Treg Cells Regulate MDSC Differentiation

In *Pdk1*$^{flox/flox}$;*CD4-Cre* mice, Treg cell function was dramatically impaired, leading to spontaneous colitis in the mice; however, colitis was not induced when the mice received adoptively transferred wild-type Treg cells (Figure 3A). In *Pdk1*$^{flox/flox}$;*CD4-Cre* mice with colitis, the subpopulation of Gr-MDSCs (CD11b$^+$Ly6G^{high}Ly6C^{low}) was enriched compared with Mo-MDSCs (CD11b$^+$Ly6G^{dim}Ly6C^{hi}) (Figure 3B). However, these changes in MDSC subpopulations reverted to normal levels upon adoptive transfer of wild-type Treg cells (Figure 3B). The proportion of Mo-MDSCs among the total MDSCs increased following the transfer of wild-type Treg cells, but the proportion of Gr-MDSCs was lower after the transfer of wild-type Treg cells into *Pdk1*$^{flox/flox}$;*CD4-Cre* mice (Figure 3B). Therefore, Treg cells modulate the differentiation of a distinct subpopulation of MDSCs.

MDSCs can differentiate from bone marrow cells in vitro by co-culture with granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, and 2-mercaptoethanol (2-ME), along with unidentified tumor-derived factors, after 3–5 days in culture (Youn et al., 2008). However, our data show that MDSCs can be generated without tumor-derived factors, and at least two distinct MDSC subpopulations, Mo-MDSCs and Gr-MDSCs, were derived from bone marrow cells (Figure 3).

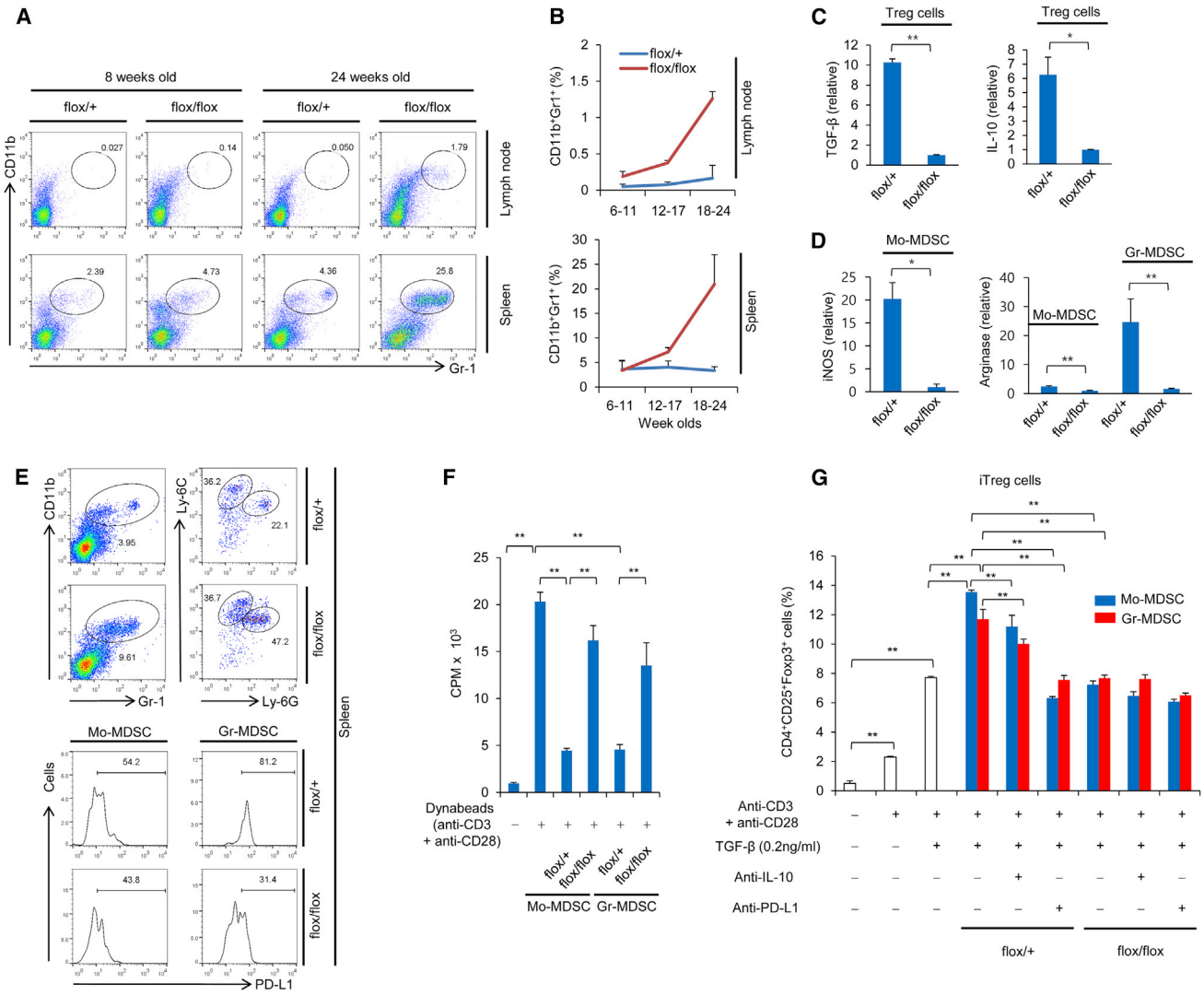


Figure 1. Immune-Suppressive Functions of MDSCs from *Pdk1*$^{flox/flox}$;*Cd4-Cre* Mice Were Reduced Compared with MDSCs from *Pdk1*$^{flox/+}$;*Cd4-Cre* Mice

(A) Flow cytometric analysis of MDSCs in the spleen and lymph nodes of *Pdk1*$^{flox/+}$;*CD4-Cre* (flox/+) and *Pdk1*$^{flox/flox}$;*CD4-Cre* (flox/flox) mice. Data are presented as a representative fluorescence-activated cell sorting (FACS) plot.

(B) The percentage of MDSCs in the lymph nodes (top) or in the spleen (bottom) by age (n = 4–5 mice/group).

(C) Relative levels of TGF-β (left) and IL-10 (right) mRNAs in Treg cells after activation with anti-CD3 and anti-CD28 antibodies. Data are representative of four independent experiments.

(D) Relative levels of iNOS mRNA (left) in splenic Mo-MDSCs and relative levels of ARG1 mRNA (right) in splenic Mo-MDSCs and Gr-MDSCs. Data are representative of three independent experiments.

(E) Flow cytometric analysis of PD-L1 expression in splenic Mo-MDSCs and Gr-MDSCs. Data are representative of two independent experiments.

(F) Suppression assay with isolated MDSCs.

(G) Flow cytometric analysis of in vitro- induced Treg cells (iTreg) with Mo-MDSCs and Gr-MDSCs isolated from the spleen. For blocking of IL-10 and PD-L1, anti-IL-10 (10 μg/mL) and anti-PD-L1 (10 μg/mL) antibodies were added to iTreg cells during differentiation.

Data are representative of three (F and G) independent experiments. Results are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$ by Student's t test (C and D) or one-way ANOVA and Tukey post-test (F and G). See also Figure S1.

In support of these findings, a recent study showed that MDSCs can be generated from bone marrow cells without tumor-derived factors (Messmann et al., 2015). To confirm the ability of Treg cells to modulate differentiation of distinct MDSC subpopulations, bone marrow cells were allowed to differentiate into MDSCs, with or without Treg cells, in the absence or presence of GM-CSF, IL-4, and 2-ME. In the absence of the cytokines, the Treg cells alone could not induce differentiation of CD11b$^+$Gr1$^+$ MDSCs (Figure 3C). However, when bone marrow-derived MDSCs were allowed to

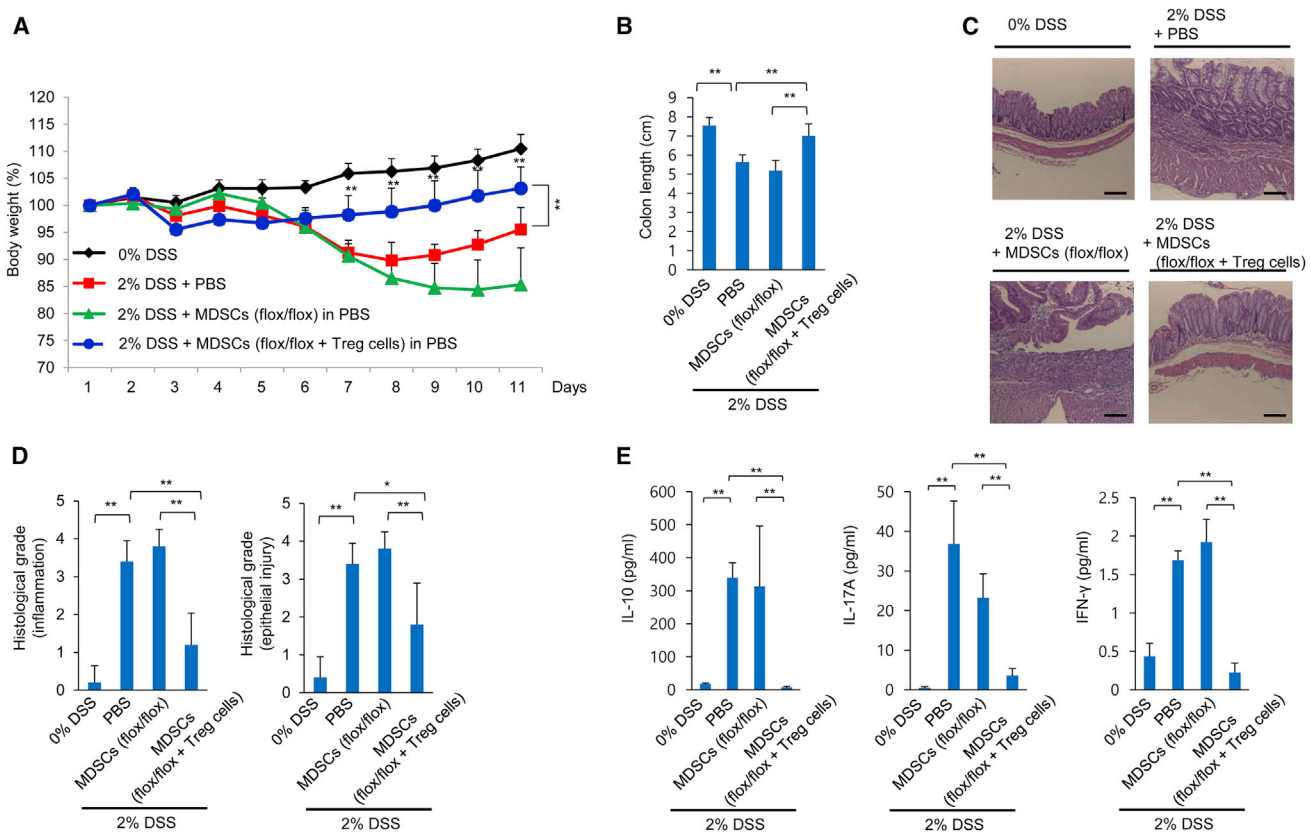


Figure 2. Adoptive Transfer of Wild-Type Treg Cells Improves the Immune-Modulatory Functions of MDSCs

(A) Body weight change (in percentage) after adoptive transfer of MDSCs (5 × 10^5 cells) from *Pdk1*$^{flox/flox}$;*CD4-Cre* (flox/flox) mice or from wild-type Treg cells (2 × 10^5 cells) transferred *Pdk1*$^{flox/flox}$;*CD4-Cre* (flox/flox + Treg cell) mice on day 6 of DSS treatment (n = 7–10 mice/group).

(B) Colon length was measured on day 11.

(C) Photomicrograph of a colon section. Scale bars, 100 μm.

(D) Histological grade of the colon sections.

(E) Measurement of cytokine levels in colon explants of DSS-treated mice on day 11 (n = 3 mice/group).

Data are representative of three (A–E) independent experiments. Results are expressed as the mean ± SD. *p < 0.05, **p < 0.01 by two-way ANOVA and Bonferroni post-test (A) or one-way ANOVA and Tukey post-test (B, D, and E). See also Figure S2.

differentiate in vitro in the presence of Treg cells with GM-CSF, IL-4, and 2-ME, MDSC differentiation skewed toward Mo-MDSCs (CD11b$^+$Ly6G^{dim}Ly6C^{hi}) (Figure 3D). This is consistent with the observation that Treg cells also modulated the differentiation of the Mo-MDSC subpopulation in *Pdk1*$^{flox/flox}$;*CD4-Cre* mice (Figure 3B).

As shown above, production of IL-10 and TGF-β was dramatically lower in PDK1-deficient Treg cells compared with wild-type Treg cells (Figure 1C). Thus, it was important to determine whether Treg cell-produced cytokines such as TGF-β and IL-10 could alter the differentiation of the two MDSC subpopulations. Addition of TGF-β-neutralizing antibodies or a selective inhibitor of the TGF-β receptor kinases, SB 431542, to bone marrow cells prevented Treg cells from modulating the differentiation of bone marrow-derived MDSCs into distinct subpopulations (i.e., it reversed the increase in Mo-MDSCs and caused a corresponding decrease in Gr-MDSCs) (Figure 3D). However, IL-10-neutralizing antibody did not affect differentiation of MDSCs in vitro (data not shown).

TGF-β Modulates the Differentiation of Bone Marrow-Derived MDSCs

Addition of TGF-β directly to in vitro-differentiating MDSCs caused the proportion of Gr-MDSCs to decrease, whereas the proportion of Mo-MDSCs increased; however, IL-10 had no effect on MDSC subpopulation differentiation (Figure 4A). Interestingly, the absolute number of Gr-MDSCs did not decrease substantially because the total number of differentiated MDSCs increased (Figures 4B and 4C), meaning that the absolute number of Mo-MDSCs did, in fact, increase significantly in response to TGF-β (Figure 4C). This is beneficial for producing large numbers of MDSCs when these differentiated cells are used for therapeutic purposes for patients suffering from inflammatory diseases. Next, bone marrow-derived Mo-MDSCs and Gr-MDSCs were isolated as they were undergoing differentiation in vitro and labeled with carboxylfluorescein succinimidyl ester (CFSE) to analyze dividing cells. Here, addition of TGF-β to the MDSCs significantly increased the proliferation of Mo-MDSCs but not Gr-MDSCs (Figure 4D). Interestingly, even though we

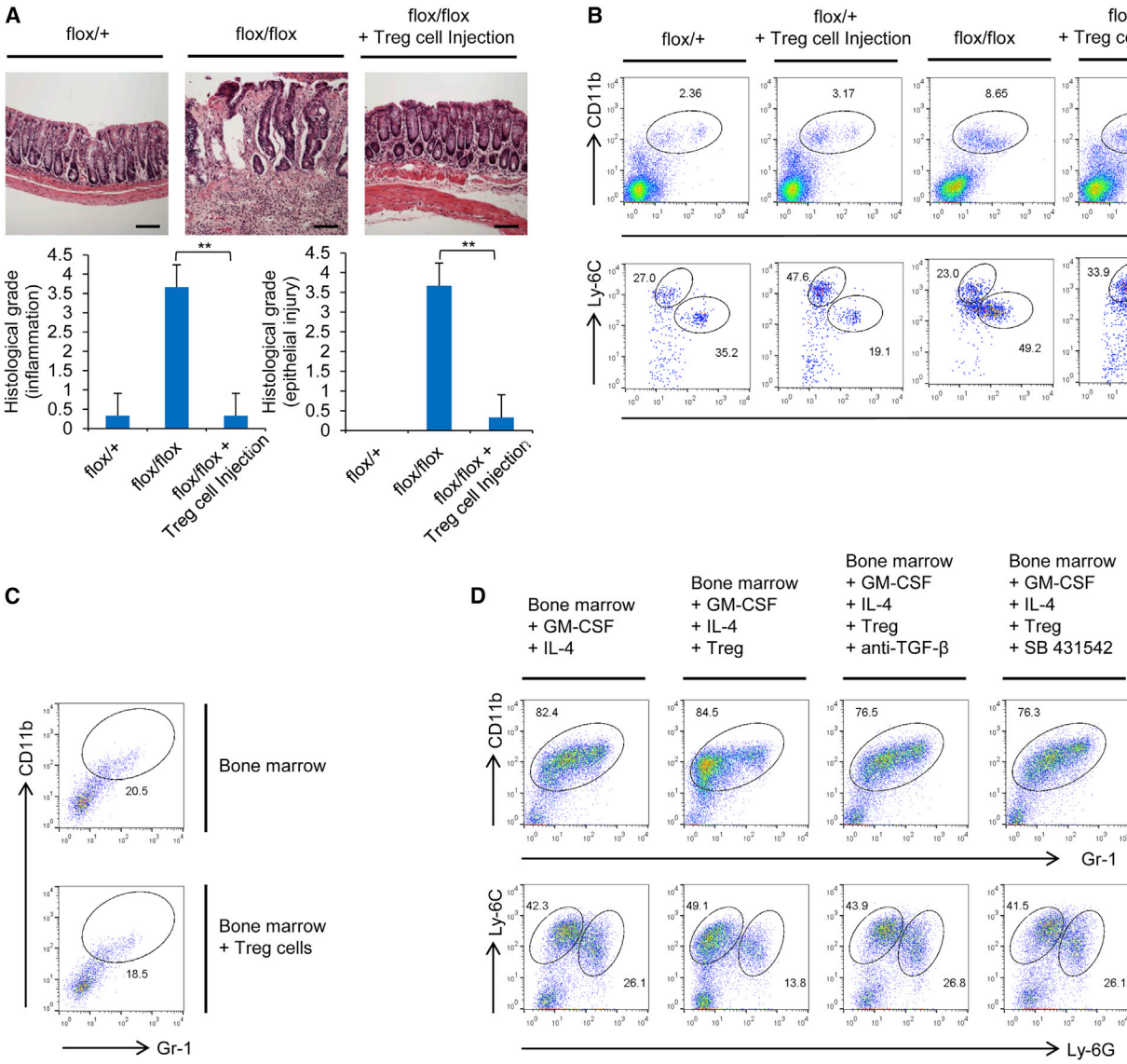


Figure 3. Adoptive Transfer of Wild-Type Treg Cells Affects MDSC Subpopulations

(A) Photomicrograph of colon sections (top) and their corresponding histological grades (bottom) of 12-week-old *Pdk1*$^{flox/flox}$;*CD4-Cre* mice (flox/flox) before or 2 weeks after three separate adoptive transfers of wild-type Treg cells (2 × 10^5 cells) and a littermate control *Pdk1*$^{flox/+}$;*CD4-Cre* (flox/+) (n = 3 mice/group). Scale bars, 100 μm.

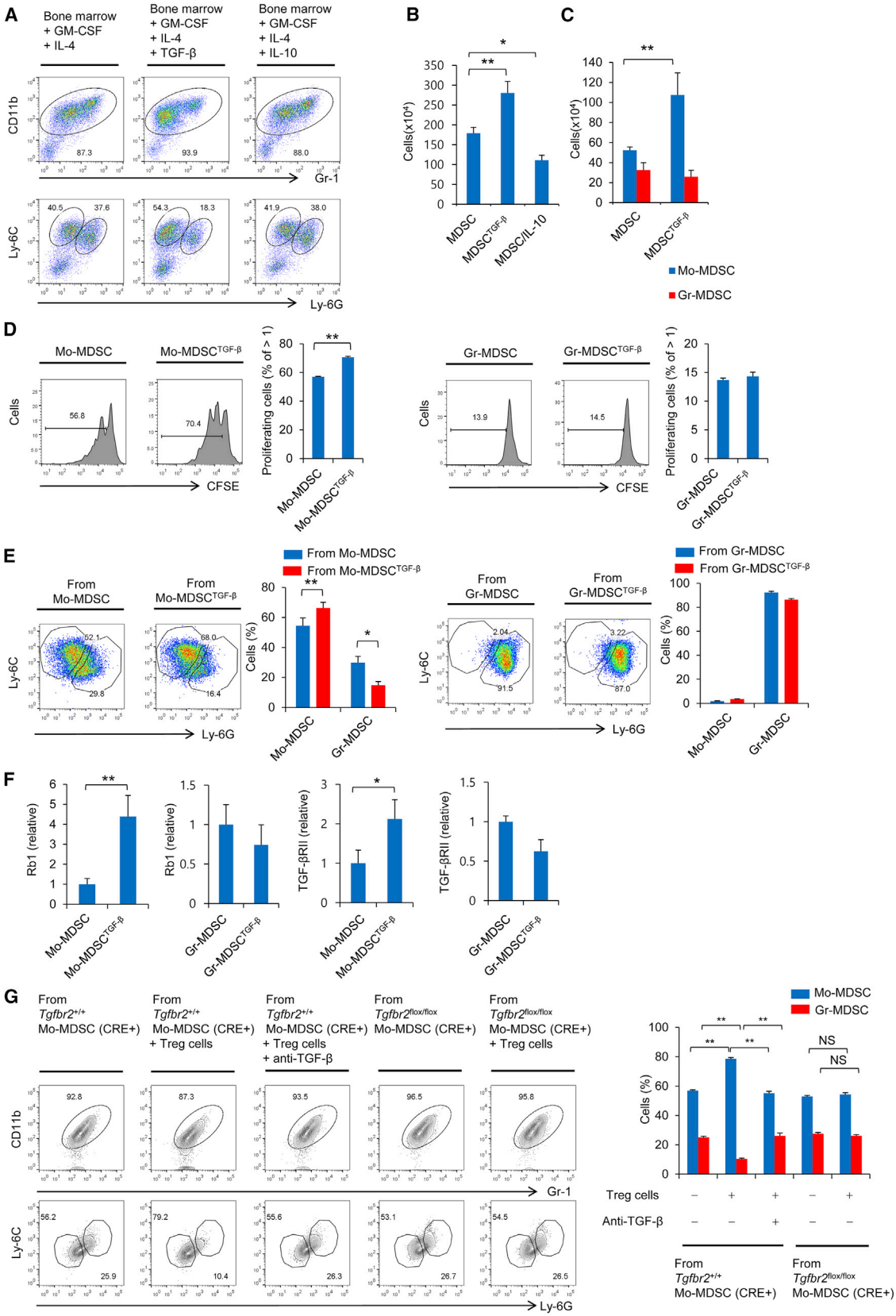
(B) Flow cytometric analysis of MDSC subpopulations in *Pdk1*$^{flox/+}$;*CD4-Cre* (flox/+) and *Pdk* $^{flox/flox}$; *CD4-Cre* (flox/flox) mice before or 3 days after adoptive transfer of wild-type Treg cells (2 × 10^5 cells).

(C) Flow cytometric analysis of bone marrow cells cultured in the absence of GM-CSF, IL-4, and 2-ME with or without Treg cells.

(D) Flow cytometric analysis of bone marrow-derived MDSCs differentiated in the presence of GM-CSF, IL-4, and 2-ME, with or without Treg cells. For blocking of TGF-β signaling, anti-TGF-β antibody (10 μg/ml) or SB 431542 (10 μM) was used.

Data are representative of three (A and B) or four (C and D) independent experiments. Results are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$ by one-way ANOVA and Bonferroni post-test (A).

isolated Mo-MDSCs as they were differentiating, some of these cells spontaneously converted into Gr-MDSCs. However, none of the Gr-MDSCs were converted into Mo-MDSCs, and the addition of TGF-β inhibited conversion of Mo-MDSCs into Gr-MDSCs (Figure 4E). Also, others have shown that retinoblastoma (Rb) inhibits conversion of Mo-MDSC into Gr-MDSC (Youn et al., 2013). Likewise, we found that TGF-β treatment increased Rb expression in Mo-MDSCs (Figure 4F). In addition, we verified TGF-β signaling in Mo-MDSCs and Gr-MDSCs to determine whether these two populations show differences in TGF-β receptor expression and phospho-Smad2/3 levels. Addition of TGF-β increased TGF-βRII (β receptor type II) expression and phospho-Smad2/3 levels in Mo-MDSCs but was unchanged in Gr-MDSCs (Figure 4F; Figures S3A and S3B). To clarify the role of



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TGF-β signaling during MDSC differentiation, we used bone marrow cells from *Tgfbr2*$^{flox/flox}$ mice and a recombinant retrovirus (murine stem cell virus [MSCV]-Cre-internal ribosome entry site [IRES]-GFP) to delete the *Tgfbr2* gene in MDSCs. Here, transduction of Mo-MDSCs was efficient because the great majority of GFP$^+$ cells were Mo-MDSCs in the MDSC population (Figure S3C). We then sorted GFP$^+$ Mo-MDSCs and determined the effect of *Tgfbr2* gene deletion in mediating conversion of Mo-MDSCs to Gr-MDSCs. We found that lack of *Tgfbr2* expression in Mo-MDSCs abolishes Treg cell-mediated inhibition of Mo-MDSC conversion into Gr-MDSCs, as did treatment of Mo-MDSCs with TGF-β-neutralizing antibody (Figure 4G).

TGF-β Increases MDSC Inhibitory Functions

Before further investigating the bone marrow-derived MDSCs that had differentiated in the presence of TGF-β in vitro (MDSCs$^{TGF-β}$), we confirmed that these cells were in an immature state by verifying the expression of the cell surface receptors F4/80 (a macrophage marker) and CD11c (a dendritic cell marker). However, very few of the bone marrow-derived MDSCs had differentiated into mature myeloid cells, regardless of TGF-β treatment (Figure 5A). In addition, differentiated MDSC and MDSC$^{TGF-β}$ were sorted, and their nuclear morphology was examined after staining with Wright-Giemsa stain. Gr-MDSCs had a clearly visible polymorphonuclear morphology, whereas Mo-MDSCs were mononuclear (Figure 5B).

The bone marrow-derived Mo-MDSCs produced molecules involved in MDSC-mediated immune suppression, including iNOS, IL-10, and TGF-β, but both Mo-MDSCs and Gr-MDSCs expressed ARG1. Interestingly, TGF-β significantly enhanced the production of iNOS, TGF-β, and IL-10 (Figure 5C) and ARG1 (Figures 5D and 5E) in MDSCs. In addition, PD-L1 is also important for MDSC-mediated immune modulation and was found to be expressed on the surface of bone marrow-derived MDSCs and particularly on Gr-MDSCs (Figure 5F). Expression of PD-L1 on the surface of Mo-MDSCs was enhanced by TGF-β, whereas the expression of PD-L1 by Gr-MDSCs was not greatly affected by TGF-β (Figure 5F), possibly because PD-L1 expression was already high on Gr-MDSCs differentiated in vitro. Next, we determined the effect of TGF-β on immune suppression by MDSCs. In our experiments, both Mo-MDSCs and Gr-MDSCs, after being differentiated with TGF-β, were more capable of suppressing CD4$^+$ T cell proliferation than Mo-MDSCs and Gr-MDSCs differentiated without TGF-β (Figure 5G).

Treg Cell-Derived TGF-β Is Crucial for Regulation of MDSCs during DSS-Induced Colitis

As DSS-induced colitis progressed over time, we observed a time-dependent (i.e., temporal) decrease in the population of Treg cells, and this was accompanied by a concomitant increase in the population of granulocytic MDSCs as the mice began to lose weight (Figure S4). It is possible that, because the absolute number of Treg cells was not significantly lower in the spleen, the decrease in Treg cell population might result from an increase in other inflammatory cells in the spleen (Figure S4A). However, during recovery from colitis, the Treg cell population reverted to a normal range, the population of granulocytic MDSCs decreased, and the monocytic MDSC population increased (Figure S4B). Based on these results, we postulated that temporal insufficiency of the Treg cell population affected the function of MDSCs. To test this hypothesis, we transferred Treg cells into mice with DSS-induced colitis at a time point just before the Treg cell population started to decrease (Figure 6; Figure S5A). When these mice received additional Treg cells under this protocol, weight gain improved dramatically (Figure 6A), and their colons were not as dramatically short compared with DSS-treated mice (Figure 6B; Figure S5B). In addition, recovery from intestinal inflammation and injury in mice with DSS-induced experimental colitis could be improved by adoptive transfer of Treg cells (Figures 6C and 6D).

To confirm this crucial role of Treg cell-derived TGF-β in regulating MDSCs, Treg cells transduced with TGF-β short hairpin RNA (shRNA) were adoptively transferred into mice with DSS-induced colitis (Figure 6A). Knockdown of TGF-β was confirmed by ELISA prior to transferring Treg cells into mice (Figure 6E). Apparently, TGF-β-deficient Treg cells cannot significantly reverse the symptoms associated with colitis, including weight loss (Figure 6A), shortening of the colon (Figure 6B; Figure S5B), intestinal inflammation and injury (Figures 6C and 6D), and production of inflammatory cytokines by intestinal cells (Figure 6F). Although wild-type Treg cells could regulate MDSC subpopulations (Figures 6G and 6H) and functions (Figure 6I) in mice with colitis, TGF-β-deficient Treg cells could regulate neither the subpopulations (Figures 6G and 6H) nor their functions (Figure 6I). Thus, our data show unequivocally that Treg cell-derived TGF-β is a key mediator for modulating differentiation of MDSCs into distinct subpopulations (Mo-MDSCs and Gr-MDSCs) as well as for regulating MDSC function. Moreover, adoptive transfer of TGF-β-deficient Treg cells into *Pdk1*$^{flox/flox}$;*CD4-Cre* mice did not contribute to differentiation

Figure 4. TGF-β Affects the Differentiation of Bone Marrow-Derived MDSCs

(A) Flow cytometric analysis of bone marrow-derived MDSCs differentiated with or without the Treg cell-related cytokines TGF-β and IL-10.

(B) The number of bone marrow-derived MDSCs with or without the Treg cell-related cytokines TGF-β and IL-10.

(C) Cell numbers of each subpopulation of bone marrow-derived MDSCs differentiated with or without TGF-β.

(D) Flow cytometric analysis of proliferating CFSE-labeled bone marrow-derived Mo-MDSCs or Gr-MDSCs differentiated with or without TGF-β.

(E) Flow cytometric analysis of Mo-MDSCs and Gr-MDSCs from purified bone marrow-derived Mo-MDSCs or Gr-MDSCs after 3 days of culture. Mo-MDSCs and Gr-MDSCs were purified from bone marrow-derived MDSCs that were differentiated with or without TGF-β. The purified Mo-MDSCs and Gr-MDSCs were further cultured for 3 days.

(F) Relative levels of Rb and TGF-βRII mRNA in bone marrow-derived Mo-MDSCs and Gr-MDSCs differentiated with or without TGF-β.

(G) Flow cytometric analysis of Mo-MDSCs and Gr-MDSCs from recombinant retrovirus (MSCV-Cre-IRES-GFP)-infected *Tgfbr2*$^{+/+}$ Mo-MDSCs or *Tgfbr2*$^{flox/flox}$ Mo-MDSC after 3 days of culture with or without Treg cells. Anti-TGF-β antibody (10 μg/mL) was used to neutralize TGF-β.

Data are representative of five (A), three (B and C), or two (D–G) independent experiments. Results are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$ by one-way ANOVA and Tukey post-test (B) or two-way ANOVA and Bonferroni post-test (C, E, and G) or Student t test (D and F). See also Figure S3.

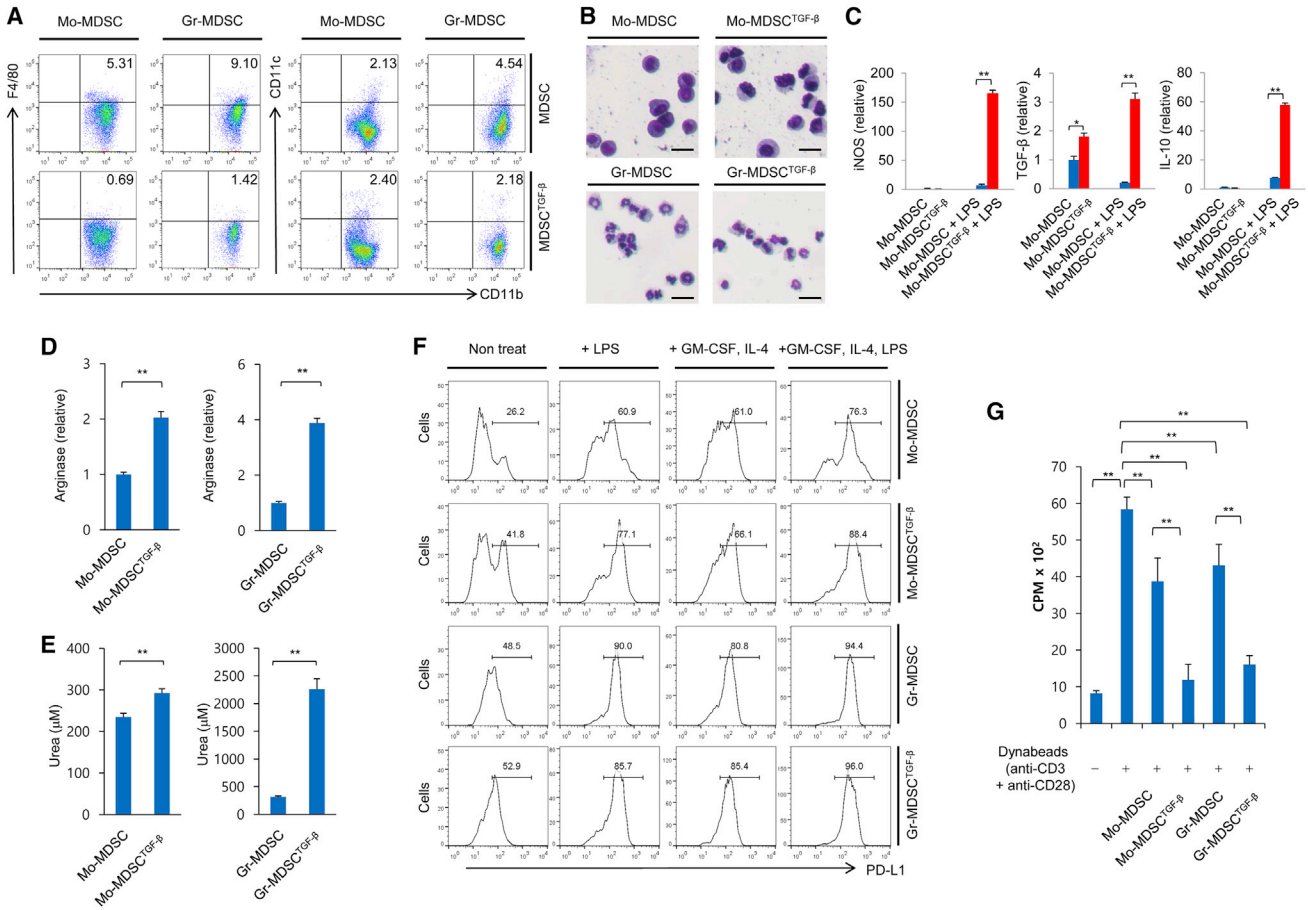

Figure 5. TGF-β Is Important for the Inhibitory Function of MDSCs

(A) Flow cytometric analysis of the expression of mature myeloid cell markers on bone marrow-derived MDSCs differentiated with or without TGF-β.

(B) Wright-Giemsa staining of bone marrow-derived MDSCs differentiated with or without TGF-β. Scale bars, 50 μm.

(C) Relative mRNA levels of iNOS (left), TGF-β (center), and IL-10 (right) in bone marrow-derived Mo-MDSCs differentiated with or without TGF-β after stimulation with lipopolysaccharide (LPS).

(D) Relative mRNA levels of ARG1 in bone marrow-derived Mo-MDSCs and Gr-MDSCs differentiated with or without TGF-β.

(E) ARG1 activity in bone marrow-derived Mo-MDSCs and Gr-MDSCs differentiated with or without TGF-β.

(F) Flow cytometric analysis of PD-L1 expression on bone marrow-derived MDSC subpopulations differentiated with or without TGF-β after treatment with LPS, GM-CSF + IL-4, or GM-CSF + IL-4 + LPS.

(G) Suppression assay with bone marrow-derived MDSCs.

Data are representative of two (A, F, and G) or three (B–E) independent experiments. Results are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$ by two-away ANOVA and Bonferroni post-test (C) or Student's t test (D and E) or one-way ANOVA and Tukey post-test (G).

of MDSC subpopulations, whereas wild-type Treg cells allowed the differentiation (Figure S5C).

Mo-MDSCs Differentiated with TGF-β Showed Enhanced Immune-Suppressive Activity in DSS-Induced Experimental Colitis

To evaluate the therapeutic effect of TGF-β-mediated, in vitro-differentiated MDSCs on inflammatory bowel disease, we examined these cells in mice with DSS-induced colitis (Figure S6A). Here, we focused on Mo-MDSCs because these cells possess more potent inhibitory activity than Gr-MDSCs (Gabrilovich and Nagaraj, 2009; Lindau et al., 2013; Solito et al., 2014). In this experiment, mice were injected intravenously with bone marrow-derived Mo-MDSC or Mo-MDSC$^{TGF-β}$ on day 1 and day 6 of DSS treatment. Our results show that weight loss was less severe in DSS-treated mice following the transfer of either Mo-MDSC or Mo-MDSC$^{TGF-β}$, and mice receiving Mo-MDSC$^{TGF-β}$ gained weight faster compared with mice receiving Mo-MDSC (Figure 7A). Also, the colon was shorter in mice with DSS-induced colitis, but, after transfer of Mo-MDSCs, the colon was not as short, whereas transfer of Mo-MDSC$^{TGF-β}$ led to nearly normal colon length (Figure 7B; Figure S6B). Therefore, transfer of Mo-MDSC$^{TGF-β}$ was more efficient at reversing intestinal inflammation and injury than the transfer of Mo-MDSCs, as evidenced by histological analysis of colon sections (Figures 7C and 7D). In addition, transferred Mo-MDSC$^{TGF-β}$ was more localized in the intestines compared with Mo-MDSCs (Figure 7E).

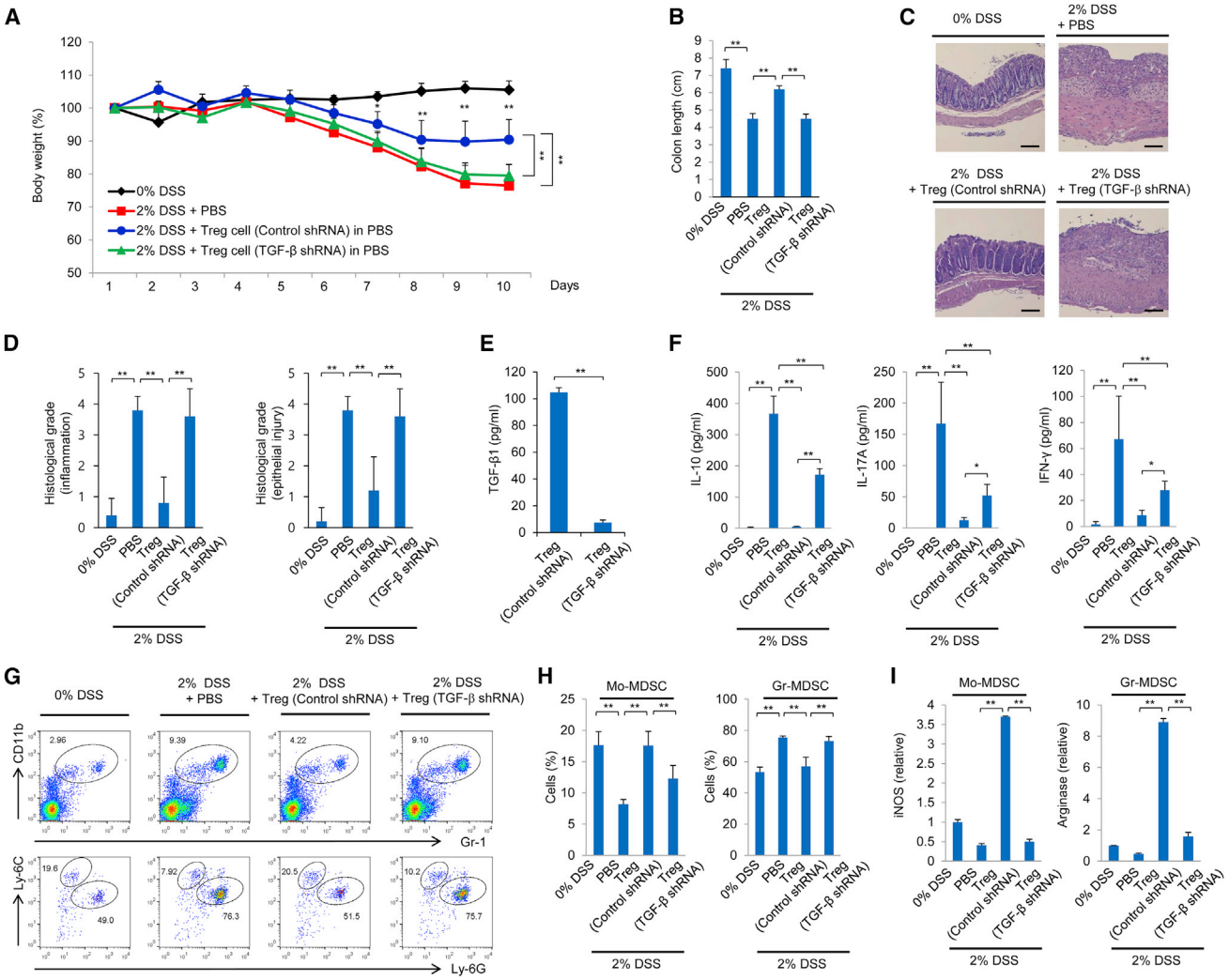

Figure 6. Treg Cell-Derived TGF-β Is Crucial for Regulation of MDSCs during DSS-Induced Colitis

(A) Body weight change (in percentage) after adoptive transfer of control shRNA-transduced or TGF-β shRNA-transduced Treg cells (2×10^5 cells) on day 5 of DSS treatment (n = 6–7 mice/group).

(B) Colon length was measured on day 10 in the DSS-treated mice.

(C) Photomicrograph of colon sections. Scale bars, 100 μm.

(D) Histological grade of the colon sections.

(E) TGF-β shRNA was introduced into Treg cells by using a recombinant lentiviruses, and the Treg cells were activated with anti-CD3 and anti-CD28 antibodies for 36 hr. Also, TGF-β production was measured by the TGF-β ELISA Ready-SET-Go system (eBioscience).

(F) Measurement of cytokine levels in colon explants from DSS-treated mice after adoptive transfer of control or TGF-β shRNA-transduced Treg cells on day 10 (n = 3 mice/group).

(G) Flow cytometric analysis of MDSC subpopulations from DSS-treated mice on day 7.

(H) The percentage of MDSC subpopulations in total MDSCs isolated from the spleen of DSS-treated mice on day 7.

(I) Relative levels of iNOS mRNA (left) in Mo-MDSCs and ARG1 mRNA (right) in Gr-MDSCs isolated from the spleens of DSS-treated mice.

Data are representative of two (A–I) independent experiments. Results are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$ by two-way ANOVA and Bonferroni post-test (A) or one-way ANOVA and Tukey post-test (B, D, H, and I), Holm-Sidak post-test (F), or Student's t test (E). See also Figures S4 and S5.

However, in other organs, including the lungs and secondary lymphoid organs, there was not much difference in localization of Mo-MDSC $^{TGF-\beta}$ and Mo-MDSCs (Figure S6C). Last, transfer of Mo-MDSC$^{TGF-\beta}$, but not Mo-MDSCs, led to large decreases in cytokines such as IL-10, IL-17A, and IFN-γ in cultured intestinal tissues (Figure 7F).

During intestinal inflammation, Treg cells are important for regulating the inflammatory response. Thus, we evaluated Treg cell populations in mice with DSS-induced colitis and found that the proportion of $CD4^+Foxp3^+$ Treg cells was low. However, when bone marrow-derived Mo-MDSC or Mo-MDSC$^{TGF-\beta}$ were transferred into these mice, the population of $CD4^+Foxp3^+$

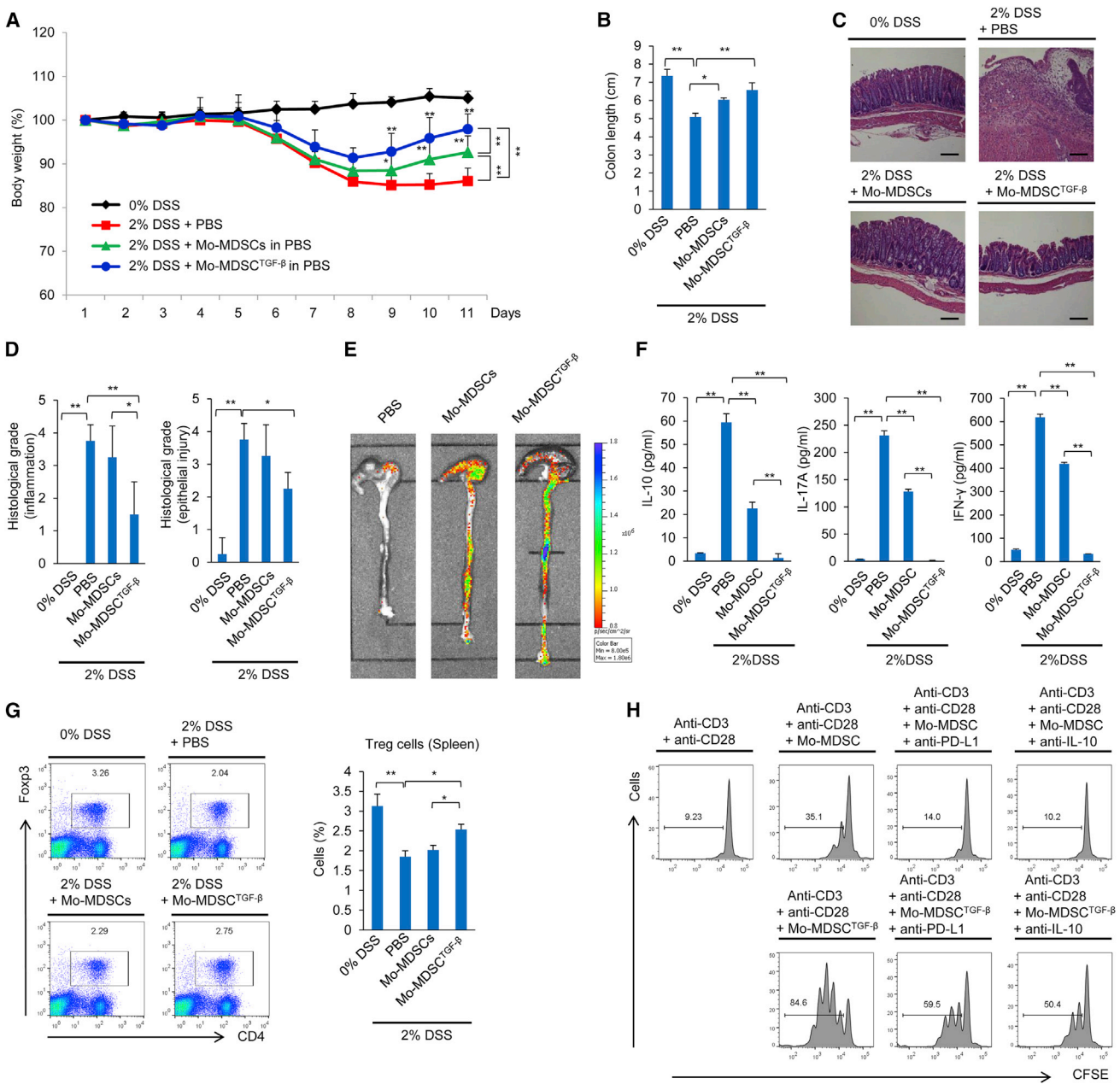

Figure 7. TGF-β-Educated MDSCs Show Enhanced Immune Suppression in DSS-Induced Colitis

(A) Body weight change (as percentage) after adoptive transfer of bone marrow-derived Mo-MDSCs or Mo-MDSC$^{TGF-β}$ (1 × 10^6 cells) on days 1 and 6 of DSS treatment (n = 9–12 mice/group).

(B) Colon length was measured on day 11.

(C) Photomicrograph of colon sections. Scale bars, 100 μm.

(D) Histological grades of the colon sections.

(E) Mice were treated with DSS for 6 days and then injected with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled bone marrow-derived Mo-MDSCs or Mo-MDSC$^{TGF-β}$ (4 × 10^6 cells). After 36 hr, fluorescence images of the intestine were obtained using the IVIS 200 imaging system.

(F) Measurement of cytokine levels in colon explants from DSS-treated mice on day 11 (n = 3 mice/group).

(G) Flow cytometric analysis of Treg cells in the spleen of DSS-treated mice on day 8.

(H) Flow cytometric analysis of proliferating CFSE-labeled Treg cells after stimulation with anti-CD3 and anti-CD28 antibodies in the presence of bone marrow-derived Mo-MDSCs or Mo-MDSC$^{TGF-β}$ with or without anti-PD-L1 antibody (10 μg/mL) and anti-IL-10 antibody (10 μg/mL).

Data are representative of three (A–G) or two (H) independent experiments. Results are expressed as the mean ± SD. *$p < 0.05$, **$p < 0.01$ by two-way ANOVA and Bonferroni post-test (A) or one-way ANOVA and Tukey post-test (B, D, F, and G). See also Figure S6.

Treg cell increased compared with DSS-treated mice (Figure 7G). Transfer of Mo-MDSC$^{TGF-\beta}$ resulted in a significant rise in total splenic Treg cells compared with the transfer of Mo-MDSCs (Figure 7G). Thus, Mo-MDSC$^{TGF-\beta}$ was more capable of inducing Treg cell expansion than Mo-MDSCs. This observation was also confirmed by assessing Treg cell proliferation in vitro. Bone marrow-derived Mo-MDSCs and Mo-MDSC$^{TGF-\beta}$ both enhanced the proliferation of Treg cells in vitro, but a greater effect was observed with Mo-MDSC$^{TGF-\beta}$ (Figure 7H). Moreover, the enhanced proliferation of Treg cells by MDSCs was significantly reduced when the function of IL-10 and PD-L1 was inhibited by adding antibodies to block IL-10 or PD-L1 (Figure 7H; Figure S6D). This suggests that both PD-L1 and IL-10 are responsible for promoting expansion of already differentiated Treg cells, whereas PD-L1, but not IL-10, is important for iTreg differentiation. In our adoptive transfer experiments, transferred Mo-MDSC or Mo-MDSC$^{TGF-\beta}$ disappeared rapidly, whereas intestinal inflammation was suppressed for longer periods. Therefore, bone marrow-derived Mo-MDSCs, especially when differentiated with TGF-β, cause Treg cells to expand in vivo and in vitro, and this is the most likely explanation for the prolonged immunosuppressive effects of Mo-MDSCs or Mo-MDSC$^{TGF-\beta}$, even though the transferred population disappeared relatively quickly.

DISCUSSION

MDSCs accumulate in the blood, lymphoid organs, spleen, and tumors under inflammatory conditions (Gabrilovich and Nagaraj, 2009; Lindau et al., 2013; Solito et al., 2014) and are involved in immune suppression by producing effector molecules such as NO and ARG1 (Gabrilovich and Nagaraj, 2009; Solito et al., 2014). In humans, MDSC populations are divided into two groups (Gr-MDSCs and Mo-MDSCs), but these populations have not been defined consistently with respect to surface marker expression, leading to controversial results (Solito et al., 2014). In mice, however, MDSCs are well defined as CD11b^{+}Gr1^{+} cells, and can be classified further as either Gr-MDSCs or Mo-MDSCs (Gabrilovich and Nagaraj, 2009; Solito et al., 2014). Although the classification of human MDSCs by cell surface marker expression is controversial, human MDSCs can be functionally characterized as Gr-MDSCs and Mo-MDSCs. This is because, under inflammatory conditions, Gr-MDSCs produce large quantities of ARG1 and ROS for immune suppression, whereas Mo-MDSCs produce NO (Gabrilovich and Nagaraj, 2009; Lindau et al., 2013; Solito et al., 2014). In addition, others have shown that Gr-MDSCs are the major subset of MDSCs to be expanded in the peripheral lymphoid organs, whereas Mo-MDSCs possess more potent inhibitory activity (Gabrilovich and Nagaraj, 2009; Lindau et al., 2013; Solito et al., 2014). In our study, the population of Gr-MDSCs was expanded in *Pdk1*$^{flox/flox}$; *CD4-Cre* mice as they developed spontaneous colitis.

Many reports have shown that MDSCs induce the expansion of Treg cells, which are involved indirectly in MDSC-mediated immune suppression (Haile et al., 2008; Luan et al., 2013; Pan et al., 2010; Serafini et al., 2008; Wood et al., 2012). In addition, it has been reported that cell surface molecules expressed on MDSCs and effector molecules secreted by MDSCs are also involved in MDSC-mediated expansion of Treg cells (Haile et al., 2008; Luan et al., 2013; Pan et al., 2010; Serafini et al., 2008; Wood et al., 2012). Thus, although MDSC-mediated Treg cell expansion is well established, its effects on MDSC differentiation and function have not been resolved. Here we show that Treg cells control MDSC differentiation and function in a murine model of colitis. The murine model of spontaneous colitis in T cell-specific *Pdk1*-deficient mice favored conditions promoting a deficiency of functional Treg cells, which allowed us to examine the relationship between Treg cells and MDSCs. Under these conditions, Treg cells had an important role in regulating Mo-MDSC expansion and in regulating the function of both Mo-MDSCs and Gr-MDSCs. In fact, in the spontaneous colitis model, the number of Gr-MDSCs increased along with disease severity. In contrast, for DSS-induced colitis, Gr-MDSCs also increased initially but decreased in number as the mice recovered from the experimentally induced colitis. Interestingly, T cell-specific *Pdk1*-deficient mice with spontaneous colitis began to recover after the transfer of wild-type Treg cells; the number of Mo-MDSCs increased but Gr-MDSCs decreased in the recipient mice, as seen in mice recovered from DSS-induced colitis. Thus, our data suggest that Treg cells are important for maintaining normal proportions of the MDSC subsets. In addition, because previous reports have shown that Mo-MDSCs possess more potent inhibitory activity than Gr-MDSCs (Gabrilovich and Nagaraj, 2009; Lindau et al., 2013; Solito et al., 2014), Treg cells may promote more immunosuppressive conditions by favoring MDSC differentiation toward Mo-MDSCs.

In addition to the interaction of Treg cells with MDSCs in mice with T cell-specific *Pdk1* gene deficiency, we found that Treg cell-derived TGF-β is important for regulating the differentiation of MDSCs into distinct subpopulations and functions. Because T cell-specific *Pdk1* gene deficiency does not represent a physiological condition, the importance of Treg cell-derived TGF-β for regulation of MDSCs was confirmed in DSS-induced colitis, a disease model used to mimic IBD (Bertrand et al., 2009; Klose et al., 2014; Moriwaki et al., 2014). During acute intestinal inflammation induced by DSS, the proportion of Treg cells decreased rapidly. At this point, adoptive transfer of wild-type Treg cells, but not of TGF-β-deficient Treg cells, helped to regulate MDSC subpopulations and functions. This finding was supported directly by experiments showing that the in vitro differentiation of MDSCs into Mo-MDSCs was affected by Treg cells, similar to what was observed in vivo. Also, Treg cell-derived TGF-β was important for the expansion of bone marrow-derived MDSCs into a Mo-MDSC subpopulation in vitro. In addition to their effects on MDSC differentiation, TGF-β was also crucial for MDSC functions. Aside from inflammatory conditions such as colitis, this interaction may also protect tumors from anti-tumor immunity; many reports have detailed a negative correlation between Treg cells and MDSCs and the prognosis of cancer patients (Arihara et al., 2013; Gabitass et al., 2011; Tazzari et al., 2014; Wu et al., 2014). Therefore, it is possible that Treg cells and MDSCs can establish a positive feedback loop during the inflammatory response and tumor environment.

In conclusion, Treg cells are capable of regulating MDSCs under pro-inflammatory conditions. There is a positive feedback

loop between MDSCs and Treg cells during the inflammatory response, where MDSCs support the expansion of Treg cells, and Treg cells modulate MDSC differentiation and effector functions via TGF-β. These observations reveal an interaction between MDSCs and Treg cells and provide insight into immune regulation. In addition, our in vitro MDSC differentiation strategy can be applied to cell-based therapeutic strategies for patients suffering from inflammatory diseases, including IBD, because TGF-β enhanced MDSC function and also increased MDSC numbers.

EXPERIMENTAL PROCEDURES

Mice and MDSC Isolation

Pdk1$^{flox/flox}$ mice were bred with *CD4-Cre* transgenic mice, and the offspring were bred with *Pdk1*$^{flox/flox}$ mice to generate *Pdk1*$^{flox/flox}$;*CD4-Cre* and *Pdk1*$^{flox/+}$;*CD4-Cre*. All mice were kept under specific pathogen-free conditions in the animal care facility at the Gwangju Institute of Science and Technology (GIST). All experiments using mice were approved by the Institutional Animal Care and Use Committee of GIST. MDSCs were stained with MDSC-specific surface markers and isolated with a FACSAria III instrument (BD Biosciences) after cell isolation using a Ficoll density gradient (GE Healthcare) to remove granulocytes.

Flow Cytometry

Isolated or differentiated cells were stained with the indicated antibodies and analyzed on a FACSCanto II (BD Biosciences) or Guava flow cytometer (Millipore). Live cells were gated by forward and side scatters for analysis. For intracellular staining to detect Foxp3, cells were fixed and permeabilized with Foxp3 staining buffer (eBioscience). The data were analyzed using FlowJo software.

Treg Cell Isolation and In Vitro Treg Cell Differentiation

$CD4^+CD25^+$ lymph node cells from *Pdk1*$^{flox/flox}$; *CD4-Cre* or *Pdk1*$^{flox/+}$; *CD4-Cre* mice were sorted on a FACSAria III instrument (BD Biosciences) after staining cells with fluorochrome-conjugated anti-mouse CD4 (GK 1.5, eBioscience) and anti-mouse CD25 (PC61.5, eBioscience) antibodies. Details of in vitro Treg cell differentiation can be found in the Supplemental Experimental Procedures.

Adoptive Transfer of Treg Cells

Wild-type Treg cells ($CD4^+CD25^+$) were isolated from lymph nodes of C57BL/6 mice with a FACSAria III instrument (BD Biosciences). Before transfer, we confirmed that more than 95% of the isolated $CD4^+CD25^+$ cells were $Foxp3^+$ cells. *Pdk1*$^{flox/flox}$; *CD4-Cre* and *Pdk1*$^{flox/+}$; *CD4-Cre* mice were injected intravenously twice with the isolated wild-type Treg cells.

qRT-PCR

Total RNA was isolated from cell pellets using the RNeasy mini kit (QIAGEN), and first-strand cDNA was subsequently synthesized using RT Drymix (Enzinomics) according to the manufacturer's instructions. The amount of each mRNA was determined by real-time PCR (Agilent Technologies) using SYBR Premix (Takara). All experiments were normalized using the glyceraldehyde-3-phospate dehydrogenase gene.

In Vitro Differentiation of Bone Marrow-Derived MDSCs

Bone marrow cells were isolated from C57BL/6 mice, and 5×10^5 cells were cultured in 0.5 mL of RPMI 1640 medium (HyClone) supplemented with 10% fetal bovine serum (FBS, HyClone), 10 ng/mL GM-CSF (eBioscience), 10 ng/mL IL-4 (eBioscience), and 50 μM 2-ME (Sigma-Aldrich). Cell cultures in 24-well plates were maintained at 37°C in a 5% CO_2-humidified atmosphere. Cells were collected on day 3 and analyzed by flow cytometry.

ARG1 Enzymatic Activity Assay

Details of the ARG1 activity measurement can be found in the Supplemental Experimental Procedures.

DSS-Induced Experimental Colitis

Colitis was induced by oral administration of 2% DSS (w/v) (MP Biomedicals, molecular weight [MW] = 36,000–50,000 Da) in the drinking water for 5 days followed by normal drinking water. Mice were checked each day for morbidity, and their body weights were recorded. After the mice were sacrificed, colons were dissected, and colon length was measured.

Colon Organ Culture and Cytokine Measurement

After mice were sacrificed, colons were dissected, and 1 × 1 cm standardized segments of colon tissue were washed in cold PBS supplemented with penicillin and streptomycin (Gibco). These segments were cultured in 24-well flat-bottom culture plates in serum-free RPMI 1640 medium (HyClone) supplemented with penicillin and streptomycin. After 24 hr, supernatants were collected to measure production of IFN-γ, IL-17A, and IL-10 using cytometric bead array kits (BD Biosciences) according to the manufacturer's instructions. A standard curve was generated using the respective lyophilized recombinant murine cytokines.

Histology and Scoring

Specimens were fixed with 4% neutral buffered formalin and embedded in paraffin. Sections were cut, deparaffinized, and stained with H&E. Details of histological grading can be found in the Supplemental Experimental Procedures.

Proliferation Assay for Treg Cells and MDSCs

Cells were isolated via a FACSAria III instrument (BD Biosciences) on the basis of cell surface marker staining. The isolated cells were stained with 5 μM of CFSE (eBioscience) for 5 min, and then the labeling was stopped by the addition of RPMI 1640 medium (HyClone) supplemented with 10% FBS (HyClone). After several washes, CFSE-labeled cells were incubated for 48–72 hr.

TGF-β Knockdown in Treg Cells

Wild-type Treg cells ($CD4^+CD25^+$) were isolated from lymph nodes of C57BL/6 mice with a FACSAria III instrument (BD Biosciences). Details of TGF-β knockdown in Treg cells can be found in the Supplemental Experimental Procedures.

In Vitro Suppression Assays

Details of the $CD4^+$ T cell suppression assay can be found in the Supplemental Experimental Procedures.

Fluorescence Imaging

Details of fluorescence labeling and the fluorescence imaging method can be found in the Supplemental Experimental Procedures.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7. All data are presented as the mean ± SD. A Student's t test was performed to analyze two sets of data. For analysis of multiple datasets, one-way ANOVA was used with Bonferroni, Tukey, or Holm-Sidak post-test, and two-way ANOVA was used with Bonferroni post-test. For all statistical analyses, $p < 0.05$ was considered to be statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.11.062.

AUTHOR CONTRIBUTIONS

C.R.L., Y.K., T.Y., J.H.H., S.H.P., W.L., K.Y.S., and J.A.K. performed the experiments and analyzed and interpreted the data. M.B.Y., Y.C.K., and

S.K.M. helped to design the experiments. C.R.L., M.B.Y., S.M.K., and S.G.P. wrote the paper. S.G.P. conceived the study, designed the experiments, and interpreted the data.

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