Myeloid-Derived Suppressor Cells in Inflammatory Bowel Disease: A New Immunoregulatory Pathway

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Background & Aims: CD11b+Gr-1+ myeloid-derived suppressor cells (MDSCs) have been shown to cause T-cell tolerance in tumor-bearing mice; however, little is known about the role of MDSCs in chronic inflammation. Here, for the first time, we have identified and analyzed their role in inflammatory bowel disease (IBD). Methods: Repetitive adoptive transfer of clone 4/T-cell receptor (CL4-TCR) transgenic CD8+ T cells into VILLIN-hemagglutinin (HA) transgenic mice was performed on days 1, 12, and 27. Recipient mice were analyzed for immunopathology, HA-specific CD8+ T-cell responses, and CD11b+Gr-1+ MDSCs (frequency, phenotype, expression analysis, and in vitro as well as in vivo function). In addition, peripheral blood from patients with active Crohn’s disease and ulcerative colitis was examined for the presence and function of human MDSCs denoted as CD14+/HLA-DR−/low cells. Results: Repetitive transfer of HA-specific CD8+ T cells prevented VILLIN-HA recipient mice from development of severe enterocolitis, which is seen after a single transfer of T cells. Repeated transfer of antigen-specific T cells led to an increase in the frequency of nitric oxide synthase 2 and arginase-expressing CD11b+Gr-1+ MDSCs in spleen and intestine of VILLIN-HA mice with immunosuppressive function. Cotransfer of MDSCs with HA-specific CD8+ T cells into naïve VILLIN-HA mice ameliorated enterocolitis, indicating a direct immune regulatory effect of MDSCs on induction of IBD by antigen-specific T cells. Finally, an increase in the frequency of human MDSCs with suppressor function was observed in peripheral blood from patients with IBD. Conclusions: These results identify MDSCs as a new immune regulatory pathway in IBD.

Substantial advances in the understanding of the molecular pathogenesis of inflammatory bowel disease (IBD) have been made in the past few years.1 Murine models, which mimic many features of IBD,2 have shown that IBD results from an imbalance between effector and regulatory T cells.3 Mucosal inflammation has been suggested to result from an excessive effector cell function directed against mucosal antigens as well as lack of regulatory responses to these antigens.4

The use of transgenic mice expressing model antigens in enterocytes facilitates the analysis of mucosal inflammation of the gastrointestinal tract induced by self-reactive T cells.5–7 We have previously described a new mouse model in which hemagglutinin (HA) is specifically expressed in the intestine and for which HA-specific CD4+ as well as CD8+ T-cell receptor (TCR) transgenic mice are available. This allows for analysis of the role of antigen-specific CD4+, CD8+, and regulatory T cells in murine enterocolitis.

Myeloid-derived suppressor cells (MDSCs), characterized by the coexpression of Gr-1 and CD11b, have been shown to inhibit T-cell activation in different tumor models, including inflammation-induced carcinomas.8–11 MDSCs are a heterogeneous population of cells, composed of precursors of macrophages, granulocytes, dendritic cells, and myeloid cells at earlier stages of differentiation.12–14 The inhibitory properties of MDSCs are proposed to be mediated by the expression of inducible NOS2 and ARG1, which are both involved in the metabolism of L-arginine.15 In murine studies, a small number of CD11b+Gr-1+ cells (<4%) can be found in the blood and spleen of naïve wild-type mice. However, the frequency of MDSCs increases dramatically under different pathologic conditions such as tumor growth and graft-versus-host disease.15 Although the direct mechanism for the generation of MDSCs is not known so far, it has been observed that different cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), inter...
leukin (IL)-3, macrophage colony-stimulating factor, and IL-6, are involved in the recruitment of MDSCs to peripheral organs. Recently, it has been shown that MDSCs expand through a MyD88-dependent mechanism in a murine model of sepsis.

We hypothesized that MDSCs could suppress T-cell responses in T cell–dependent autoimmune diseases. Here, we analyzed the role of MDSCs in a CD8+ T cell–mediated model of IBD, which we have recently described. Our results show that repetitive transfer of CD8+ T cells specific for a model antigen expressed in enterocytes leads to an increase in the frequency of MDSCs. MDSCs inhibit T cell–mediated intestinal injury and protect mice from T cell–mediated chronic enterocolitis. Finally, we show an increase in arginase (ARG) 1–expressing MDSCs with immunosuppressive function in patients with IBD, showing the significance of MDSCs in these patients.

Materials and Methods

Mice

VILLIN-HA mice express the A/PR8/34 HA from influenza virus A under control of the enterocyte-specific VILLIN promoter as described previously. Clone 4 (CL4)-TCR transgenic mice express the α/β-TCR that recognizes an epitope of the HA protein presented by major histocompatibility complex class I (H-2Kd: HA512–520 complex) and were bred on a BALB/c background.

Adoptive Transfer

A total of 3.5–5 × 10⁴ splenocytes from CL4-TCR transgenic mice were injected intravenously into naive VILLIN-HA recipients on day 0. Mice were injected again with 1 × 10⁷ splenocytes on days 12 and 27 for the second and third times, respectively. Mice were killed on day 31 for further analysis. For cotransfer experiments, naive splenocytes from CL4-TCR mice were transferred alone or together with sorted Gr-1+CD11b+ cells obtained from VILLIN-HA mice after 3 transfers of splenocytes from CL4-TCR mice.

Histology

The entire intestine was removed immediately after the mice were killed. Tissue was fixed in formalin and embedded in paraffin. Slides were cut longitudinally at 3-μm thickness and stained with H&E. Histologic sections from duodenum, jejunum, ileum, cecum, and colon were analyzed. Two sections each obtained at 100-μm distances were evaluated. Mice were scored individually, with each score representing the mean of 3 sections. An independent investigator blinded to the type of treatment performed histologic examination. Morphology was graded as described in the supplementary materials (see supplemental material online at www.gastrojournal.org).

Results

Repeated Transfer of Splenocytes From CL4-TCR Mice Into VILLIN-HA Mice Ameliorates Enterocolitis

We have previously shown that adoptive transfer of 3–4 × 10⁶ CD8+ T cells isolated from CL4-TCR mice into VILLIN-HA mice caused weight loss and intestinal inflammation in recipient mice. In an effort to analyze the role of antigen-specific T cells in chronic intestinal inflammation, we performed repetitive transfer of CL4-TCR splenocytes into VILLIN-HA mice. Therefore, 3–5 × 10⁶ splenocytes from CL4-TCR mice were transferred into VILLIN-HA mice on day 0. This transfer resulted in a mild enterocolitis and a maximum of 10% loss in body weight within 5 days after transfer with a subsequent recovery in body weight. After 12 and 27 days, VILLIN-HA mice were rechallenged with 1 × 10⁷ splenocytes from CL4-TCR mice. Unexpectedly, although VILLIN-HA mice received a 20- to 35-fold higher number of antigen-specific naive CD8+ T cells with the second and third cell transfer, they showed almost no signs of enterocolitis and no significant loss in body weight. In contrast, age-matched naive VILLIN-HA mice developed severe weight loss (>15%), bloody diarrhea, and wasting symptoms within 5 days after transfer of 1 × 10⁷ cells (Figure 1A). All mice were killed 5 days after cell transfer, and the intestine was examined. As shown in Figure 1B, mice with one transfer of 1 × 10⁷ transgenic splenocytes showed macroscopic signs of acute inflammation, including thickening of the wall and mucosa damage. In contrast, no significant differences were observed when the intestine from mice after 3 transfers was compared with the intestine from naive mice (Figure 1B). Histologic analysis of the jejunum and colon from mice after one cell transfer and 3 cell transfers was performed and showed a marked epithelial destruction with erosive and focally ulcerative mucosal inflammation and hyperemia of capillaries. Additionally, an increased mitotic activity as a sign of crypt regeneration could be observed (Figure 1C). Taken together, these changes correspond to an inflammation score of 20 in mice after one cell transfer (Figure 1D). In contrast, inflammation was less severe in mice after 3 cell transfers, corresponding to an inflammation score of 4 (Figure 1D).

We next investigated the function of HA-specific CD8+ T cells in these mice. Antigen-specific interferon (IFN)-γ responses were reduced in mesenteric lymph nodes (MLN) derived from mice after 3 cell transfers (3.8%) in comparison with mice after one cell transfer (9.4%) (Figure 2A). However, no differences in proliferation of antigen-specific CD8+ T cells in VILLIN-HA mice was observed after one transfer or 3 transfers of splenocytes (Figure 2B).
Repeated Transfer of Splenocytes From CL4-TCR Mice Into VILLIN-HA Mice Increases the Frequency of MDSCs With Immune Suppressor Function in Spleen and Intestine

We hypothesized that MDSCs also accumulate during intestinal inflammation and determined the frequency of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in naive VILLIN-HA mice after one T-cell transfer or 3 T-cell transfers. The frequency of CD11b<sup>+</sup>Gr-1<sup>+</sup> cells in spleen increased from 1.6% in naive VILLIN-HA mice to 3.4% after one T-cell transfer and 14.2% after 3 T-cell transfers (Figure 3A). In parallel, an increase in frequency of MDSCs from 0.03% to 3.1% was also detected in the intestine of mice after one transfer and 3 transfers of splenocytes from CL4-TCR transgenic mice (100- and 400-fold magnification). Data from one of at least 8 independent experiments are shown.  

Similarly, no signs of colitis (data not shown) and an increase of MDSCs were seen after transfer of sorted CD8<sup>+</sup> T cells from CL4-TCR mice (Supplementary Figure 1; see supplemental material online at www.gastrojournal.org). We also determined the number of CD4<sup>+</sup> regulatory T cells, which previously have been shown to play an important role in IBD. An increase in the percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells was observed in MLN but not in spleen after one transfer, which did not further increase after 3 transfers (Supplementary Figure 2; see supplemental material online at www.gastrojournal.org). The frequency of CD8<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells also did not change significantly in MLN and spleen from mice after 3 cell transfers (data not shown). Phenotypic analysis showed similar expression of CD11a, CD44, CD62L, CD16/32, CD45, and F4/80 on MDSCs from naive mice and mice after 3 cell transfers. MDSCs isolated from mice after 3 cell transfers expressed higher levels of CD31 (Figure 3C). In addition, IL-4Ra (CD124) and Ly6C<sup>high</sup> expression was seen on MDSCs in mice after 3 transfers as markers of functionally active subpopulations (Figure 3D).
MDSCs are best characterized by their functional ability to inhibit T-cell activation and the expression of ARG. Therefore, we analyzed the inhibitory effect of MDSCs isolated from mice after 3 transfers. An increasing number of MDSCs led to reduction of T-cell proliferation (68% with 1:1 ratio) (Figure 3).

**MDSCs Are Increased Only in T Cell–Dependent Colitis**

MDSCs were analyzed in 2 other models of colitis. In a T cell–independent model (dextran sulfate sodium [DSS] treatment), the frequency of CD11b*Gr-1* cells did not change in spleen or MLN (Figure 4A). In contrast, adoptive transfer of cologenic CD4*CD45RB* T cells (T cell–dependent colitis) resulted in a marked increase in CD11b*Gr-1* cells in the spleen (Figure 4B). We next compared the cytokine profile of the small intestine, colon, MLN, and serum from VILLIN-HA mice after cell transfer and upon DSS treatment. Increased GM-CSF and IFN-γ concentrations were only detected in small intestine and colon of mice after cell transfer but not with DSS treatment (Figure 4C and D), suggesting that these cytokines can be involved in MDSC generation as it was shown for tumor-bearing mice. No significant differences were found for IL-6, IL-10, and IL-12 (Supplementary Figure 3; see supplemental material online at www.gastrojournal.org). Finally, IL-23 was detected in small intestine and colon of mice after transfer of splenocytes from CL4-TCR, further showing the relevance of the model used in this study (Supplementary Figure 3; see supplemental material online at www.gastrojournal.org).

**Figure 2.** HA-specific IFN-γ response is reduced upon repetitive transfer of antigen-specific splenocytes into VILLIN-HA mice. (A) MLN were isolated from VILLIN-HA mice 5 days after one cell transfer or 3 cell transfers and stimulated in vitro with the HA512–520 peptide. Intracellular IFN-γ responses were analyzed by fluorescence-activated cell sorting. Data are representative of 2 independent experiments with 2 mice per group. (B) In vivo proliferation of HA-specific transgenic CD8* T cells in spleen (SPL) and MLN of VILLIN-HA mice. Carboxyfluorescein succinimidyl ester–labeled splenocytes from CL4-TCR transgenic mice were adoptively transferred to naive mice and mice after 3 cell transfers. Data are derived from 2 independent experiments with 2 mice per group.

**Functional Analysis of MDSCs in Mice After Repetitive Transfer of CL4-TCR Splenocyte**

We analyzed the expression of NOS2 and ARG1 in MDSCs from VILLIN-HA mice after repetitive cell transfer. As shown in Figure 5A, NOS2 and ARG1 messenger RNA were detected in naive MDSCs and MDSCs from VILLIN-HA mice after 3 cell transfers. In an effort to further analyze the function of MDSCs, we coincubated CD11b*Gr-1* MDSCs obtained from 3-transfer VILLIN-HA mice together with HA-peptide pulsed CL4-TCR transgenic splenocytes. A total of 15 μmol/L NO was detected in the supernatants after this coincubation (Figure 5B), suggesting that MDSCs can suppress T cells through NO-mediated mechanisms. In contrast, no NO was detected when CD11b*Gr-1* or CD11b*Gr-1* cells were incubated alone. Only 2 μmol/L NO was detected in supernatant from CL4-TCR splenocytes alone (which also contain CD11b*Gr-1* cells) or in the supernatant from CL4-TCR splenocytes incubated together with CD11b*Gr-1* cells. Furthermore, functional ARG activity was shown in MDSCs (Figure 5C), showing that CD11b*Gr-1* cells in mice induced by repetitive transfer of antigen-specific T cells have a similar function and phenotype as MDSCs previously described in tumor-bearing mice. Coincubation of MDSCs with antigen-specific CD8* T cells induced NO release, which was more pronounced when MDSCs and CD8* T cells were in direct cell contact (Figure 5D, top). Furthermore, transwell experiments showed that no cell contact was needed for inhibition of proliferation of CD8* T cells (Figure 5D, bottom). Only the NO synthase inhibitor L-NAME but not the ARG inhibitor NG-hydroxy-L-arginine was able to reverse the inhibition of CD8* T-cell proliferation (Figure 5E), suggesting that NO is pivotal for MDSC-mediated suppression in our model. Finally, annexin V/7-aminoactinomycin D staining showed that CD11b*Gr-1* cells induced apoptosis in the CD8* T-cell population upon coculture (Figure 5F).
MDSCs Inhibit Antigen-Specific CD8+ T Cell–Mediated Enterocolitis in VILLIN-HA Mice

To demonstrate a direct effect of MDSCs on T cell–mediated enterocolitis, we isolated MDSCs from mice after 3 transfers and coinjected them with CL4-TCR splenocytes into naive VILLIN-HA mice after one cell transfer and 3 cell transfers. Data shown are representative of 3 independent experiments. (B) MDSCs accumulate in the intestine after 3 transfers of HA-specific splenocytes into VILLIN-HA mice. CD11b+Gr-1+ cells from intestine were identified by fluorescence-activated cell sorting. (C and D) MDSCs from naive mice and mice after 3 cell transfers were further characterized using additional surface markers. The panels are gated on CD11b+Gr-1+ cells. Filled histograms show isotype controls. Data are from one of 2 independent experiments. (E) MDSCs from VILLIN-HA mice after 3 transfers have a suppressive function on antigen-specific CD8+ T cells in vitro. MDSCs from VILLIN-HA mice after 3 transfers were incubated with HA512–520 peptide stimulated transgenic splenocytes from CL4-TCR mice. Data from one of 2 independent experiments with similar results are shown. *P < .05 and **P < .01 as determined by Student’s t test.
mice after cotransfer of MDSCs with CL4-TCR cells (Figure 6B). Analysis of the entire intestine revealed an inflammation score of 6 for VILLIN-HA mice after cotransfer, in contrast to an inflammation score of 15 for recipients, which received CL4-TCR cells alone (Figure 6C). Interestingly, depletion of CD25+ cells before cell transfer had no effect on induction of enterocolitis, with no signs of body weight change but a small decrease in the frequency of splenic MDSCs (Supplementary Figure 4; see supplemental material online at www.gastrojournal.org). In addition, transfer of CD11b+Gr-1+ cells could not prevent enterocolitis after transfer of CL4-TCR cells (data not shown).

**MDSCs With Suppressor Function Are Found in Peripheral Blood From Patients With IBD**

Having demonstrated the role of MDSCs in intestinal inflammation in mice, we next tested whether MDSCs could also be detected in patients with IBD. A few studies have described MDSCs in patients with cancer. However, human MDSCs express different markers than murine MDSCs, and until today limited data have been available on them. Recently, we have shown an increase in the frequency of CD14+CD19−HLA-DR−/low MDSCs in patients with hepatocellular carcinoma, which had the typical features of murine MDSCs. We therefore analyzed peripheral blood from patients with ulcerative colitis, patients with Crohn’s disease, and healthy controls (Table 1 and supplementary patient information; see supplemental material online at www.gastrojournal.org). There was no difference in the frequency of CD14+ cells in peripheral blood from patients with IBD and healthy controls (Figure 7A). However, we could demonstrate an increase in the frequency of CD14+HLA-DR−/low MDSCs in both patients with active ulcerative colitis (33.6% ± 4.2%, n = 18) and Crohn’s disease (27.3% ± 3.3%, n = 21) in contrast to healthy controls (3.1% ± 1.0%, n = 12) (Figure 7B). A slightly lower frequency of CD14+HLA-DR−/low cells was observed in patients with IBD in remission; however, additional studies with more patients will be needed for this comparison. Further analysis of MDSCs isolated from peripheral blood of patients with IBD and healthy controls showed similar ARG1 activity (Figure 7C). Finally, we tested the immunosuppressive function of CD14+ cells from patients with IBD and healthy controls. They were added at different ratios to anti-CD3/CD28−stimulated autologous peripheral blood mononuclear cells (PBMCs), and proliferation as well as IFN-γ release were analyzed. CD14+ cells from both healthy controls and patients with IBD suppressed the proliferation of PBMCs in a dose-dependent manner (almost by 100% suppression at CD14+ cells; PBMC ratio of 20:1) (Figure 7D).
IFN-γ release by PBMCs was more significantly suppressed by CD14+ cells derived from patients with IBD than from healthy controls (Figure 7E).

Discussion

Treatment of patients with IBD remains a major challenge in gastroenterology. Analysis of immune responses in different animal models has helped to understand the pathophysiology of this disease and develop new therapeutic strategies. Mouse studies, in which antigens expressed in enterocytes are recognized by antigen-specific CD8+ T cells, have shown that CD8+ T cells could initiate intestinal inflammation. Because IBD is characterized by chronic inflammation, we transferred repeatedly HA-specific CD8+ T cells into VILLIN-HA mice to induce chronic enterocolitis. As expected, the first transfer of cells resulted in a marked loss of body weight within 5 days after transfer as well as histologic signs of intestinal inflammation. Unexpectedly, after a second or third transfer of CD8+ T cells, mice did not show any significant loss in body weight or signs of severe intestinal inflammation.
Different immunoregulatory mechanisms have been shown in IBD, including CD4+ and CD8+ regulatory T cells. In our previous study, we showed an increase in the number of CD8+FoxP3+ regulatory T cells in VILLIN-HA CL4-TCR transgenic T cells in acute enterocolitis; however, our present study shows a lower increase of CD8+ regulatory T cells than MDSCs after repetitive transfer. Most importantly, cotransfer of MDSCs together with CL4-TCR transgenic T cells impaired inflammation in VILLIN-HA mice, clearly showing that MDSCs could suppress HA-specific CD8+ T cells, while depletion of CD25+ regulatory T cells had no effect on the induction of enterocolitis.

Further evidence showing the significance of MDSCs in enterocolitis comes from studies performed on LysMcre/Stat3flox−/− mice. These mice have a targeted knockout of the Stat3 gene in macrophages and neutrophils and as recently shown in MDSCs. These mice develop spontaneous T cell–mediated colitis, which is aborted if they are crossed with RAG knockout mice. These data indicate that targeted disruption of Stat3 in MDSCs leads to uncontrolled T-cell activation and triggers intestinal inflammation, clearly showing the importance of MDSCs in T-cell regulation.

Dramatic expansion of the Gr-1+CD11b+ cells in response to intestinal inflammation is very similar to those previously described in mice with actively growing tumors. It has been shown that GM-CSF–expressing tumors support MDSC generation together with other cytokines. It is also known that GM-CSF is secreted by CD4+ T cells and Paneth cells of the intestinal epithelium. Therefore, increased concentrations of this cytokine, seen in intestine after 3 transfers but not with DSS treatment, might be an important factor for the expansion of MDSCs in colitis. Interestingly, GM-CSF treatment has been shown to decrease disease severity and improve quality of life in a randomized placebo-controlled trial for patients with Crohn’s disease through a yet unknown mechanism. Therefore, we suggest that future studies should include analysis of MDSCs in GM-CSF–treated patients with Crohn’s disease.

A number of different mechanisms have been described as to how MDSCs exert their inhibitory functions, ranging from direct cell-cell contact to modification of the microenvironment. MDSCs freshly isolated from the spleens of tumor-bearing mice were originally shown to suppress the functional activity of CD8+ T cells through the generation of reactive oxygen species. In a recent study, it was shown that T-cell tolerance can be induced through reactive oxygen species and peroxynitrite nitration of the TCR-CD8 complex, which aborts antigen recognition and IFN-γ response of affected CD8+ T cells. At this point our studies do not clearly

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NOTE. Detailed patient information is available as supplementary material (see supplemental material online at www.gastrojournal.org).
show the exact mechanism as to how MDSCs exert their function in this model. However, our in vitro results indicate a potential role of NO, which is generated after incubation of MDSCs with antigen-stimulated T cells. Further studies are needed to fully investigate the in vivo function of MDSCs in mice with colitis.

So far, a number of reports describe MDSCs in patients with different types of tumors such as non-small cell lung, head and neck cancer, breast, and renal carcinoma. We have recently identified a subset of CD14+ or CD14+ HLA-DR–low cells in patients with hepatocellular carcinoma. These cells were unable to stimulate an

**Figure 7.** Patients with IBD demonstrate higher frequency of MDSCs in peripheral blood. (A) Frequency of CD14+ cells in peripheral blood from patients with ulcerative colitis, patients with Crohn’s disease, and healthy controls. (B) MDSCs were detected by CD14/HLA-DR staining as described. *P < .05 as shown by Student t test. (C) CD14+ cells from patients with IBD and healthy donors demonstrate ARG activity. ARG activity was determined from isolated CD14+ cells as described. Data shown are from healthy donors (n = 3) and patients with IBD (n = 3). (D) Purified MDSCs from patients with IBD suppress proliferation and cytokine secretion by autologous-stimulated PBMCs cells in a dose-dependent manner. The CD14+ population was sorted from PBMCs of patients with IBD or healthy donors and tested with autologous PBMCs in a suppression assay. Data are derived from 3 patients and 2 healthy donors. Proliferation was determined in duplicate cultures by 3H incorporation. **P < .01 and ***P < .001 versus control as determined by Student t test. (E) Supernatants from the proliferation assay were removed after 48 hours and measured for IFN-γ production by enzyme-linked immunosorbent assay. Data are derived from patients with IBD (n = 3) and healthy donors (n = 2). *P < .05, **P < .01, and ***P < .001 versus control as determined by Student t test.
allogeneic T-cell response, suppressed autologous T-cell proliferation, and had high ARG activity, a hallmark characteristic of MDSCs. This allowed us to investigate for the first time the role of MDSCs in patients with IBD, where we have found an increase in the frequency of these cells during active disease. However, more specific markers are needed to characterize and examine their function in detail. Finally, because MDSCs impair inflammation in our enterocolitis model but have also been shown to suppress antitumor immune responses, it will be important to understand their role in the context of inflammation-induced carcinogenesis.

Our mouse studies have shown an increase in the frequency of MDSCs, which protect mice from the development of severe enterocolitis. In contrast, we have observed an increase in the frequency of MDSCs in the peripheral blood from patients with active chronic colitis. We propose that active colitis is causing an increase in the frequency of immunosuppressive MDSCs, thereby stopping the development of a more severe and possibly fatal inflammation. Therefore, such immunosuppressive MDSCs could potentially become a therapeutic option for patients with active chronic IBD.

In summary, our results show that the frequency of Gr-1$^-$/CD11b$^+$ MDSCs increases dramatically during intestinal inflammation in mice and these cells suppress IFN-γ release by T cells. Cotransfer of transgenic T cells with MDSCs ameliorated intestinal inflammation. Analysis of peripheral blood from patients with IBD also revealed an increase in the frequency of MDSC-like cells with a suppressor activity. Therefore, these studies are not only the first to show that repetitive transfer of antigen-specific T cells in mice induces amplification of MDSCs with immunosuppressive function, but also that MDSCs play an important role in T cell–dependent mouse models of intestinal inflammation and most importantly in patients with IBD. Further studies are needed to investigate the role of MDSCs in the pathogenesis of IBD as well as to evaluate whether these cells might become a therapeutic option for patients with IBD.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.06.032.

References

Supplementary Materials and Methods

Mice
BALB/c mice (6–8 weeks old) were obtained from Charles River Laboratories (Charles River, Sulzfeld, Germany). All experiments were performed according to the institutional guidelines.

Histologic Grading of Intestinal Sections
Histologic grading was as follows. Epithelium: 0, normal morphology; 1, loss of goblet cells and <10% hyperproliferation; 2, 10%–50% loss of goblet cells; 3, 50%–90% loss of goblet cells; 4, complete loss of crypts and epithelium intact; 5, small and medium size (<10 crypts) ulcer; 6, big ulcer (>10 crypts). Infiltration of mucosa: 0, no infiltrate; 1, slight infiltration; 2, moderate infiltration; 3, severe infiltration. Infiltration of submucosa: 0, no infiltrate; 1, slight and moderate infiltration; 2, severe infiltration. Infiltration of muscularis: 0, no infiltrate; 1, slight and moderate infiltration; 2, severe infiltration. Infiltration of lamina propria: 0, no infiltrate; 1, slight infiltration; 2, moderate infiltration; 3, severe infiltration. Infiltration of muscularis mucosa: 0, no infiltrate; 1, slight infiltration; 2, moderate infiltration; 3, severe infiltration. Infiltration of submucosa: 0, no infiltrate; 1, slight and moderate infiltration; 2, severe infiltration. Infiltration of muscularis: 0, no infiltrate; 1, slight and moderate infiltration; 2, severe infiltration. The total histologic score represents the sum of the epithelium and each infiltration score for the duodenum, jejunum, ileum, cecum, and colon and thus ranges from 0 to 60.

T-Cell Transfer Model Using CD45RB^{high} Cells
A total of 5 × 10^5 fluorescence-activated cell sorted CD4^{+}CD45RB^{high} cells (purity >90%) were injected intraperitoneally into SCID mice. Mice were followed up after transfer and killed when they develop wasting disease and/or diarrhea as determined by body weight loss.

Flow Cytometry
Flow cytometry was performed on a Becton Dickinson FACS Calibur using CellQuest software (Becton Dickinson, Heidelberg, Germany). Antibodies included anti-mouse CD4 (GK 1.5), CD8 (53-6.7), CD11a (M17/4), CD11b (M1/70.15), CD16/32 (93), CD31 (390), CD44 (1M7), CD45 (30-F11), CD44 (IM7), CD62L (MEL-14), CD124 (hIL4R-M57), Gr-1 (RB6-8C5), F4/80 (BM8), Ly6C (1G7.G10) and IFN-γ (XMG1.2), Foxp3 (FJK-16s) and anti-human CD14 (TU4), CD19 (SJ25-C1), and HLA-DR (HL-39). Annexin V and 7-aminoactinomycin D staining was performed to detect apoptotic T cells in vitro cultures. Data analysis was performed using FlowJo software (Tree Star Inc, Ashland, OR). Isotype-matched antibodies were used with all the samples as controls. Gr-1^{+}CD11b^{+} cells were purified from naive or 3-transfer VILLIN-HA mice spleens using the BD FACS Aria cell sorting system (Becton Dickinson). The purity of the MDSCs was shown to be ≥95%.

Quantitation of MDSCs in the Intestine.
The intestine was opened longitudinally and rinsed with cold phosphate-buffered saline. The mucosa was scraped off and treated with 1 mmol/L dithiothreitol at 37°C in RPMI medium (GIBCO-Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum. The cells were then resuspended in buffer and stained with the appropriate antibodies.

In Vivo Proliferation Assay
Splenocytes from CL4-TCR mice were labeled with carboxyfluorescein succinimidyl ester (Molecular Probes, Göttingen, Germany) at 37°C. The reaction was stopped by addition of 25 mL fetal calf serum. After washing, VILLIN-HA mice were injected with a total of 1 × 10^7 cells intravenously. After 5 days, percentages of dividing cells in the spleen and MLN were identified by fluorescence-activated cell sorter analysis.

Intracellular Staining for IFN-γ
Single-cell suspensions were obtained by desegregation of MLN. A total of 1 × 10^6 cells were restimulated with HA512–520 peptide. Intracellular cytokine analysis of IFN-γ was performed using a kit from BD Pharmingen (Heidelberg, Germany). The percentage of IFN-γ-secreting CD8^{+} T cells was determined after gating on carboxyfluorescein succinimidyl ester-positive cells.

Cytokine Analysis
Small pieces of colon and small intestine were cut, rinsed in phosphate-buffered saline, and weighed. Small tissue pieces were cultured for 48 hours in 24-well tissue culture plates (Greiner Bio-one, Frickenhausen, Germany) in 400 μL complete RPMI 1640 at 37°C and 5% CO2. After centrifugation at 10,000g to pellet the debris, culture supernatants were transferred to fresh tubes and stored at -20°C. Supernatant was checked by enzyme-linked immunosorbent assay for GM-CSF (eBioscience, San Diego, CA) and IFN-γ (R&D Systems, Wiesbaden, Germany), IL-6, IL-12 (p40), and IL-10 (BioLegend, San Diego, CA) according to the manufacturer’s instructions. The cytokine levels were normalized to weight of small intestine or colon.

Suppression Assays
Fluorescence-activated cell sorted Gr-1^{+}CD11b^{+} MDSCs were coincubated in different ratios with CL-4 TCR splenocytes. For human samples, CD14^{+} cells were purified as described previously. A total of 10^6 PBMCs were stimulated using a T-cell activation and expansion kit (Miltenyi, Bergisch Gladbach, Germany) and incubated with CD14^{+} cells at different ratios. Nω-monomethyl-L-arginine (Sigma) and Nω-hydroxy-L-arginine (Sigma) were used as NOS and ARG enzyme inhibitor, respectively. [3H]Thymidine (Amersham, Freiburg, Germany) was added to the cultures and proliferation was measured after 72 hours by [3H] incorporation. Radioactivity was measured using a scintillation counter (Wallac, Turka, Finland).
Determination of ARG Activity

The ARG activity of MDSCs was determined as described previously.1 Briefly, cells were lysed with 100 μL of lysis buffer (0.1% Triton X-100 plus one tablet of protease inhibitor mixture; Roche, Basel, Switzerland). After 30 minutes at 37°C, 100 μL of 25 mmol/L Tris-HCl and 10 μL of 10 mmol/L MnCl₂ were added. The ARG was activated by heating for 10 minutes at 56°C. The lysate was incubated with 100 μL of 0.5 mol/L l-arginine (pH 9.7) for 1 hour at 37°C. The reaction was stopped by the addition of 800 μL stop solution (96% H₂SO₄/85% H₃PO₄/H₂O, ratio 1:3:7). A total of 40 μL α-isonitroso-propiophenone (dissolved in 100% ethanol) was added and heated at 100°C for 1 hour. A standard curve consisting of serial dilutions of urea was run in parallel. The urea concentration was measured at 540 nm.

Messenger RNA Expression Analysis

MDSCs were isolated from the spleen of naive and 3-transfer VILLIN-HA mice. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized using Superscript II First-Strand Synthesis System (Invitrogen, Karlsruhe, Germany). NOS2, ARG1, and glyceraldehyde-3-phosphate dehydrogenase messenger RNA expression was visualized by electrophoresis through 1% agarose gel. The primers used were as follows: NOS2 forward 5′-CCTTGTTCAAGCCTTCC-3′, NOS2 reverse 5′-CCAAAGTTGTTGTCACCAC-3′, ARG 1 forward 5′-AGGCTGTGGATAAA-3′, ARG 1 reverse 5′-ACAGACCGTGGTCTTTCACC-3′, glyceraldehyde-3-phosphate forward 5′-CCTGCACCCACCAACTGCTTA-3′, glyceraldehyde-3-phosphate reverse 5′-TCATGAGCCCTTCCACAATG-3′.

NO Detection

Purified CD11b⁺Gr-1⁺ cells were cultured with HA-pulsed CL4-TCR splenocytes for 48 hours. The production of NO was determined from the culture supernatant using iT High-Sensitivity Nitrite Assay Kit according to the manufacturer’s protocol (Molecular Probes, Eugene, OR).

Cell Isolation and Sorting of Human PBMCs

PBMCs were isolated from freshly obtained blood by Ficoll density gradient (Biochrom, Berlin, Germany). For isolation of CD14⁺ monocytes, PBMCs were further separated using CD14 Microbeads (Miltenyi Biotech) and AutoMACS separation unit (Miltenyi Biotech) according to the manufacturer’s instructions. Purified monocytes (>98%) were used for further assays.

Enzyme-Linked Immunosorbent Assay

Culture supernatant from the suppression assays was removed after 48 hours and tested for IFN-γ (ImmunoTools, Friesoythe, Germany) production according to the manufacturer’s instructions.

Statistical Analysis

Data are expressed as mean ± SD. Statistical analysis was performed using Student’s t test to assess differences between the different study groups. P < .05 was considered statistically significant.

Reference

Supplementary Figure 1. Adoptive transfer of purified CD8+ T cells from Cl-4 TCR mice induce an increase in frequency of CD11b+ Gr-1+ cells in the spleen. CD8+ T cells from CL-4 TCR transgenic mice were adoptively transferred three times (2x10^5 cells on day 0, 1x10^6 cells on day 12 and 27) into VILLIN-HA mice. Five days after last transfer, all mice were sacrificed and splenocytes were stained for Gr-1 and CD11b and analyzed with flow cytometry. * P < .05 versus naive control.

Supplementary Figure 2. The percentage of CD4+Foxp3+ regulatory T cells is increased in mesenteric lymph nodes but not in spleen after one transfer, however, three transfers did not further increase the frequency of CD4+ cells. CD4+Foxp3+ Cells were analyzed by flow cytometry in the spleen and MLN from either naive mice or after 1 and three transfers of CL-4 TCR spleocytes into VILLIN-HA mice. Two mice are analyzed individually in two independent experiments.
Supplementary Figure 3. No significant differences were found for IL-6, IL-10 and IL-12 after DSS treatment and CD8⁺ T cell transfer. (A) Colitis is induced in VILLIN-HA mice by the addition of DSS in drinking water on days 0,15, 27. Mice were treated with DSS in drinking water or adoptively transferred with CL-4 TCR splenocytes. Intestine was put into culture in complete media and supernatants were collected from 48 hours culture of the colon, small intestine and MLN as well, as serum and checked by ELISA for IL-6, IL-10, IL-23 (B, C). The cytokine levels were normalized to weight of small intestine or colon.

Supplementary Figure 4. Depletion of regulatory T cells does not reverse the attenuated intestinal inflammation observed after third transfer. Mice were depleted of regulatory T cells starting at 5 days before the third transfer and antigen-specific splenocytes from CL-4 TCR transgenic mice were transferred into the VILLIN-HA mice on day 27. (A) Mice were monitored for signs of colitis and the body weight was monitored. (B) Five days after cell transfer all mice were sacrificed and the frequency of splenic MDSCs was determined.
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M, male; F, female; UC, ulcerative colitis; CD, Crohn’s disease; A, active disease; R, disease in remission; %, no treatment.