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The Role of Gr1+ Cells after Anti-CD20 Treatment in Type 1 Diabetes in Nonobese Diabetic Mice

Changyun Hu,* Wei Du,* Xiaojun Zhang,* F. Susan Wong,† and Li Wen*

Studies suggest that Gr1+CD11b+ cells have immunoregulatory function, and these cells may play an important role in autoimmune diseases. In this study, we investigated the regulatory role of Gr1+CD11b+ cells in protecting against type 1 diabetes in NOD mice. In this study, we showed that temporary B cell depletion induced the expansion of Gr1+CD11b+ cells. Gr1+CD11b+ cells not only directly suppress diabetogenic T cell function but also can induce regulatory T cell differentiation in a TGF-β-dependent manner. Furthermore, we found that Gr1+CD11b+ cells could suppress diabetogenic CD4 and CD8 T cell function in an IL-10–, NO–, and cell contact-dependent manner. Interestingly, single anti-Gr1 mAb treatment can also induce a transient expansion of Gr1+CD11b+ cells that delayed diabetes development in NOD mice. Our data suggest that Gr1+CD11b+ cells contribute to the establishment of immune tolerance to pancreatic islet autoimmunity. Manipulation of Gr1+CD11b+ cells could be considered as a novel immunotherapy for the prevention of type 1 diabetes.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells that are Gr1+CD11b+ (1). Gr1+CD11b+ cells, as part of a myeloid macropopulation, make up at least two subsets of polymorphonuclear and monocytic cells with different immunosuppressive properties (2). They have been studied in tumor immunology (3) and other diseases such as graft-versus-host disease (4), sepsis, and trauma (5). Recently, the immunosuppressive function of Gr1+CD11b+ cells has also been recognized in autoimmune diseases (6–10). In experimental induced organ-specific autoimmune disease, Gr1+CD11b+ cells can be found in the spleen and in target organs, and they may play a role in limiting the T cell response to autoantigens in the target tissue (8). CD11b+Ly-6Chigh cells induced during EAE priming are powerful suppressors of activated T cells (6). When B10.RIII mice are immunized to induce experimental autoimmune uveoretinitis, Gr1+CD11b+ cells accumulate in large numbers at the peak of disease (9). Iwata et al. (10) reported the involvement of Gr1+CD11b+ cells in the autoimmune disorder in MRL-Fas+ mice via the regulation of CCL2/CCR2 signaling. In skin transplantation models, adoptive transfer of Gr1+CD11b+ cells and M-CSF–induced Gr1+CD11b+ cells can prolong allogeneic graft survival (11, 12). Transplantation tolerance induced by anti-CD28 treatment was associated with the accumulation of Gr1+CD11b+ cells in rat kidney allografts (13). Mobilization of bone marrow CD11b+CD115+Gr1+ monocytes could lead to indefinite cardiac allograft survival (14).

In an allogeneic islet transplantation model, adoptive transfer of bone marrow–derived Gr1+CD11b+ cells protected recipients from recurrent diabetes (15). Using tumor-derived MDSCs, Yin et al. (16) showed that CD115+Gr1+ MDSCs efficiently prevented the onset of hemagglutinin-specific T cell–induced diabetes in INS- HA/RAG−/− recipient mice. Furthermore, in a spontaneous diabetes model, adoptive transfer of Gr1+CD11b+ cells, generated using GM-CSF– and TGF-β–stimulated bone marrow cells from transgenic mice expressing proinsulin driven by the class II promoter, protected against diabetes in NOD mouse (17). However, whether the expansion of endogenous Gr1+CD11b+ cells by mAb treatment can control pancreatic islet-specific autoimmunity and induce immune tolerance is not known. This is of interest because we found that temporary B cell depletion induced regulatory T cells (Tregs) and regulatory B cells in the human (h)CD20/NOD mouse model (18). Moreover, in the current study, we found that B cell depletion also expanded a subset of Gr1+CD11b+ cells with characteristics of MDSCs. We have further investigated the role of Gr1+CD11b+ cells in β cell autoimmune tolerance in spontaneous diabetes. We found that Gr1+CD11b+ cells prevented type 1 diabetes (T1D) in NOD mice through multiple immune tolerance pathways.

Materials and Methods

Mice

The NOD/Caj mice have been maintained at Yale University (New Haven, CT) for >20 y. All the mice were kept in specific pathogen-free conditions in a 12-h dark/light cycle and housed in individually ventilated filter cages with autoclaved food. hCD20-transgenic NOD (hCD20/NOD) mice were generated as described previously (18). The use of the animals in this study was approved by the Yale University Institutional Animal Care and Use Committee.

Abs and reagents

All fluorochrome-conjugated mAbs used in this study were purchased from eBioscience. All the hybridoma supernatants containing different mAbs were generously provided by the late Charles Janeway (Yale University). Affinity-purified anti-hCD20 mAb 2H7 was prepared as described previously (18). Anti-Gr1 mAb (clone RB6-8C5) that binds particularly to mouse CD11b+Ly-6Chigh cells isotype control 28–8–16–13 and F.11). The Journal of Immunology, 2012, 188: 294–301.

The online version of this article contains supplemental material.
X Cell. Control mouse or rat IgG used in the in vivo studies was purchased from Rockland.

B cell depletion

Temporary B cell depletion in hCD20/NOD mice, using anti-human CD20 mAb (clone 2H7), was performed as described previously (18). Briefly, 9-wk-old hCD20/NOD mice were injected i.v. with 2H7 or control IgG at 0.5 mg/mouse for the first injection (day 0), followed by three injections of 0.25 mg/mouse at 3-d intervals.

Anti-Gr1 treatment and its effect on spontaneous diabetes development

Prediabetic female NOD mice (9 wk old) were treated with a single dose of anti-Gr1 mAb (0.25 mg/mouse) i.v. A group of age- and sex-matched mice were treated with rat IgG as controls. All the treated mice were observed for diabetes development up to 35 wk. They were screened for glycosuria twice a week. Diabetes was confirmed by blood glucose measurement >250 mg/dl (13.9 mmol/l).

T cell purification

T cells were isolated using MACS cell isolation kits following the manufacturer’s instructions (Miltenyi Biotec). In some experiments, T cells were also isolated by negative selection to remove B cells (anti-mouse Igs) and macrophages (clone F4/80) with magnetic beads. CD4+ T cells were purified by removing CD8+ T cells (clone TIB 105), B cells (anti-mouse Igs), and MHC class II+ cells (clone 10.2.16) using mAbs and magnetic beads. CD8+ T cells were purified by removing CD4+ T cells (clone GK1.5), B cells, and MHC class II+ cells. The purity of the cells was routinely >90%, analyzed by FACS.

T cell proliferation and inhibition assays

The following T cells were used in the assays: purified BDC2.5 TCR-transgenic CD4 or NY8.3 TCR-transgenic CD8 T cells or insulin-reactive CD8 T cell clone 6426. The cells (1 × 10^6/well) were cocultured with irradiated peritoneal cells (2 × 10^5/well), as APCs, in the presence of BDC2.5 mimotope (19), IGRP peptide (20), or insulin B15-23 peptide (21), respectively, at 37°C, 5% CO2, for 4 d. [3H]thymidine was added during the last 16 h of culture. To study the suppressive function of Gr1+CD11b+ cells, FACS-sorted Gr1+CD11b+ cells (2 × 10^5/well) from 2H7 and/or control IgG-treated mice were added to the culture. To study the role of soluble immune regulatory molecules, such as IL-10 and NO, we performed in vitro cultures as described above in the presence or absence of anti–IL-10 (clone JESS-2A5), anti–IL-10R (clone 1B1.3A), and the NO synthase inhibitor L-NAME (Cayman Chemical), respectively. To test whether the suppressive function was cell contact dependent, we performed the assays with Gr1+CD11b+ cells in a 0.2-μm Transwell culture system. All the assays were performed in triplicate for each of the culture conditions.

We also used CFSE dilution assay to study the inhibitory function of Gr1+CD11b+ cells in vivo by transferring CFSE-labeled BDC2.5 CD4 or NY8.3 CD8 T cells into NOD recipients treated with anti-Gr1 or control IgG 1 wk earlier.

Treg induction in the presence of Gr1+CD11b+ cells

FACS-sorted naive CD4 T cells (CD4+CD25−CD44−/−CD62L−) from NOD mice were stimulated with anti-CD3 (2C11 hybridoma supernatant, 1/200) and anti-CD28 (37.51 hybridoma supernatant, 1/300) and cocultured with purified Gr1+CD11b+ cells from anti-CD20 and/or control IgG-treated mice at a ratio of 5:1 in the presence of mouse M-CSF (50 ng/ml; PeproTech) at 37°C and 5% CO2. After 5 d, cells were collected for quantitative PCR (qPCR) analysis of Foxp3 mRNA expression (5′-primer, CAGAGTATGACGTGAGAGACCAC; 3′-primer, CAGGTTGTCATCTTGAGGGAA; 3′-primer, CAGCTTGTCTACTT-CAGTCAATGGAG) and intracellular staining of Foxp3 protein expression using an anti-mouse/rat Foxp3 staining kit from eBioscience.

Histopathology and insulin score

Pancreata were fixed in 10% buffered formalin and then paraffin embedded. Tissues were sectioned and stained with H&E. Insulitis was scored under light microscopy using the following grading: 0, no insulitis; 1, insulitis affecting <25% of the islet; 2, insulitis affecting 25–75% of the islet; and 3, >75% islet infiltration. A total of 107 islets from the control group (mice, n = 5) and 124 islets from the anti-Gr1–treated group (mice, n = 5) was scored.

Statistics

Statistical analysis was performed using GraphPad Prism software.

Results

Expansion of Gr1+CD11b+ cells that express IL-4 and IL-10 after B cell depletion

We have previously shown that temporary B cell depletion by anti-human CD20 mAb 2H7 prevented and reversed T1D in hCD20/NOD mice (18). To further characterize the mechanisms of immune tolerance induced by anti-CD20 treatment, we performed intracellular immunoregulatory cytokine staining for IL-4 and IL-10. Surprisingly, we found that B cell depletion expanded a population of IL-4- and IL-10–producing cells that were Gr1+ (Fig. 1A). These cells were also CD11b+ (Fig. 1B). The expansion of Gr1+CD11b+ cells was seen only in mice treated with 2H7 but not in IgG-treated control mice (Fig. 1B, 1C).

![FIGURE 1. B cell depletion induced regulatory Gr1+ cells that express IL-4 and IL-10. A. Splenocytes from anti-CD20 (2H7) or control IgG-treated hCD20/NOD mice (n = 5/group) were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for 5 h in the presence of GolgiStop (BD Biosciences) and then stained with mAbs against the surface markers CD4, CD11b, and Gr1, followed by intracellular staining with anti–IL-4 and anti–IL-10 or isotype control Ab. One representative set of FACS plots is shown in A with gating on CD4+ cells. B. Splenocytes from mice treated with 2H7 or control mouse IgG were stained with mAbs against the surface markers Gr1 and CD11b. C. Gr1+CD11b+ cell number was enumerated in splenocytes from 2H7 or control mouse IgG-treated mice (n = 4/group, Student t test, *p = 0.011).](http://www.jimmunol.org/)

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Gr1+CD11b+ cells suppress T cell function in an IL-10–dependent manner

To test whether Gr1+CD11b+ cells could suppress T cell function directly in our system, we cocultured purified diabetogenic CD4 or CD8 T cells with purified Gr1+CD11b+ cells from 2H7-treated hCD20/NOD mice (18) in the presence or absence of antigenic peptides. Interestingly, Gr1+CD11b+ cells suppressed both diabetogenic CD4 and CD8 T cell proliferation with a greater effect on CD8 T cells (Fig. 2A). The suppressive function was not restricted to anti-CD20 treatment because Gr1+CD11b+ cells from control IgG-treated mice also showed similar suppression to the diabetogenic CD4 or CD8 T cells (Fig. 2D, 2E). This suggests that B cell depletion induces the expansion of a subset of pre-existing Gr1+CD11b+ cells with immunosuppressive function in hCD20/NOD mice. To further test the suppressive function of Gr1+CD11b+ cells in vivo, we adoptively transferred CFSE-labeled purified BDC2.5 TCR transgenic CD4+ T cells or purified NY8.3 TCR transgenic CD8+ T cells into NOD recipients that were pretreated with anti-Gr1 Ab (which would lead to the expansion of Gr1+CD11b+ cells, see below) or control IgG. Consistently, the proliferation of both BDC2.5 and NY8.3 T cells, which was indicated by CFSE dilution, was suppressed in the anti-Gr1–treated recipients, compared with the IgG-treated mice (Fig. 2B).

We had shown earlier that Gr1+CD11b+ cells express the regulatory cytokine IL-10. To test whether IL-10 was responsible for the suppressive function of Gr1+CD11b+ cells, we cultured purified BDC2.5 CD4+ T cells or NY8.3 CD8+ T cells with antigenic peptides together with purified Gr1+CD11b+ cells from 2H7-treated hCD20/NOD mice in the presence or absence of anti–IL-10 blocking mAb (clone JESS-2A5). As shown in Fig. 2C, the addition of anti–IL-10 Ab partially restored the proliferation of T cells. We also tested the role of IL-4 in the same culture system. However, the addition of IL-4 blocking Ab (clone 11B11) could not reverse the suppression (data not shown), although Gr1+CD11b+ cells also expressed IL-4. Similar results were also observed when using purified Gr1+CD11b+ cells from control IgG-treated hCD20/NOD mice (data not shown).

**FIGURE 2.** Gr1+CD11b+ cells suppressed diabetogenic T cell proliferation in an IL-10–dependent manner. A, In MLCs, purified Gr1+CD11b+ cells (2×10^4/well) suppressed the proliferation of BDC2.5 CD4 T cells (10^5/well) designated as CD4 or insulin-reactive CD8 T cell clone 6426 (10^5/well), designated as CD8. One representative data set is shown from at least three experiments. B, CFSE-labeled BDC2.5 CD4 or NY8.3 CD8 T cells were adoptively transferred into NOD recipients (6×10^6/mouse). NOD recipients were treated with 0.25 mg/mouse anti-Gr1 (gray area) or control IgG (dark line) mAb at the time of T cell transfer. Cells from pancreatic lymph nodes were collected 7 d later for FACS analysis of CFSE dilution (n = 5/group). C, The addition of neutralizing anti–IL-10 mAb (clone JESS-2A5; 10 μg/ml) partially reversed the suppression by Gr1+CD11b+ cells of BDC2.5 CD4 or insulin-reactive CD8 T cell clone 6426 proliferation. One representative data set is shown from at least three experiments. Gr1+CD11b+ cells (2×10^4/well) purified from both 2H7 and control IgG-treated mice showed comparable suppression of the proliferation of diabetogenic BDC2.5 CD4+ (10^5/well) (D) and NY8.3 CD8+ T cells (10^5/well) (E). One representative data set is shown from three experiments. The baseline proliferation, measured by [3H]thymidine incorporation, for all assays was between 100 and 800 cpm. Error bars represent SEM. *p < 0.05, **p < 0.001, ***p < 0.0001
The suppressive function of Gr1^CD11b^ cells is NO and cell contact dependent

Previous studies have shown the involvement of NO in the suppressive function of myeloid-derived suppressor cells (22). To investigate whether NO plays a role in T cell suppression by Gr1^CD11b^ cells in our model system, we performed in vitro T cell culture, as described above, in the presence or absence of the pan-NO synthase inhibitor L-NAME. Our results showed that in the presence of L-NAME, the suppressive function of Gr1^CD11b^ cells was reversed for CD4 T cells and partially reversed for CD8 T cells (Fig. 3A). To test whether cell contact was required for suppression of T cells in our system, we placed Gr1^CD11b^ cells in a 0.2-μm Transwell in the above T cell culture system. The presence of the Transwell reduced the suppressive function of Gr1^CD11b^ cells (Fig. 3B). The NO- and cell contact-dependent suppression were also detected using Gr1^CD11b^ cells that were isolated from control IgG-treated hCD20/NOD mice (data not shown).

Our results, thus far, suggest that B cell depletion induces the expansion of a subset of pre-existing Gr1^CD11b^ cells, which have characteristics of myeloid-derived suppressor cells, rather than modulating their function.

Anti-Gr1 treatment prevents diabetes development in NOD mice through transient expansion of Gr1^CD11b^ cells

Because B cell depletion induced Gr1^CD11b^ cells that could contribute to the delay and reduction of diabetes onset in hCD20/ NOD mice, we expected that removal of Gr1^CD11b^ cells would accelerate diabetes development in NOD mice. To test this hypothesis, we injected (i.v.) a group of prediabetic NOD mice at 9 wk of age with 0.25 mg depleting anti-Gr1 mAb (clone RB6-8C5). To our surprise, instead of accelerating the disease, we found that anti-Gr1 treatment significantly delayed and prevented diabetes development compared with the control rat IgG-treated mice (p < 0.05; Fig. 4A). To test whether anti-Gr1 treatment affected the cellular infiltrate of pancreatic islets, some mice were randomly...
selected and sacrificed 1 mo after treatment for insulitis analysis. There was significantly less infiltration in mice treated with anti-Gr1 compared with the control rat IgG-treated group as shown in Fig. 4B and 4C (p < 0.03).

To examine the efficiency of Gr1+CD11b+ cell depletion, we injected anti-Gr1 Ab (0.25 mg/mouse) i.v. in an additional group of NOD mice and analyzed the Gr1+CD11b+ cells in peripheral blood over time using a different anti-Gr1 (clone 1A8, which recognizes a different epitope of Ly6G) and anti-CD11b Abs. As shown in Fig. 5A, 1 h after the RB6-8C5 treatment, we observed a significant decrease in the number of Gr1+CD11b+ cells. The depletion of Gr1+CD11b+ cells was maintained for 4 d, at which time Gr1+CD11b+ cells gradually regenerated and increased to a higher level compared with the IgG-treated control mice 7 d after treatment. This higher level of Gr1+CD11b+ cells persisted for ~10 d; the cell frequency and number were restored to normal by 18 d after anti-Gr1 treatment. The depletion dynamics demonstrated that anti-Gr1 treatment resulted in a short period of removal of Gr1+CD11b+ cells but was followed by a longer period of transient increase of Gr1+CD11b+ cells, which possibly explains the observed diabetes protection shown in Fig. 4. We also observed expansion of Gr1+CD11b+ cells in spleen and lymph nodes from different anatomical sites 7 d after anti-Gr1 treatment (Fig. 5B). Our results support the finding by Bronte and colleagues (1), who showed that the discontinuation of anti-Gr1 treatment in a tumor model system resulted in increased numbers of myeloid-derived suppressor cells.

To confirm that the expansion of Gr1+CD11b+ cells was responsible for diabetes protection by a single anti-Gr1 Ab injection, we tried to deplete Gr1+CD11b+ cells by multiple anti-Gr1 injections. We treated 9-wk-old female NOD mice with four weekly injections of anti-Gr1 Ab (0.25 mg/mouse i.p.), such that reconstitution of the Gr1+CD11b+ cells was prevented during a critical period of diabetes protection. It is interesting that the continued depletion of Gr1+CD11b+ cells and more importantly, inhibition of the expansion of these cells over a longer period of time, abrogated diabetes protection (Fig. 6). The data further support the notion that diabetes protection was mediated by the expansion of Gr1+CD11b+ cells.

**TGF-β is required for the induction of Foxp3+ Tregs by anti-Gr1 treatment**

To test the hypothesis that anti-Gr1 treatment could promote activity of other regulatory cells, including Tregs, we analyzed Foxp3+ Tregs in NOD mice 1 mo after a single anti-Gr1 or rat IgG injection. It is interesting that we found a consistent increase in Foxp3+ Tregs in the anti-Gr1 Ab-treated mice compared with IgG-treated control mice, which suggested that anti-Gr1 treatment-induced Tregs (Fig. 7A). The increase of CD4+Foxp3+ Tregs...
was more evident at later time points, seen at 35 wk of age, when the experiments were terminated (Fig. 7B). We further tested whether Gr1+CD11b+ cells could induce Foxp3+ cells in vitro. We sorted naive splenic CD4 T cells and Gr1+CD11b+ cells by FACS and cocultured them for 5 d. Foxp3+ cells were then analyzed by intracellular staining. We found that a significant number of naive T cells differentiated into Foxp3+ cells when they were cocultured in the presence of Gr1+CD11b+ cells (Fig. 7C). The expression of the Foxp3 gene was further confirmed at transcriptional level by qPCR (Fig. 7D). It is known that TGF-β is essential for Treg differentiation. To understand whether TGF-β was involved in Treg induction by Gr1+CD11b+ cells, we measured the TGF-β level in the supernatant from the above coculture experiments by ELISA and found an increased level of bioactive TGF-β in supernatants of cocultured T cells and Gr1+CD11b+ cells (Fig. 8A). The upregulation of TGF-β was also confirmed at transcriptional level by qPCR (Fig. 8B). To test the role of TGF-β in the induction of Foxp3 expression, we added neutralizing anti–TGF-β mAb (clone 1D11) to the coculture system. The presence of neutralizing anti–TGF-β mAb completely abrogated the induction of Foxp3 expression (Fig. 8C). To investigate the effect on Treg induction in vivo, we treated groups of NOD mice with anti-Gr1 Ab (single injection) with or without neutralizing anti–TGF-β Ab. We found that anti–TGF-β–treated mice had fewer Foxp3+ Tregs in the spleen compared with control mice treated with anti-Gr1 alone (Fig. 8D). Furthermore, the administration of anti–TGF-β Ab in vivo also abolished the protective effect of a single anti-Gr1 injection on diabetes development (Fig. 8E).

**Discussion**

In this study, we have shown that B cell depletion expanded an immunosuppressive cell population that is Gr1+CD11b+ and can produce IL-10. We further showed that these Gr1+CD11b+ cells could suppress T cell function in an IL-10, NO, and cell contact-dependent manner. Single anti-Gr1 treatment could also transiently expand Gr1+CD11b+ cells, which induced Foxp3+ Tregs in vivo.
a TGF-β–dependent manner. The novelty of our study lies in the induction method of Gr1+CD11b+ cells where we found that both B cell depletion and anti-Gr1 treatment can each expand Gr1+CD11b+ cells. This expansion of the Gr1+CD11b+ cells is different from previous reports using tumor-bearing mice or bone marrow as the source of Gr1+CD11b+ cells. This builds on our earlier study showing that temporary B cell depletion could prevent and reverse T1D in hCD20/NOD mice through several mechanisms including induction of Tregs and regulatory B cells, and suppression of Ag-presenting function of macrophages and dendritic cells (18). This highlights the fact that depleting a major subset of immune cells leads to an altered balance of cell populations as the cells repopulate with an increase in a number of regulatory cell types with immunosuppressive functions. We believe that all of these immune suppressive functions contribute to the prevention of T1D development in transiently B cell-depleted NOD mice.

Although Gr1+CD11b+ MDSCs are present in tumors and a variety of chronic inflammatory states (6–8, 11), their presence will have a number of different consequences. In cancer, the immunosuppressive effects prevent an antitumor T cell response. In this setting, these cells have a deleterious effect, and reducing or removing them could be of therapeutic value. In autoimmunity, however, increasing the Gr1+CD11b+ cells and activating them could be of considerable importance, given their ability to reduce T cell responses directly and induce the expansion of Tregs. In this paper, we showed that reconstitution of cells after both B cell depletion and anti-Gr1 treatment could also transiently increase Gr1+CD11b+ cells. One possible explanation for the expansion of Gr1+CD11b+ cells is that Ab treatment leads to Gr1 cell apoptosis. In response to these apoptotic cells, the immune system generates more immature myeloid cells, which later become phagocytic cells including neutrophils, macrophages, and dendritic cells to phagocytose the dead cells. This was supported by the fact that when we adoptively transferred apoptotic syngeneic splenocytes, NOD recipients had expansion of Gr1+CD11b+ cells, which led to delayed diabetes development (data not shown). The detailed

FIGURE 8. Gr1+CD11b+ cells induce Treg differentiation in a TGF-β–dependent manner. A, Secreted TGF-β from culture supernatants (Fig. 7C) was tested by ELISA (BD Biosciences). Purified naive T cells were cocultured with purified Gr1+CD11b+ cells (T+Gr1) compared with T cells alone (T). ***p < 0.0001. B, TGF-β expression was detected at transcriptional level by qPCR (5′-primer, TGGAGCCCTGGACAC-ACAGTA; 3′-primer, GTTGGACAACTGCTCCACCT). qPCR was performed in triplicate in three separate experiments. Data from one of three experiments are shown. ***p < 0.0001. C, Purified Gr1+CD11b+ cells were cocultured with purified naive T cells at a 1:5 ratio for 5 d in the presence or absence of neutralizing anti–TGF-β (clone 1D11; 10 µg/ml). The differentiation of naive T cells into Tregs was determined by flow cytometry. Three independent experiments were performed. D and E, Nine-week-old female NOD mice were injected with one dose of 0.25 mg anti-Gr1 or control IgG Ab i.v. together with or without neutralizing anti–TGF-β (three injections of 0.25 mg/mouse, 3-d intervals). Foxp3+CD4+ cells were examined at 35 wk of age in both Ab- and control IgG-treated mice (D, percentage on the left and absolute numbers on the right, *p < 0.05) together with the development of diabetes (E). Diabetes development was monitored by testing for glycosuria twice a week until 35 wk of age. Disease was confirmed by a blood glucose level >250 mg/dl. p = 0.035 for anti-Gr1 versus anti-Gr1 + anti–TGF-β groups, p = 0.331 for control IgG versus anti-Gr1 + anti–TGF-β groups, p = 0.045 for anti-Gr1 versus control IgG groups.
mechanism(s) is under further investigation. It is, however, in their immature state that Gr1+CD11b+ cells exert their suppressive function.

Why are these immature myeloid cells present in these chronic inflammatory states? Delano et al. (23), using a model of sepsis, showed that TLR signaling is important in expansion of this group of cells. Tissue damage induced in chronic inflammation may be important in inducing suppressive cells to reduce the inflammation. Clearly, under normal circumstances in diabetes, their presence in this context is not sufficient to stop the inflammation and β cell destruction. Maintaining the cells in an immature state, while also activating them to carry out immunosuppressive functions over a longer period of time, would be an important therapeutic challenge. This is evidenced by transfer experiments where purified bone marrow-derived Gr1+CD11b+ cells were shown to delay but not completely protect NOD.SCID recipients from diabetes when cotransferred with diabetogenic NOD splenocytes (Supplemental Fig. 1).

Interestingly, although the Gr1+CD11b+ cells in this study were induced by B cell depletion or single anti-Gr1 treatment, they exhibit immunosuppressive function through similar mechanisms to those of MDSCs found in tumor environments. Firstly, the suppressive function requires the production of NO by Gr1+CD11b+ cells and inhibition of NO synthase partially blocked their function. Secondly, cell–cell contact is essential for the regulatory function, as our in vitro experiments showed that the presence of a Transwell completely abrogated the immunosuppressive function of Gr1+CD11b+ cells. The upregulation of iNOS and Arginase1 gene expression in Gr1+CD11b+ cells further supported the role of NO for the immunosuppression (Supplemental Fig. 2). However, Gr1+CD11b+ cells induced by B cell depletion can suppress both CD4 and CD8 T cell proliferation, whereas MDSCs from tumor environments appeared to mainly affect CD8 T cells (22).

Our previous study showed an increase of Tregs after B cell depletion (18). It was not clear how the depletion of B cells induced Tregs. Our current study indicates that Treg induction is likely to be mediated by Gr1+CD11b+ cells after B cell depletion. This occurred in a TGF-β–dependent manner. We suggest that B cell depletion induces Gr1+CD11b+ cells that not only function by producing NO and reactive oxygen mediators but also produce IL-10 and TGF-β. These cytokines will induce the differentiation of Tregs and could further stimulate both CD4 T cells and Gr1+CD11b+ cells to produce more IL-10 and TGF-β.

Thus, in our study, we have reported the role of Gr1+CD11b+ cells in the development of T1D in the NOD mouse model of T1D. This adds another regulatory population, both endogenously as well as by adoptive transfer, that could be therapeutically exploited to increase immune regulation in diabetes. It is striking that a single injection of anti-Gr1 Ab in prediabetic NOD mice promoted long-lasting immune regulation leading to diabetes protection. It is clear that immune regulation mediated by Gr1+CD11b+ cells is complex and more than one mechanism is involved in the regulatory function. Further understanding of how Gr1+CD11b+ cells function in situ in the pancreatic islet to modulate autoreactive T cells will provide new insight into pancreatic autoimmunity.

Disclosures

The authors have no financial conflicts of interest.

References

Corrections


The primer sequence for Foxp3 mRNA detection in the Materials and Methods under the heading “Treg induction in the presence of Gr1\(^+\) CD11b\(^+\) cells” is incorrect.

The correct Foxp3 primer pair sequence is:

5′-CAGCTGCCTACAGTGCCCCTAG-3′

5′-CATTTGCCAGCAGTGGGTAG-3′

The authors sincerely apologize for this error.

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Supplementary Figure 1. Gr1⁺CD11b⁺ cells delayed diabetes development in adoptive transfer. Bone marrow derived Gr1⁺CD11b⁺ cells were cultured as described (11). Briefly, bone marrow cells were flushed from the femurs and tibiae of NOD mice. Bone marrow cells were depleted of red cells and cultured in Bruff’s medium (Invitrogen) with 5% FCS in the presence of GM-CSF (1% supernatant from J558L cells transfected with mouse GM-CSF construct) and recombinant IL-6 (20 ng/ml, Peprotech). Four days later, cells were harvested and Gr1⁺CD11b⁺ cells were purified by FACS sorting. A) 2 x 10⁶ bone marrow derived Gr1⁺CD11b⁺ cells were co-transferred with 6 x 10⁶ splenocytes from diabetic NOD mice into 6-week old NOD.SCID mice (n=5 for each group). Diabetes development was monitored by glycosuria tested twice a week. Diabetes was confirmed by blood glucose measurement greater than 250 mg/dl (13.9mmol/l). Bone marrow derived Gr1⁺CD11b⁺ cells significantly delayed the onset of diabetes when cotransferred with splenocytes from diabetic NOD mice (Diab-SPL) compared with the control group (Log-rank test, p=0.0127). FACS sorted Gr1⁺CD11b⁺ cells (2 x 10⁴/well) from untreated (BM) or GM-CSF/IL-6 treated (G-BM) bone marrow cells were cultured with BDC2.5 CD4 T cells (10⁵/well) (B) or NY8.3 CD8 T cells (10⁵/well) (C) and proliferation with BM-Gr1⁺ cells (W/BM-Gr1⁺) or G-BM-Gr1⁺ cells (W/G-BM-Gr1⁺) is shown compared to T cells cultured in the absence of GR1⁺ cells (W/O Gr1⁺). The background CPM counts were between 400 and 600. P values were calculated by Student’s t test. ** P<0.001.

Supplementary Figure 2. iNOS and Arg1 gene expression. Purified Gr1⁺CD11b⁺ cells were co-cultured with naïve T cells at 1:5 ratio for 5 days. Arg1 and iNOS mRNA were detected by quantitative PCR in the Gr1⁺CD11b⁺ cell co-cultures (Gr1⁺T) compared with naïve T cells alone (T). **p<0.001, ***p<0.0001.