PD-1 deletion restores susceptibility to experimental autoimmune encephalomyelitis in miR-155-deficient mice

Jinyu Zhang¹² and Michel Y. Braun¹

¹Institute for Medical Immunology, Université Libre de Bruxelles, Gosselies 1420, Belgium
²Department of Clinical Microbiology and Immunology, College of Medical Laboratory Science, Third Military Medical University, Chongqing 400038, People’s Republic of China

Correspondence to: M. Y. Braun; E-mail: mbraun@ulb.ac.be

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Abstract

MiR-155−/− mice are highly resistant to experimental autoimmune encephalomyelitis (EAE), while Pdcd1−/− mice develop a more severe form of the disease. To determine the conflicting roles of these two molecules in the disease, we generated miR-155−/−Pdcd1−/− double knockout (DKO) mice. We found that ablation of programmed cell death protein 1 (PD-1) expression in miR-155-deficient mice restored the susceptibility to EAE. The increased severity of the disease in DKO mice was accompanied by an enhanced T-cell infiltration into the brain as well as an increased production of pro-inflammatory cytokines IFN-γ and IL-17. Furthermore, the major contribution of the DKO to EAE was T-cell intrinsic since adoptive transfer of CD4+ T cells from DKO donors promoted the disease in lymphopenic recipients. These results define PD-1 deficiency in miR-155−/− mice as a promoting factor of autoimmune inflammation by increasing antigen-driven T-cell expansion and infiltration.

Keywords: autoimmunity, IFN-γ, IL-17, inflammation, microRNA

Introduction

MicroRNAs (miRNA) are small endogenous non-coding RNA molecules of 21–25 nucleotides in length. MiRNAs regulate gene expression by targeting complementary miRNAs, which results in translational repression or increased degradation of target mRNAs (1, 2). It is well established that miRNAs play crucial roles in the process of maturation, proliferation, migration, differentiation and function in the immune system. Among the most abundant miRNAs present in immune cells, miR-155 was shown to be critical for T-cell fitness and effector functions and deletion of miR-155 leads to severe defects in immune responses (3–5). MiR-155 is best characterized as a pro-inflammatory miRNA because it was shown to enhance the production of pro-inflammatory cytokines in immune cells. Uncommitted miR-155-deficient T cells showed a reduced capacity to produce inflammatory TNF-1 cytokines, such as IL-2 and IFN-γ, after in vitro stimulation (5). Accordingly, miR-155-deficient mice failed to control efficiently bacterial and virus infection (6–10). MiR-155 was also implicated in autoimmune diseases. MiR-155−/− mice did not develop clinical or histological signs of collagen-induced arthritis and had decreased IL-6 and IL-17 production (11, 12). In experimental autoimmune encephalomyelitis (EAE), miR-155 was up-regulated upon myelin oligodendrocyte glycoprotein peptide fragment 35-55 (MOG35-55) immunization, and miR-155-deficient mice were highly resistant to EAE (3, 13). EAE resistance in these animals was linked to a T-cell intrinsic defect in T0.1 and T0.17 cell development. Moreover, silencing miR-155 using synthetic oligonucleotides reduced the number of T0.1 and T0.17 cells as well as the clinical severity of EAE in miR-155-sufficient mice. These observations clearly indicate the crucial importance for miR-155 activity in promoting the development of inflammatory T cells.

Programmed cell death protein 1 (PD-1) is a protein that is encoded by the Pdcd1 gene on chromosomes 1 and 2, in mice and in humans, respectively. Interactions between PD-1 and its two ligands, PD-L1 and PD-L2, attenuate T-cell activation and effector function (14). The PD-1 inhibitory pathway has been demonstrated to operate in the control of various chronic infectious diseases and cancer and in the control of autoimmune T cells (15–22). Consequently, the modulation of this inhibitory pathway is generally considered as a promising therapeutic means for the control of T-cell function or to enhance immune responses. The PD-1 pathway has also been shown to play an important role in regulating inflammatory processes during EAE (23). The expressions of PD-1 and PD-L1 were progressively increased within the central nervous system (CNS) of mice with
PD-1-mediated regulation of EAE in miR-155-deficient mice

EAE. Consequently, PD-L1 blockade led to the expansion of MOG-reactive T cells, increased lymphocytic infiltration of the CNS and, ultimately, accelerated disease onset and severity (21). Studies with PD-1- or PD-L1-deficient mice indicate that PD-1 interaction with PD-L1, but not PD-L2, is predominantly responsible for regulating disease severity (20, 24–26). Re-stimulation of T cells from PD-1−/− or PD-L1−/− mice with active disease resulted in a burst of IL-17 and IFN-γ production, suggesting that T cells with defective PD-1 signalling may be preferentially polarized toward effector T-cell differentiation (20). Recently, PD-1 deficiency in macrophages was shown to enhance the production of the pro-inflammatory cytokine IL-6 during EAE, leading to the development of inflammatory T cells and disease exacerbation (27).

Thus, miR-155 and PD-1 appear to have opposite impacts on inflammatory responses carried out by T lymphocytes in the context of autoimmunity, and little is known about the cross-regulation of autoimmunity by miR-155 and PD-1. In our study, we generated miR-155−/− Pdcd1−/− double knockout (DKO) mice to investigate the effects exerted by both miR-155 and PD-1 during the development of EAE.

Methods

Mice

Rag1−/−, Cd3−/− and B6 congenic mice were purchased from Charles River and bred at the Institute for Medical Immunology, Université Libre de Bruxelles, Belgium. Pdcd1−/− B6 mice were a kind gift from T. Honjo (Kyoto University, Japan). MiR-155−/− B6 mice were purchased from Jackson Laboratories. MiR-155−/− Pdcd1−/− DKO mice were generated by crossing Pdcd1−/− with miR-155−/− mice. All age- and sex-matched mice used in this study were between 6- and 10-weeks old and were bred in individually ventilated cages on the same rack under specific pathogen-free conditions (FELASA) at the Institute for Medical Immunology.

Experimental autoimmune encephalomyelitis

Mice were immunized s.c. with 100 μg per mouse MOG peptide (Sigma–Aldrich) emulsified in Complete Freund’s Adjuvant (Sigma–Aldrich) supplemented with 400 μg per mouse Mycobacterium tuberculosis (DIFCO Laboratories). Mice received Pertussis Toxin (Sigma–Aldrich) 250 μg per mouse (DIFCO Laboratories) under specific pathogen-free conditions (FELASA) at the Institute for Medical Immunology.

Cell culture supernatants were used to measure IFN-γ and IL-17 secretion by ELISA.

In some experiment, spleen CD4+ T cells were isolated 17 days after immunization with the Dynal mouse CD4 negative isolation kit (Invitrogen). T-cell purity assessed by flow cytometry was ≥97%. Peritoneal macrophages (PMs) from T-cell-deficient Cd3−/− mice were prepared as described previously and used as antigen-presenting cells (29). Spleen CD4+ T cells (1 x 10^6 cells per well) were incubated with PMs (5 x 10^5 cells per well) and MOG peptide (20 μg ml^-1). Seventy-two hours later, supernatants were collected for detecting IFN-γ and IL-17 secretion by ELISA.

Enzyme-linked immunosorbent assay

Cell culture supernatants were used to measure IFN-γ and IL-17 concentrations by ELISA (R&D Systems) according to the manufacturer’s instructions.

Statistical analysis

An unpaired Student’s t-test or analysis of variance was used to determine the degree of significance. Data are presented as mean ± SEM. A P value <0.05 was considered statistically significant.
Results

Deletion of PD-1 promotes EAE in miR-155−/− mice

MiR-155+/+, miR-155−/−, Pdcd1−/− and DKO mice were immunized with MOG35–55, respectively and disease development was followed over time. As depicted in Fig. 1(A), DKO mice exhibited a more pronounced pathology than miR-155−/− mice, the onset of clinical symptoms and kinetics of disease development being similar to those of miR-155+/+ and Pdcd1−/− mice. MiR-155+/+ and Pdcd1−/− mice displayed neurologic symptoms approximately 12 days after immunization, with peak disease severity on day 17, and 100% disease incidence. As previously reported by others (21), disease severity symptoms in Pdcd1−/− mice were more pronounced than those observed in miR-155+/+ mice. In contrast, miR-155−/− mice exhibited a later onset of symptoms on day 15. By day 17, they displayed a low average clinical score of 0.4, which was consistent with previous reports (3). Unlike Pdcd1−/− mice, DKO mice began to display neurologic symptoms at day 12 post immunization. By day 15, 100% disease incidence was observed and, at the end of the experiment, 66% of DKO mice developed a clinical score more than 2.5. These results indicated that deletion of PD-1 expression could restore the susceptibility to EAE in miR-155−/− mice.

The pathogenesis of EAE initiates with immune cell infiltration into the brain (3). On day 17 after immunization, mice from the indicated four groups were sacrificed and mononuclear cells were isolated from spleen, draining LNs and brain. As depicted in Fig. 1(B), total cell numbers in spleen and draining LNs were in general not significantly altered in the different groups of mice. The situation in brain was, however, very different. Leukocyte numbers in the brain of miR-155−/− mice were the lowest among the four experimental groups, which was consistent with the reduced disease observed in these animals. On the contrary, leukocyte numbers in the brain of DKO mice were comparable to those of miR-155+/+ and Pdcd1−/− mice. Taken together, these results demonstrated that ablation of PD-1 expression in miR-155−/− mice promoted immune cell infiltration into the brain, resulting in the development of an aggravated disease.

PD-1 deficiency promotes T-cell infiltration in the brain of miR-155−/− mice immunized with MOG35–55 peptide

To investigate the possible mechanism underlying the more severe EAE observed in DKO mice, the cellular contents of spleen, draining LNs and brain isolated from the different groups of mice were characterized by flow cytometry. As depicted in Fig. 2, the cellularity of spleen or LNs was in general not significantly altered in the different groups of mice. However, the composition of cellular infiltrates in brain was again very different. Consistent with a low disease severity, the lack of miR-155 expression in brain was again very different. Consistent with a low disease severity, the lack of miR-155 expression correlated with fewer CD3+ T cells (Fig. 2A), CD11b+...
myeloid cells (Fig. 2B), and B220+ B cells (Fig. 2C) within the brain of MOG_{35-55}-immunized mice. However, deletion of PD-1 expression in miR-155−/− mice restored brain cellular infiltrates at levels comparable to those observed in Pdcd1−/− miR-155+/+ counterparts, supporting a role for PD-1 in regulating the inflammation of the CNS regardless of the presence of miR-155.

**PD-1 deficiency promotes brain inflammation by T_{h1} and T_{h17} cells during EAE in miR-155−/− mice**

It is known that brain inflammation in EAE is mediated by both MOG-specific T_{h1} and T_{h17} cells (30, 31). We then examined spleen, LNs and brain for the presence of T_{h1} or T_{h17} cells during EAE in our experimental settings. Intracellular labelling results presented in Fig. 3 show that both the percentages of T_{h1} and T_{h17} cells are increased in miR-155−/− mice compared to wild-type controls.
and Th17 cells in spleen and LNs were in general very similar among the four groups. However, though Th1 and Th17 cell percentages in the brain of miR-155−/− mice were lower than those observed in miR-155+/+ mice, this is consistent with previous data. Th1 and Th17 percentages in the brain of DKO mice were not significantly changed compared with those of miR-155−/− mice (Fig. 3B). Altogether, these observations supported the idea that blockade of PD-1 pathway in miR-155−/− mice did not alter the differentiation of Th1 or Th17 responses. However, the absolute number of T cells present within the brain of DKO mice was always much higher than that observed in Pdcd1−/− mice (Fig. 3C). Thus, increased T-cell infiltration in brain could promote the aggravated disease observed in DKO mice.

To further investigate the mechanisms underlying the more severe EAE observed in DKO mice, the recall T-cell response to the MOG35–55 peptide was also assessed. Splenocytes, LN cells and lymphoid cells isolated from brain were re-stimulated in vitro with 20 μg ml−1 of MOG35–55 peptide. After 72h, culture supernatants were collected and their content of IL-17 or IFN-γ was assessed by ELISA (Fig. 4). Spleen or
LN cells from miR-155−/− mice showed minimal production of both cytokines regardless of the expression of a functional PD-1 molecule (Fig. 4A and B). This was also generally observed when a fixed number of purified splenic T cells were stimulated with antigen-presenting cells pulsed with MOG 35–55 peptides (Fig. 4C). Though the amount of IFN-γ produced by purified DKO T cells was significantly more important than that secreted by miR-155−/− T cells, it never reached levels
observed with miR-155+/− T cells. A very different situation was, however, observed for lymphoid cells isolated from brain. The addition of MOG_{35-55} peptides to cultures of DKO spleen cells indeed stimulated the secretion of both cytokines (Fig. 4A and B). Moreover, the level of inflammatory cytokines produced by DKO brain cells was similar to those observed in cultures of miR-155+/− and Pdcd1−/− cells. Thus, PD-1 deficiency appear to restore the capacity of antigen-specific miR-155−/− T_{h1} or T_{h17} cells to migrate into the brain and promote inflammation.

T-cell intrinsic role for PD-1 deficiency in restoring EAE in miR-155−/− mice

To test whether promoting brain inflammation during EAE in DKO mice was intrinsically linked to T-cell activity, we adoptively transferred 1 × 10^6 purified CD4+ T cells from miR-155+/+, miR-155−/−, Pdcd1−/− and DKO mice into Rag1−/− recipients and induced EAE 24 h later. Contrarily to what was observed in our experiments involving the direct immunization of immunocompetent mice, we only observed a very minor T-cell intrinsic effect of PD-1 on disease in mice adoptively transferred with miR-155+/+ T cells (Fig. 5A). This is perhaps because, as shown in the figure, disease severity was low in transfer experiments and probably not sufficient to reveal the impact of PD-1 deficiency in miR-155−/− T cells. More importantly, however, mice receiving CD4+ T cells from miR-155−/−, Pdcd1−/− and DKO mice exhibited a substantially more severe and accelerated course of disease than mice reconstituted with miR-155−/− CD4+ T cells (Fig. 5A). Compared with mice that received miR-155−/− CD4+ T cells, animals adoptively transferred with DKO CD4+ T cells had a significant increase of leukocyte numbers in their brain (Fig. 5B). In addition, DKO T cells, but not miR-155−/− T cells, were found in high numbers in the brain of recipient mice (Fig. 5C). Thus, the capacity to promote EAE in DKO mice appears to be, at least in part, intrinsic to T cells.

Discussion

MiR-155 is abundant in activated T cells and recent evidence implicates miR-155 in the regulation of lymphocyte proliferation and homeostasis (32). In EAE, miR-155 was shown to promote autoimmune inflammation by enhancing inflammatory T-cell development (3). Here we show that the lack of T-cell inflammatory activity in miR-155-deficient mice can be restored by the absence of PD-1-mediated inhibitory signaling in T cells, and miR-155−/− Pdcd1−/− mice developed EAE with intensity comparable to that seen in miR-155+/+ Pdcd1−/− mice.
animals. One obvious explanation for this situation would be to postulate that PD-1 and miR-155, though developing opposite effects, control the expression of the same molecules involved in the regulation of T-cell inflammatory function. Among molecules whose expression is targeted by miR-155, the suppressor of cytokine signalling 1 (SOCS1) functions downstream of cytokine receptors and participates in regulating cytokine signalling and T-cell function (33). It is known that high expression of SOCS1 in T regulatory cells suppresses IFN-γ and IL-17 production stimulated by the signal transducer and activator of transcription 1 and 3 (STAT1/3) pathway (34). Thus, miR-155 could control T-cell function by inhibiting SOCS1-mediated regulation. Interestingly, several studies have shown that blocking PD-1-dependent modulation of TCR signalling down-regulated Soc1 gene expression in antigen-stimulated T cells (35). Thus, in the absence of PD-1 expression in T cells, increased TCR signalling could counterbalance the effect that miR-155 deficiency has on SOCS1 expression and promote inflammatory function in T cells.

Our results also indicate that PD-1 appears to regulate directly the capacity of T cells to infiltrate the brain of mice with EAE rather than the differentiation of MOG-specific T\(_h\) subsets. This is clearly visible in the brain of DKO mice where numbers of infiltrating T\(_h\) or T\(_b\) cells increase with disease development, whereas in lymphoid organs these T-cell populations remain as numerous as in miR-155\(^{-/-}\) mice. The impact of PD-1 engagement on the capacity of autoimmune T cells to infiltrate organs has been previously addressed. Fife et al. have shown that interactions between PD-1 and PD-L1 promote T-cell unresponsiveness by blocking the TCR-induced stop signal, resulting in higher T-cell motility in infiltrated organs and maintenance of peripheral tolerance (36). Thus, inhibition of PD-1–PD-L1 interaction in DKO mice would allow inflammatory MOG-specific miR-155-deficient CD4\(^+\) T cells to prolong contacts with antigen-presenting cells present within the brain. The organ would then act as a trap where activated T cells accumulate, leading to the development of organ-specific autoimmunity.

We also confirm previous results that miR-155 deficiency compromises the development of T\(_h\) cells and there are less MOG\(_{95-58}\)-specific T\(_b\) and T\(_h\) CD4\(^+\) T cells present in immunized miR-155\(^{-/-}\) mice than in miR-155\(^{-/-}\) counterparts (3). It has been suggested that T\(_h\) differentiation can be regulated by TCR signalling: strong antigenic stimulation would lead to T\(_b\) or T\(_h\) cell derivation, whereas weaker signals would derive T\(_h\) cells from naive precursors (37). Since PD-1 inhibition of T-cell function targets mainly TCR signallng (38), it could be hypothesized that stronger early TCR signals generated in the absence of PD-1 could favour fate decision of naive T cells toward T\(_b\) or T\(_h\) subsets in miR-155\(^{-/-}\) mice as it has been shown in normal T cells (39). However, in our experiments, deleting PD-1 on T cells did not increase the emergence of T\(_b\) or T\(_h\) cells after immunization with MOG\(_{95-58}\) in miR-155-deficient mice. Thus, the pathways by which miR-155 promotes T\(_b\) and T\(_h\) cell differentiation is not regulated by PD-1-mediated regulation of TCR signalling. One could conclude that miR-155 regulation of T\(_h\) differentiation does not result from how T cells are activated by their antigen.

Our results clearly show that PD-1-mediated inhibition of T cells controls autoimmunity in miR-155\(^{-/-}\) mice and participates in the maintenance of immune unresponsiveness that characterizes these animals. Further studies will be needed to evaluate the mechanism by which PD-1 deletion promotes T-cell-mediated EAE in miR-155\(^{-/-}\) mice, which could help to explore novel and effective therapies against autoimmune diseases. Moreover, it is not clear how these molecules interact at the molecular levels in T cells. Is PD-1 a direct target of miR-155? Does PD-1 signalling regulate miR-155 expression? These questions should be addressed further.

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