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Arginase-1–dependent promotion of T_H17 differentiation and disease progression by MDSCs in systemic lupus erythematosus

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Abstract

Expansion of myeloid-derived suppressor cells (MDSCs) has been documented in some murine models and patients with autoimmune diseases, but the exact role of MDSCs in this process remains largely unknown. The current study investigates this question in patients with systemic lupus erythematosus (SLE). Patients with active SLE showed a significant increase in HLA-DR⁺CD11b⁺CD33⁺ MDSCs, including both CD14⁺CD66b⁺ monocytic and CD14⁺CD66b⁺ granulocytic MDSCs, in the peripheral blood compared to healthy controls (HCs). The frequency of MDSCs was positively correlated with the levels of serum arginase-1 (Arg-1) activity, T helper 17 (T_H17) responses, and disease severity in SLE patients. Consistently, in comparison with MDSCs from HCs, MDSCs from SLE patients exhibited significantly elevated Arg-1 production and increased potential to promote T_H17 differentiation in vitro in an Arg-1–dependent manner. Moreover, in a humanized SLE model, MDSCs were essential for the induction of T_H17 responses

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SUPPLEMENTARY MATERIALS

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Fig. S1. Correlation between MDSC numbers and SLEDAI scores in SLE patients.

Fig. S2. Correlation between serum Arg-1 activity and MDSC numbers in SLE patients.

Fig. S3. Serum levels of IL-17A and IL-17F correlate positively with the frequencies of MDSCs in PBMCs and serum levels of Arg-1 in SLE patients.

Fig. S4. Suppression of T cell proliferation by MDSCs from SLE patients.

Fig. S5. Nor-NOHA does not inhibit T_H17 differentiation in the absence of MDSCs.

Fig. S6. Correlation between the ability of SLE patient PBMCs to induce proteinuria in mice and the disease severity of donor patients.

Fig. S7. Isotype controls and gating strategy for flow cytometry analysis.

Fig. S8. Isotype controls for immunofluorescence.

Table S1. Patients' characteristics at diagnosis (*n* = 32).

Table S2. Original data and statistics (*P* values) (provided as a separate Excel file). Reference (67)

and the associated renal injuries, and the effect of MDSCs was Arg-1–dependent. Our data provide direct evidence demonstrating a pathogenic role for MDSCs in human SLE. This study also provides a molecular mechanism of the pathogenesis of SLE by demonstrating an Arg-1–dependent effect of MDSCs in the development of T_H17 cell–associated autoimmunity, and suggests that targeting MDSCs or Arg-1 may offer potential therapeutic strategies for the treatment of SLE and other T_H17 cell–mediated autoimmune diseases.

INTRODUCTION

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature cells derived from myeloid progenitors with immunosuppressive functions (¹). Human MDSCs are CD11b⁺CD33⁺HLA-DR[−] and can be further classified into two major subsets, CD14⁺ monocytic MDSCs (M-MDSCs) and CD15⁺CD66b⁺ granulocytic MDSCs (G-MDSCs) (^{1, 2}). Murine MDSCs are characterized by coexpression of Gr-1 and CD11b, and can be further subdivided into CD11b⁺Gr-1^{high} G-MDSCs and CD11b⁺Gr-1^{low} M-MDSCs (³). Although MDSCs were found to suppress T cell responses in the context of tumor-associated inflammation (^{4, 5}), the role of MDSCs in autoimmune diseases is still controversial (⁶). In murine models of autoimmune disease, MDSCs were found to attenuate the disease severity in some studies (^{7–10}), whereas others reported a deleterious role of MDSCs in autoimmune disease progression (^{11–13}).

T helper 17 (T_H17) cells, a subset of CD4⁺ T_H cells that produce interleukin-17A (IL-17A), IL-17F, and other proinflammatory cytokines (^{14, 15}), have been shown to play a critical role in the pathogenesis of a range of autoimmune diseases, including systemic lupus erythematosus (SLE) (^{16, 17}), systemic sclerosis (¹⁸), multiple sclerosis (MS) (¹⁹), and rheumatoid arthritis (RA) (^{20, 21}). Recent studies showed that mouse CD11b⁺Gr-1⁺ MDSCs may promote T_H17 cell differentiation in vitro in the presence of IL-6 and transforming growth factor-β (TGF-β) (^{11, 13}). Similarly, mouse MDSCs isolated from tumors also promoted naïve CD4⁺ T cell differentiation into T_H17 cells in vitro (²²). However, the role of MDSCs in T_H17 differentiation and pathogenesis of autoimmune diseases in human is relatively unknown. Here, we seek to address these questions in patients with SLE. We show that SLE patients had a significant increase in MDSCs that correlated positively with disease activity. MDSCs from SLE patients were more potent than those from healthy controls (HCs) in promoting T_H17 cell differentiation in vitro. Moreover, MDSC depletion markedly attenuated the disease progression in a humanized SLE model. Furthermore, the ability of MDSCs to augment T_H17 differentiation and disease activity was arginase-1 (Arg-1)–dependent.

RESULTS

Positive correlation between number of circulating MDSCs and disease activity in SLE patients

We first measured the frequency of MDSCs and their subsets isolated from the peripheral blood mononuclear cells (PBMCs) of SLE patients using flow cytometry. PBMCs were collected from a total of 32 patients (2 males and 30 females, aged 17 to 65 years) and 25

HCs (3 males and 22 females, aged 17 to 64 years). All patients were diagnosed with active SLE according to the SLE Disease Activity Index (SLEDAI) scores (²³) ranging between 8 and 23. Detailed clinical and laboratory characteristics of these patients are presented in table S1. MDSCs were defined as CD11b⁺CD33⁺HLA-DR⁻, which were further divided into SSC^{low}CD14⁺CD66b⁻ M-MDSC and SSC^{high}CD14⁻CD66b⁺ G-MDSC subsets (Fig. 1A and fig. S1). Hematoxylin and eosin (H&E) staining of sorted M-MDSCs and G-MDSCs revealed no detectable difference in morphology between SLE patients and HCs (fig. S1A). Compared to HCs, SLE patients showed significant increases in both the percentages ($11.468 \pm 5.745\%$ versus $2.175 \pm 1.0364\%$; Fig. 1B) and numbers (10.674 ± 6.030 versus 2.668 ± 1.141 ; fig. S1B) of MDSCs, which were positively correlated with the disease status by SLEDAI scores. All patients showed a significant increase in M-MDSCs (Fig. 1C and fig. S1C), and about half of them also showed a marked increase in G-MDSCs (Fig. 1D and fig. S1D). In addition, the numbers of both subsets showed positive correlation with the disease activity (fig. S1, C and D).

Increased serum Arg-1 activity and Arg-1 production by MDSCs in SLE patients

MDSCs were reported to be a major source of Arg-1 production in cancer patients (²⁴); therefore, we next measured serum Arg-1 activity in SLE patients and in HCs. A significant increase in serum Arg-1 activity, which was also positively correlated with the disease activity, was detected in all SLE patients compared to HCs (9.961 ± 3.858 U/liter versus 3.218 ± 1.600 U/liter; Fig. 2A). The positive correlation between the level of serum Arg-1 activity and the number of circulating MDSCs in SLE patients indicated that MDSCs may be an important source of Arg-1 in SLE patients (fig. S2). In support of this possibility, intracellular staining demonstrated a marked increase in Arg-1 production by MDSCs from SLE patients compared to those isolated from HCs (Fig. 2B). G-MDSCs produced more Arg-1 compared to M-MDSCs in both HCs and SLE patients, and only G-MDSCs from the SLE patients showed a significantly increased Arg-1 production (Fig. 2, C and D).

IL-6 was reported to induce Arg-1 production in mouse macrophages (²⁵). To determine whether IL-6 is involved in the observed up-regulation of Arg-1 in MDSCs from the SLE patients, we compared the serum levels of IL-6 between SLE patients and HCs. Similar to previous reports (^{26, 27}), we found a significant elevation in serum IL-6 in SLE patients (Fig. 2E). Furthermore, real-time quantitative polymerase chain reaction (qPCR) revealed a significant increase in *arg-1* mRNA expression in MDSCs after treatment with IL-6 (Fig. 2F). Because SLE patients also showed significantly increased IL-17 production (see Fig. 3 below), we next determined the effect of IL-17 on *arg-1* expression by MDSCs. As shown in Fig. 2G, incubation with IL-17 failed to increase *arg-1* expression in human MDSCs. These results indicate that elevated IL-6 production is likely to contribute to the up-regulation of Arg-1 production in MDSCs from SLE patients.

Increased number of T_H17 cells and serum level of T_H17 cytokines in SLE patients

PBMCs were prepared from SLE patients and HCs, and T_H17 cell frequencies (that is, CD4⁺ T cells producing IL-17A/IL-17F) were determined by flow cytometry after a short stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin. Patients with SLE had a significantly increased frequency of IL-17A⁺CD4⁺ cells compared to HCs (Fig. 3, A

and B). In agreement with that, serum levels of IL-17A and IL-17F in SLE patients were found to be significantly higher compared to those in HCs (Fig. 3C) and correlated positively with the SLEDAI scores (Fig. 3D), MDSC frequencies (fig. S3A), and serum Arg-1 levels (fig. S3B). Immunohistologic staining revealed the presence of IL-17A and IL-17F in the kidney biopsy samples from all SLE patients examined ($n = 5$; Fig. 3E). Both IL-17A and IL-17F were primarily detected in the glomeruli (Fig. 3E), where mesangium proliferation and inflammatory cell infiltration in interstitial space were detected by periodic acid–Schiff (PAS) and H&E staining, respectively (Fig. 3F). Collectively, our data implicate that T_H17 cells and cytokines are likely to play an important role in the pathogenesis of SLE.

Increased potential of MDSCs from SLE patients to promote T_H17 cell differentiation via an Arg-1–dependent mechanism

We then assessed the effect of MDSCs on T_H17 cell differentiation from anti-CD3/CD28–activated naïve (that is, $CD4^+CD45RA^+$) T cells in T_H17 polarizing medium consisting of IL-6, TGF- β , IL-23, IL-1 β , anti–interferon- γ (IFN- γ), and anti–IL-4 monoclonal antibodies (mAbs) (28). In vitro suppression assay revealed that MDSCs from SLE patients were much more potent than those from HCs in suppressing anti-CD3/CD28–induced polyclonal T cell proliferation (fig. S4). We first assessed the role of MDSCs from HCs in T_H17 cell differentiation and found that MDSCs can significantly promote the generation of $CD4^+$ T cells that produce IL-17A or IL-17F (Fig. 4, A to C). However, MDSC-mediated T_H17 differentiation was completely diminished in the presence of nor-NOHA, a selective Arg-1 inhibitor, suggesting that the effect of MDSCs is mediated by their production of Arg-1 (Fig. 4, A to C). In further support of this conclusion is the observation that nor-NOHA failed to inhibit T_H17 differentiation in the absence of MDSCs (fig. S5).

T_H17 differentiation and function are regulated by the transcription factor retinoic acid–related orphan receptor γ t (ROR γ t; RORc in humans) (29). Aryl hydrocarbon receptor (AHR) is also highly expressed in T_H17 cells and may promote T_H17 differentiation (30). We found that MDSCs markedly enhanced the expression of ROR γ t and AHR in CD4 T cells under the T_H17 polarizing condition, but only MDSC-induced ROR γ t up-regulation was effectively prevented by nor-NOHA (Fig. 4D). The data suggest a possibility that MDSCs may induce ROR γ t expression through Arg-1 and thus promote T_H17 differentiation.

Arg-1 has been suggested to regulate T cells through pathways that involve the kinases general control nondepressible 2 (GCN2) and mammalian target of rapamycin (mTOR) (31). Under the T_H17 polarizing condition, MDSCs were found to up-regulate both GCN2 and mTOR expression in CD4 T cells, and nor-NOHA countervailed this effect (Fig. 4E). GCN2 is the main protein kinase responsible for the phosphorylation of eukaryotic initiation factor 2 α (eIF2 α) on Ser⁵¹, resulting in repression of general protein translation (32, 33) while enhancing the synthesis of stress-responsive proteins (34, 35). Thus, we next assessed the effect of MDSCs on eIF2 α phosphorylation in CD4 T cells during T_H17 differentiation. Compared to the controls with CD4 T cells only, addition of MDSCs markedly increased the level of EIF2S1 (phosphorylated eIF2 α) in CD4 T cells (Fig. 4F). Such an effect of MDSCs is largely Arg-1–dependent because addition of nor-NOHA greatly suppressed eIF2 α

phosphorylation (Fig. 4F). The level of IL-17A expression was correlated with the level of EIF2S1 in CD4 T cells (Fig. 4F). Our data suggest that mTOR and GCN2-eIF2 α signaling are likely to be involved in Arg-1–dependent induction of T_H17 differentiation by MDSCs.

We then evaluated the effect of MDSCs from SLE patients on T_H17 differentiation in comparison with MDSCs from the HCs. As elucidated in Fig. 4G, MDSCs from SLE patients were significantly more potent than those from HCs in promoting T_H17 cell differentiation, and their effect was also Arg-1–dependent.

Mitigation of T_H17 cell–mediated renal injury and proteinuria in humanized SLE mice by depletion of MDSCs or inhibition of Arg-1 activity

We further assessed the role of MDSCs in the pathogenesis of SLE in a humanized mouse model (³⁶). This model was created by intravenous injection of PBMCs isolated from the SLE patients with active disease [SLEDAI, 9; double-stranded DNA (dsDNA), 1:10] into immunodeficient nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice. To determine the role of MDSCs and Arg-1 in the disease development, NOD/SCID mice were administered unaltered PBMCs, MDSC-depleted PBMCs, or unaltered PBMCs plus the Arg-1 inhibitor nor-NOHA (Fig. 5A). All mice receiving unaltered PBMCs developed lupus nephritis–like symptoms within 4 to 5 weeks, such as the production of human anti-dsDNA antibodies (Fig. 5B) and development of proteinuria (Fig. 5C). The potential of PBMCs to induce proteinuria (measured as the albumin/creatinine ratio in urine) in humanized mice correlated positively with the SLEDAI scores of patients from whom PBMCs were obtained (Fig. 5D). However, mice receiving MDSC-depleted PBMCs showed markedly less severe symptom, as shown by significantly lower levels of serum human anti-dsDNA antibodies (Fig. 5B) and proteinuria (Fig. 5C), indicating that MDSCs are essential for the disease pathogenesis in vivo. The disease-promoting role of MDSCs is likely to be dependent on Arg-1 because treatment with the Arg-1 inhibitor nor-NOHA inhibited the disease progression in NOD/SCID mice to a similar extent as MDSC depletion (Fig. 5, B and C). Compared to mice receiving unaltered PBMCs, mice that received MDSC-depleted PBMCs or unaltered PBMCs plus nor-NOHA showed markedly reduced *il-17a* expression in the spleen and kidney (Fig. 5D). Histological analysis of kidney tissue revealed reduced IL-17A and human immunoglobulin G (IgG) deposition (Fig. 5E) and reduced mesangial cell proliferation (Fig. 5F) in the glomeruli of the mice receiving MDSC-depleted PBMCs or unaltered PBMCs plus nor-NOHA compared to those injected with unaltered PBMCs.

DISCUSSION

Given that MDSCs suppress immune responses, researchers have attempted to exploit the potential of these cells for the treatment of autoimmune diseases (^{7, 9, 10, 37}). However, we show that MDSCs may also exacerbate T_H17-driven autoimmune diseases. There was a significant increase in Arg-1–producing MDSCs and a strong correlation of the disease severity with the MDSC numbers and serum Arg-1 activity in patients with SLE. Furthermore, using a combination of in vitro assay and humanized SLE mouse model, we demonstrated that MDSCs from SLE patients are highly potent in promoting T_H17 cell differentiation and disease progression in an Arg-1–dependent manner.

Although SLE patients showed increase in the number of T_H17 cells (38, 39) and the serum level of T_H17 cytokines (17, 38–40), previous studies have suggested that there is no correlation between T_H17 responses and SLE (16, 40–43). This is in agreement with previous reports indicating that neither IL-17A deficiency nor IL-17A neutralization affected the clinical course of nephritis in murine models of SLE (44). However, other studies have paradoxically shown that the levels of T_H17 cytokines are increased in SLE patients (38, 39) and mouse models of SLE (45, 46), and reduced disease progression has been seen in mice deficient in IL-17 (46) or IL-23 (45) receptor. Our data showed a positive correlation between the serum IL-17 levels and disease activity in SLE patients. Furthermore, preferential deposition of IL-17A and IL-17F in the glomerular areas with mesangial proliferation and inflammation was detected in both SLE patients and humanized SLE mice. Together, these results support a pathogenic role for T_H17 in SLE.

Arg-1 is an enzyme that hydrolyzes the amino acid L-arginine to ornithine and urea (31). Besides hepatocytes, Arg-1 can be produced by MDSCs, alternatively activated macrophage (M2), and neutrophils (47). Here, we showed that, although Arg-1 was detectable in both M-MDSCs and G-MDSCs, the latter population produces significantly more Arg-1 in both HCs and SLE patients. Furthermore, a significant increase in Arg-1 production was detected in G-MDSCs but not in M-MDSCs of the patients with SLE compared to MDSCs isolated from HCs. This indicates that G-MDSCs are the major contributors of elevated Arg-1 levels in the serum in SLE patients.

Cytokines play an important role in MDSC expansion under pathological conditions, such as cancer, trauma, and chronic inflammation (48). IL-17A has been shown to promote GM-CSF-mediated neutrophilia (49) and MDSC infiltration into tumor site (50, 51). Although IL-17 may potentially contribute to the observed MDSC expansion in SLE patients, our data showed that this cytokine does not stimulate Arg-1 expression in MDSCs. Instead, we found that IL-6, which is also elevated in SLE patients, can significantly increase Arg-1 production in MDSCs. This is consistent with a previous report showing that IL-6 induces Arg-1 production in mouse macrophages through signal transducer and activator of transcription 3 (STAT3) and CCAAT/enhancer binding protein β (C/EBP β) (25).

Arg-1 has been reported to negatively regulate immunity (31, 47, 52–55), but in murine autoimmune model of MS, increased Arg-1 production was paradoxically detected in the spinal cord and inhibition of Arg-1 resulted in amelioration of the disease (31, 56). To date, there is no report that directly links Arg-1 to T_H17 responses. Our data show that the high level of serum Arg-1 activity was correlated positively with IL-17 activity in SLE patients. Furthermore, Arg-1 was found to enhance T_H17 differentiation both in vitro and in vivo (that is, humanized SLE mice). Although IL-1 β produced by MDSCs has been reported to play a role in promoting T_H17 differentiation in mouse models of autoimmune diseases (11, 13, 21), Arg-1 inhibition does not affect IL-1 β secretion by MDSCs (13). Together, these data suggest that Arg-1 may facilitate T_H17 immunity via an IL-1 β -independent mechanism.

We showed that MDSCs up-regulate ROR γ t, mTOR, and GCN2 via an Arg-1-dependent mechanism. mTOR promotes T_H17 differentiation through multiple mechanisms including phosphorylation and activation of STAT3 (57), suppression of growth factor independent 1

(⁵⁸), direct promotion of ROR γ t translocation (⁵⁹), and induction of hypoxia-inducible factor 1 α expression (⁶⁰). The cytoplasmic kinase GCN2 is activated under multiple stress stimuli including nutrient deprivation, which in turn phosphorylates eIF2 α at Ser51 (³², ³³). Thus, the observed MDSC-induced GCN2 activation is likely mediated by Arg-1–induced amino acid starvation. Although stress-induced phosphorylation of eIF2 α suppresses general translation initiation, it may enhance the synthesis of stress-responsive proteins, for example, activating transcription factor 4 (³⁴, ³⁵). It has been reported that stress, such as hypoxia, up-regulates the expression of ROR α (⁶¹), a cotranscription factor for T_H17 development (⁶²). Together, these results suggest that Arg-1–dependent stimulation of T_H17 responses by MDSCs is likely mediated by multiple mechanisms involving ROR γ t, ROR α , and mTOR.

The poor long-term survival of human myeloid cells in immunodeficient mice presents a potential limitation for the humanized SLE model. Moreover, the lack of specific markers for identifying functional MDSCs makes it difficult to track these cells in vivo. However, previous studies suggest that long-term survival is not required for the immunoregulatory effects of adoptively transferred MDSCs because donor MDSCs normally execute their immune regulatory effects within a few days after transfer (⁷, ¹³, ²¹, ⁶³, ⁶⁴). We found that NOD/SCID mice receiving unmanipulated, but not MDSC-depleted, PBMCs from SLE patients showed the development of lupus nephritis–like symptoms and the associated T_H17 cell activation. The observed significant inhibition of disease progression by depletion of MDSCs from the PBMC inoculum is considered a confirmation of the functional status of adoptively transferred human MDSCs in the NOD/SCID mouse recipients.

In summary, the present study reveals a previously unknown role for MDSC-derived Arg-1 in promoting T_H17 cell differentiation in the context of inflammatory condition and autoimmune disease. Our data highlight the importance of the MDSC–T cell interaction in shaping of autoimmune T cell responses and suggest that MDSCs may provide a promising target to develop an efficacious treatment for T_H17-driven autoimmune disorders such as SLE.

MATERIALS AND METHODS

Study design

Thirty-two patients fulfilling the 1982 American College of Rheumatology criteria (⁶⁵) for the classification of SLE were randomly recruited between May 2012 and Jun 2014 from the First Hospital of Jilin University (table S1). Thirty-one of them had lupus nephritis. Disease activity at the time of blood drawn was evaluated with the SLEDAI (²³). The exclusion criteria were pregnancy and acute infection. PBMCs from patient nos. 27 to 32 with lupus nephritis were used to construct humanized SLE mice. Healthy volunteers were randomly recruited as HCs with an effort to match age and gender of SLE patients. For all the experiments using clinical samples, we have ensured blinded outcome assessment. All participants were given written informed consent, and all procedures in this study were approved by the ethics committee of the First Hospital of Jilin University.

Cell preparations

PBMCs were prepared by density gradient centrifugation using Histopaque-1077 (Sigma). Naïve CD4⁺ T cells were isolated from PBMCs using naïve CD4⁺ T cell isolation kit II (Miltenyi Biotec) according to the manufacturer's instruction, and the purity of the cells after separation was >98%. MDSCs were isolated or depleted from PBMCs by cell sorting using a cell sorter (Influx, Becton Dickinson).

Flow cytometric analysis

Flow cytometry was used to determine the phenotypes of human MDSCs and T cells using various combinations of the following fluorochrome-conjugated mAbs: anti-human HLA-DR (TU36), CD11b (ICRF44), CD33 (WM53), CD14 (M5E2), CD4 (RPA-T4), IL-17A (SCPL1362), and IL-17F (O33-782) from BD Pharmingen; anti-human arginase-1 (658922, R&D Systems); CD14 (61D3, eBioscience); CD33 (WM53, eBioscience); and CD66b (G10F5, BioLegend). Isotype controls used include mouse IgG1 (X40, MOPC-21), IgG2a (G155-178), and IgG2b (27-35) from BD Pharmingen and mouse IgG2b (MPC-11) from BioLegend. For intracellular staining, cells were first stained for surface antigens, fixed, and permeabilized with intracellular fixation and permeabilization buffer (eBioscience), followed by staining with fluorochrome-conjugated mAb against respective intracellular proteins. All samples were collected on a fluorescence-activated cell sorter (LSRFortessa, Becton Dickinson) and analyzed by FlowJo software (Tree Star). Isotype controls and gating strategy for flow cytometric analyses are presented in the Supplementary Materials (fig. S7).

Humanized mouse SLE model

Female NOD/SCID mice (4 to 5 weeks old) were obtained from Vital River Laboratories and housed in specific pathogen-free conditions. Humanized NOD/SCID mice with SLE-like syndrome were prepared as previously described⁽³⁶⁾. Briefly, PBMCs prepared from SLE patients with active disease (SLEDAI, 9; dsDNA, 1:10) and lupus nephritis were either unmanipulated or depleted of MDSCs (that is, CD11b⁺HLA-DR⁻ cells) by cell sorting and intravenously injected into NOD/SCID mice (5×10^6 to 10×10^6 cells per mouse). Some mice were treated with intraperitoneal injection of nor-NOHA (400 µg/day, 5 days/week; Cayman) for 4 weeks.

T_H17 polarization

Purified CD4⁺CD45RA⁺ naïve T cells (2×10^5 to 5×10^5 per well) were cultured for 6 or 7 days under T_H17 differentiation condition with plate-bound anti-human CD3 (OKT-3, BioXcell), soluble antihuman CD28 mAb (CD28.2, eBioscience), human TGF-β (5 ng/ml), human IL-6 (20 ng/ml), human IL-23 (10 ng/ml), human IL-1β (10 ng/ml) (PeproTech), anti-IFN-γ (5 µg/ml) (NIB42, eBioscience), and anti-IL-4 (MP4-25D2, eBioscience). In some experiments, MDSCs (at a 1:1 ratio to the naïve CD4 T cells) and nor-NOHA (at 300 µM) were added to determine the role of MDSCs and Arg-1 in T_H17 cell differentiation. The cells were further stimulated with PMA (300 ng/ml) plus ionomycin (1 µg/ml) (Sigma) for the last 5 hours in the presence of brefeldin A (BD Pharmingen) and then stained intracellularly with anti-IL-17A, anti-IL-17F, anti-mTOR (eBioscience), anti-RORγt (BD Pharmingen), anti-GCN2 (ab134053, Abcam), anti-EIF2S1 (eIF2α-P; Abcam), or anti-AHR

(eBioscience). IL-17A and IL-17F concentrations in the culture supernatants were measured in duplicate by human IL-17A (BioLegend) and IL-17F (eBioscience) ELISA kits, respectively.

Quantification of serum arginase activity and IL-6

Arginase activity was quantified by QuantiChrom arginase assay kit (Bioassay Systems) according to the manufacturer's instructions, and data are expressed as enzyme activity (units per liter). Serum levels of human IL-6 were measured using an ELISA kit according to the manufacturer's instructions (R&D Systems).

Detection of serum human anti-dsDNA antibody in mice

Serum samples were collected from humanized SLE mice 4 to 5 weeks after injection of patient PBMCs and examined for anti-dsDNA antibodies using an anti-dsDNA Ab ELISA kit (YHLO Biotech).

Measurement of urinary albumin and creatinine

Urine samples were collected from humanized SLE mice, and the levels of albumin and creatinine were measured by Albuwell M and Creatinine Companion kit (Exocell), respectively, according to the manufacturer's instructions. Proteinuria is expressed as the ratio of the urinary albumin (microgram) to urinary creatinine concentration (milligram).

Histology

Renal tissues from SLE patients and humanized SLE mice were fixed in 10% buffered formalin or cryopreserved in optimal cutting temperature (OCT) compound. Paraffin sections were prepared and stained with H&E and PAS. For immunofluorescence, frozen tissue sections were prepared and stained with rabbit anti-IL-17A (Abcam) or mouse anti-IL-17F (mouse IgG1; Santa Cruz Biotechnology) antibodies, or isotype control antibodies (that is, rabbit IgG and mouse IgG1 for IL-17A and IL-17F, respectively), followed by staining with Alexa Fluor 594-conjugated anti-rabbit IgG or Alexa Fluor 488-conjugated anti-mouse IgG, respectively. Human IgG deposition in tissues was determined on frozen sections by staining with Alexa Fluor 488-conjugated anti-human IgG (Dako). Immunofluorescence staining with isotype control antibodies is presented in the Supplementary Materials (fig. S8).

Quantitative real-time PCR

RNA was extracted and complementary DNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen). All PCRs were triplicated and carried out on an ABI StepOnePlus system (Applied Biosystems) with TransScript Green Two-Step qRT-PCR SuperMix (TransGen Biotech). mRNA expression levels were quantified using primers for *il17a* or *arginase-1*, and *actin* was used as an internal control for normalization by standard 2^{-CT} calculation as described previously (⁶⁶). The primer sequences are as follows: *il17a*, 5'-ACCAATCCCAAAGGTCCTC-3' (forward) and 5'-GGGGACAGAGTTCATGTGGT-3' (reverse); *harginase-1*, 5'-GTTTCTCAAGCAGACCAGCC-3' (forward) and 5'-GCTCAAGTGCAGCAAAGAGA-3'

(reverse); and *hmactin*, 5'-TTCAACACCCCAGCCATG-3' (forward) and 5'-CCTCGTAGATGGGCACAGT-3' (reverse).

Statistical analysis

Statistical analyses were performed on GraphPad Prism 5.0 software. Data are expressed as means $\pm$ SD. Between-group comparisons were performed using two-tailed *t* test, whereas multiple-group comparisons were performed using one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. The Spearman rank test was used for analysis of correlation. A *P* value of <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. MDSC expansion in PBMCs correlated with disease activity in patients with SLE
(A) Staining profiles of MDSCs (CD11b⁺CD33⁺HLA-DR⁻), M-MDSCs (CD11b⁺CD33⁺HLA-DR⁻CD14⁺CD66b⁻), and G-MDSCs (CD11b⁺CD33⁺HLA-DR⁻CD14⁻CD66b⁺) from a representative HC and a representative SLE patient. **(B to D)** Percentages of MDSCs ($P = 0.0001$, t test) **(B)**, M-MDSCs ($P = 0.0001$, t test) **(C)**, and G-MDSCs ($P = 0.0001$, t test) **(D)** in HCs ($n = 25$) and SLE patients ($n = 26$) (left panel), and the correlation analysis between the indicated cell frequency and disease activity (that is, SLEDAI score) in SLE patients (right panel) (MDSCs, $P = 0.0001$; M-MDSCs, $P = 0.0001$; G-MDSCs, $P = 0.0006$).

Fig. 2. Serum Arg-1 activity is correlated with disease activity in patients with SLE

(A) Arginase activity in sera from HCs ($n = 19$) and SLE patients ($n = 26$) ($P = 0.0001$, t test; left panel) and correlation analysis between serum arginase activity and disease activity in SLE ($r^2 = 0.3853$, $P = 0.0007$; right panel). (B) Levels of Arg-1 expression in PBMCs. Left panel shows staining profiles of MDSCs from a representative HC (blue filled) and a representative SLE patient (red line); the gray filled represents isotype control. Data (mean fluorescence intensity; means $\pm$ SD; $n = 3$ per group; $P = 0.0493$, t test) shown in the right panel are a representative of three independent experiments with similar results. (C) Arg-1 expression in M-MDSCs ($n = 3$ per group; $P = 0.45$, t test) and G-MDSCs ($n = 3$ per group; $P = 0.0328$, t test) from HCs and SLE patients. Left panel shows staining profiles of the cells from a representative HC (blue filled) and a representative SLE patient (red line); the gray filled represents isotype control. Data shown in the right panel are a representative of three

independent experiments with similar results. **(D)** Representative images of immunofluorescence staining for Arg-1 in M-MDSCs and G-MDSCs from an HC and an SLE patient. Original magnification, $\times 400$. **(E)** Serum IL-6 in SLE patients and HCs [$n = 14$ (SLE) and $n = 17$ (HC); $P = 0.0002$, t test]. **(F and G)** MDSCs were cultured for 6 to 12 hours with granulocyte-macrophage colony-stimulating factor (GM-CSF; 20 ng/ml), then human IL-6 (10 ng/ml) (F) or human IL-17 (100 ng/ml) (G) was added, and levels of Arg-1 mRNA were measured by qPCR [$n = 3$; $P = 0.0001$ and $P = 0.2268$ for (F) and (G), respectively; t test]. Representative results of three independent experiments are shown.

Fig. 3. TH17 responses in SLE patients

(A and B) PBMCs from HCs and SLE patients were stimulated with PMA and ionomycin and analyzed for IL-17A and IL-17F by intracellular staining. Shown are representative staining profiles (A) and percentages (B) of IL-17A⁺ and IL-17F⁺ cells in gated CD4⁺ T cells [IL-17A: $n = 8$ (SLE) and $n = 8$ (HC); $P = 0.0003$; IL-17F: $n = 5$ (SLE) and $n = 7$ (HC); $P = 0.0004$, t test]. Data shown are a representative of two independent experiments. (C) Serum levels of IL-17A [$n = 22$ (HC) and $n = 26$ (SLE); $P = 0.0001$, t test] and IL-17F [$n = 17$ (HC) and $n = 20$ (SLE); $P = 0.0015$, t test] in HCs and SLE patients. Each symbol

represents an individual HC or patient. **(D)** Correlation analysis between serum IL-17A or IL-17F and disease activity in SLE patients. Each symbol represents an individual patient (IL-17A: $n = 26$, $r^2 = 0.2642$, $P = 0.007$; IL-17F: $n = 20$, $r^2 = 0.2152$, $P = 0.039$). **(E and F)** Histological analysis of kidney biopsy samples from SLE patients ($n = 5$). Renal biopsy samples from two patients with minimal change disease and a healthy subject were used as controls (Ctrl; $n = 3$). **(E)** Immunofluorescence staining of IL-17A and IL-17F in kidney tissue sections. **(F)** Mesangium proliferation in glomerulus (arrow) identified by PAS staining and focal inflammatory cell infiltration in interstitial space (arrow) in H&E-stained section. Shown are representative images. Original magnification, $\times 400$.

Fig. 4. MDSCs facilitate T_H17 cell differentiation in vitro in an Arg-1-dependent manner (A to F) Naïve CD4 T cells from HCs were cultured under T_H17 differentiation without MDSCs, with autologous MDSCs, or with autologous MDSCs + nor-NOHA and analyzed for the expression of T_H17 cytokines and relevant transcription and signaling factors. Shown are representative staining profiles (A) and percentages (B) of IL-17A⁺ ($n = 3$; T_H17 versus MDSC, $P = 0.0007$; MDSC versus MDSC + nor-NOHA, $P = 0.0013$, t test) and IL-17F⁺ ($n = 3$; T_H17 versus MDSC, $P = 0.0004$; MDSC versus MDSC + nor-NOHA, $P = 0.0004$, t test) cells in CD4⁺ T cells, levels of IL-17A ($n = 3$; T_H17 versus MDSC, $P = 0.0055$; MDSC

versus MDSC + nor-NOHA, $P = 0.0044$, t test) and IL-17F ($n = 3$; T_H17 versus MDSC, $P = 0.0048$; MDSC versus MDSC + nor-NOHA, $P = 0.0051$, t test) in the culture supernatants measured by enzyme-linked immunosorbent assay (ELISA) (C), as well as the representative flow cytometry profiles depicting the expression of ROR γ t and AHR (D), GCN2 and mTOR (E), and IL-17A and EIF2S1 (F) in CD4 T cells. Data are from one representative of three independent experiments. (G) Naïve CD4⁺ T cells from HCs and SLE patients were cultured under T_H17 differentiation without MDSCs, with autologous MDSCs, or with autologous MDSCs plus nor-NOHA, and the levels of IL-17A [$n = 3$; T_H17 versus MDSC (HC), $P = 0.0030$; MDSC (HC) versus MDSC (HC) + nor-NOHA, $P = 0.0106$; T_H17 versus MDSC (SLE), $P = 0.0034$; MDSC (HC) versus MDSC (SLE), $P = 0.0102$; MDSC (SLE) versus MDSC (SLE) + nor-NOHA, $P = 0.0027$; t test] and IL-17F [$n = 3$; T_H17 versus MDSC (HC), $P = 0.0045$; MDSC (HC) versus MDSC (HC) + nor-NOHA, $P = 0.0089$; T_H17 versus MDSC (SLE), $P = 0.0032$; MDSC (HC) versus MDSC (SLE), $P = 0.0075$; MDSC (SLE) versus MDSC (SLE) + nor-NOHA, $P = 0.0023$; t test] in the culture supernatants were quantified by ELISA. Data from one representative of three independent experiments are shown. * $P < 0.05$, ** $P < 0.01$.

Fig. 5. MDSC depletion or Arg-1 inhibition suppresses TH17 responses and proteinuria in NOD/SCID mice receiving PBMCs from SLE patients

(A) Schematics of adoptive transfer of PBMCs from SLE patients to NOD/SCID mice (5×10^6 to 10×10^6 PBMCs per mouse). Three groups of mice each received unmanipulated PBMCs, MDSC-depleted PBMCs, or unmanipulated PBMCs + nor-NOHA treatment (400 µg/day intraperitoneally, 5 days/week), respectively. (B) Serum levels of human anti-dsDNA antibodies ($n = 6$; PBMC versus MDSC-depleted PBMC, $P = 0.0057$; PBMC versus PBMC + nor-NOHA, $P = 0.0370$, t test). (C) Urinary proteinuria levels [presented as the ratios of urinary albumin to creatinine (A/C)] ($n = 5$; PBMC versus MDSC-depleted PBMC, $P = 0.0229$; PBMC versus PBMC + nor-NOHA, $P = 0.0234$, t test). (D) Levels of human IL-17A

mRNA in humanized mouse spleen ($n = 6$; PBMC versus MDSC-depleted PBMC, $P = 0.0001$; PBMC versus PBMC + nor-NOHA, $P = 0.0001$, t test) (top) and kidney ($n = 6$; PBMC versus MDSC-depleted PBMC, $P = 0.0001$; PBMC versus PBMC + nor-NOHA, $P = 0.0001$, t test) (bottom). (**E** and **F**) Immunofluorescence staining for human IL-17A and IgG (**E**) and PAS staining (**F**) (arrowhead indicates mesangial proliferation) of kidney sections from representative humanized mice.