Low expression of CD39 on regulatory T cells as a biomarker for resistance to methotrexate therapy in rheumatoid arthritis

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Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterized by joint destruction and severe morbidity. Methotrexate (MTX) is the standard first-line therapy of RA. However, about 40% of RA patients are unresponsive to MTX treatment. Regulatory T cells (Tregs, $CD4^+CD25^+FoxP3^+$) are thought to play an important role in attenuating RA. To investigate the role of Tregs in MTX resistance, we recruited 122 RA patients (53 responsive, R-MTX; 69 unresponsive, UR-MTX) and 33 healthy controls. Three months after MTX treatment, R-MTX but not UR-MTX showed higher frequency of peripheral blood $CD39^+CD4^+CD25^+FoxP3^+$ Tregs than the healthy controls. Tregs produce adenosine (ADO) through ATP degradation by sequential actions of two cell surface ectonucleotidases: CD39 and CD73. Tregs from UR-MTX expressed a lower density of CD39, produced less ADO, and had reduced suppressive activity than Tregs from R-MTX. In a prospective study, before MTX treatment, UR-MTX expressed a lower density of CD39 on Tregs than those of R-MTX or control ($P < 0.01$). In a murine model of arthritis, CD39 blockade reversed the antiarthritic effects of MTX treatment. Our results demonstrate that MTX unresponsiveness in RA is associated with low expression of CD39 on Tregs and the decreased suppressive activity of these cells through reduced ADO production. Our findings thus provide hitherto unrecognized mechanism of immune regulation in RA and on mode of action of MTX. Furthermore, our data suggest that low expression of CD39 on Tregs could be a noninvasive biomarker for identifying MTX-resistant RA patients.

methotrexate | rheumatoid arthritis | adenosine | biomarker | ectonucleotidases

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterized by chronic and deforming destructive polyarthritis and occasionally extraarticular features accompanied by systemic dysfunctions in many patients. Multiple clinical features of RA and heterogeneous therapeutic responses may be influenced by genetic, environmental, and immunologic factors (1). Although some of these features have been used for disease stratification and prognostic (2, 3), they have failed to predict the response to treatments. The first-line pharmacotherapy for RA is low-dose methotrexate (MTX), an antimetabolic drug and an inhibitor of dihydrofolate reductase/folic acid metabolism (4). However, a significant percentage of RA patients are resistant to MTX treatment, which compels the later use of other therapeutic strategies, especially immunobiologics, such as TNF-α targeting (5). The reasons for the unresponsiveness to MTX therapy remain unknown. Understanding MTX resistance would be important, and a biomarker identifying unresponsive MTX (UR-MTX) patients would be valuable to start alternative effective therapies without undue delay.

In addition to its anti-folate effect, MTX also dampens inflammation by maintaining high levels of extracellular adenosine (ADO) (4, 6). MTX inhibits the enzyme 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformilase, leading to the accumulation of AICAR. AICAR accumulation culminates in the release of ATP to extracellular compartment. The ectonucleoside triphosphate diphosphohydrolase-1 (CD39/ENTP1) hydrolyzes ATP and ADP to AMP, and subsequently, the ecto-5′-nucleotidase (CD73) degrades AMP to ADO (7). Blockage of adenosine receptors reduces the anti-inflammatory effect of MTX in humans and in a murine model of arthritis (8, 9). Furthermore, MTX failed to inhibit carrageenan-induced inflammation in CD73-deficient mice (10).

Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells critical for immune homeostasis, preventing the onset of autoimmunity

Significance

Methotrexate (MTX) is the first-line therapy for rheumatoid arthritis (RA). However, about 40% of patients are resistant to MTX. Furthermore, MTX resistance is only apparent after a prolonged continuous MTX treatment (>3 mo), by which time the disease of the nonresponders would have aggravated. Thus, there is a considerable unmet need for a biomarker to select MTX-resistant patients and place them immediately on alternative therapy. We found here that the low density of CD39 on peripheral regulatory T cells in RA patients is a rapid, convenient, and reliable ($P < 0.01$) biomarker for MTX resistance. Our findings also provide previously unrecognized information on aspects of immune regulation in RA and the mechanism of action of MTX.

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diseases. Human and murine Tregs are identified by high expression of CD25 (IL-2α chain receptor) and the master transcription factor forkhead box P3 (FoxP3), which controls Treg development and function (11–13). Tregs suppress the activation, proliferation, and effector functions of a wide range of immune cells via multiple mechanisms (14). One of these mechanisms is via the production of extracellular ADO mediated by CD39/CD73, which are highly expressed on the Treg surface (7). ADO suppresses effector T-cell function through activation of adenosine receptor 2a (A2aR) and adenosine receptor 2b (A2bR) (15, 16), which promote the blockade of cell proliferation, release of cytotoxic granules, expression of FasL, and proinflammatory cytokines secretion (17). Furthermore, activation of A2aR by ADO also enhances the generation of induced Tregs (iTregs) by inhibiting IL-6 expression and enhancing TGF-β secretion (18). In addition, ADO can also affect dendritic cell (DC) function, modulating their maturation and consequently driving them to a tolerogenic phenotype (19, 20).

Given that the anti-inflammatory effects of MTX are associated with ADO generation by CD39/CD73 and the importance of these ectonucleotidases in Treg suppressive function, we investigated the possibility that MTX unresponsiveness in RA patients is associated with a deficiency of ADO generation by Tregs due to a depressed expression of CD39/CD73 on Tregs. We found that after MTX therapy, responsive patients (R-MTX), but not UR-MTX patients, presented enhanced frequency of Tregs compared with healthy donors. Furthermore, Tregs from UR-MTX patients had impaired ADO production compared with Tregs from R-MTX or healthy controls. This impairment was associated with lower CD39 expression on the Treg surface before and after MTX. Moreover, Tregs from UR-MTX patients are less effective in suppressing T-effector cell proliferation compared with those from R-MTX or healthy controls via reduced production of ADO. Taken together, our results provide a hitherto unrecognized mechanism of immune regulation in RA and on mode of action of methotrexate, in addition to the potential value for prediction of response to methotrexate.

Results

Characterization of RA Patients. We recruited 122 random RA patients who received MTX monotherapy at doses from 15 to 20 mg/wk, maintained for at least 4 wk before peripheral blood collection. In some patients, blood samples were also collected before MTX monotherapy. Disease activity was measured using disease activity score, including a 28-joint count (DAS28). RA patients were stratified according to their response to MTX therapy following these criteria: (*i*) UR-MTX ($n = 69$), treated with MTX doses ≥15 mg/wk for at least 3 mo and still presented active disease (DAS28 > 4.0); and (*ii*) R-MTX ($n = 53$), treated with MTX for >3 mo and presented DAS28 < 3.0. We also recruited 33 healthy blood donors for the study.

The clinical and serological features of RA patients are shown in Table S1. There was no significant difference in demographic (sex and age), clinical (time of disease, smoking habits), and serological variables [rheumatoid factor (RF), anticitrullinated protein antibodies (ACPA)] between R-MTX and UR-MTX patients. Parameters related to disease activity [C-reactive protein (CRP) and DAS28] and plasma concentrations of TNF-α and IL-1β were significantly higher in UR-MTX compared with R-MTX following MTX treatment (Fig. S1 *A* and *B*).

Increased Circulating Tregs and Decreased Th17 and Th1 cells in R-MTX Patients. We first determined the level of serum inflammatory cytokines and the frequencies of blood leukocyte subtypes in peripheral mononuclear cell populations from UR-MTX and R-MTX patients 3 mo after MTX treatment. UR-MTX showed significantly higher levels of serum TNF-α and IL-1β compared with R-MTX and healthy controls, although the levels of these cytokines are generally low (10–70 pg/mL; Fig. S1*B*). R-MTX patients showed a higher frequency of IL-10–producing $CD4^+$ T cells compared with UR-MTX patients or healthy controls. The frequency of $CD4^+$ IL-10^+ cells in UR-MTX was not different from healthy controls. In contrast, the frequencies of $CD4^+IL\text{-}17^+$ (Th17) and $CD4^+IFN\text{-}\gamma^+$ (Th1) populations were higher in UR-MTX than in R-MTX or healthy controls (Fig. S1*C*). The frequencies of $CD3^+$, $CD4^+CD3^+$, and $CD8^+CD3^+$ T cells and B cells ($CD19^+CD3^-$) were similar among UR-MTX, R-MTX, and healthy controls (Fig. S2). However, the percentage of dendritic cells (DCs; $CD11b^+$ $CD11c^+$) was higher in RA patients than in healthy controls whether the patients were responsive or not to MTX therapy. Strikingly, R-MTX had markedly higher frequencies and number of peripheral Tregs ($CD4^+CD25^+FoxP3^+$) compared with UR-MTX or controls (Fig. 1 *A* and *B*). There was no difference in the frequency and number of Tregs between healthy controls and UR-MTX groups. These data suggest that the therapeutic effectiveness of MTX could be associated with an increase of circulating Tregs in RA patients.

Expression and Function of CD39 and CD73 on Tregs from RA Patients. We next determined the expression of CD39 and CD73 on the peripheral blood mononuclear cells (PBMCs) of RA patients. R-MTX had a higher frequency of $CD39^+$ $CD4^+$ T cells compared with UR-MTX or healthy controls (Fig. 1 *C* and *D*), whereas $CD73^+CD4^+$ T-cell frequencies were not different in all investigated groups (Fig. 1 *E* and *F*). There was also no difference in the percentages of CD39 or CD73 expressing $CD8^+CD3^+$ T cells, B cells, or DCs in all three groups analyzed (Fig. S3). The frequencies of $CD39^+CD4^+CD25^+$ T cells and $CD39^+CD4^+$ $CD25^+FoxP3^+$ Tregs were significantly higher in the R-MTX than those in the UR-MTX or healthy controls (Fig. 1 *G* and *H*). There was no difference in the percentage of $CD39^+$ cells on the Treg population between healthy controls and UR-MTX patients. There was also no difference in the relatively low percentage of $CD39^+$ cells in the population of $CD4^+CD25^-$ cells (effectors T lymphocytes) among the three groups (Fig. 1*I*). There was no difference in the frequency of $CD73^+$ T cells among the Treg populations (Fig. S4*A*).

We then determined the density of CD39 expression [mean fluorescent intensity (MFI)] on Tregs from RA patients. The CD39 MFI on $CD4^+CD25^+$ cells from UR-MTX patients were markedly lower compared with healthy controls or R-MTX patients (Fig. 2 *A* and *B*). This finding is specific for the $CD4^+$ $CD25^+$ cells population, because there was no difference in the relatively low MFI of CD39 expression on $CD4^+CD25^-$ cells in the three investigated groups. There was also no difference in the CD73 MFI on $CD4^+CD25^+$ (Fig. S4*B*). These results suggested an association between CD39 expression on Tregs and responsiveness to MTX. Furthermore, the data also indicate that Tregs from UR-MTX express significantly lower density of CD39 than that on the Tregs from R-MTX or healthy controls.

Next we examined whether the lower expression of CD39 on Tregs from UR-MTX patients leads to impaired conversion of ADP into extracellular ADO. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified from the peripheral blood of RA patients and healthy donors. The purity of the $CD4^+CD25^+$ were >85%, of which >77% were $FoxP3^+$ (Fig. S5). The cells were incubated with ADP, and the ADO generated in the culture supernatant was quantified by HPLC. The ability of Tregs from UR-MTX to generate ADO was markedly impaired compared with that of the Tregs from R-MTX patients or healthy individuals (Fig. 2 *C* and *D*). This difference was not observed in the relatively lower ADO production by $CD4^+CD25^-$ (effectors T cells) cells from all of the three groups analyzed (Fig. 2*D*). The conversion of ADP by ectonucleotidases was also quantified by the production of inorganic phosphate (Pi) with the colorimetric Malachite Green

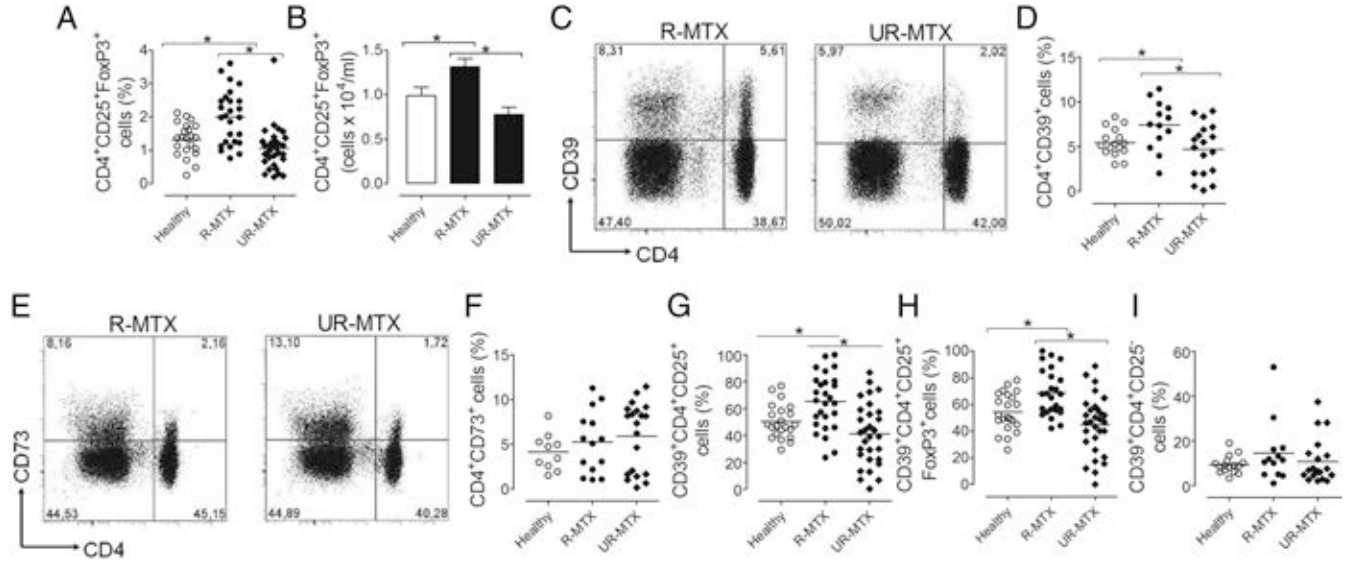

Fig. 1. Increased frequency of circulating Tregs in R-MTX patients. (*A* and *B*) Frequency and absolute number of $CD4^+CD25^+FoxP3^+$ cells from healthy controls (Healthy) (○, $n = 22$), R-MTX (●, $n = 27$), and UR-MTX (♦, $n = 30$) RA patients analyzed by flow cytometry. (*C*) Representative dot plot showing frequency of $CD4^+CD39^+$ cells from R-MTX and UR-MTX patients. (*D*) Percentage of $CD4^+CD39^+$ cells from healthy donors (○, $n = 16$), R-MTX (●, $n = 13$), and UR-MTX (♦, $n = 20$). (*E*) Representative dot plot showing percentage of $CD4^+CD73^+$ cells from R-MTX and UR-MTX patients. (*F*) Percentage of $CD4^+CD73^+$ cells from healthy donors (○, $n = 10$), R-MTX (●, $n = 13$), and UR-MTX (♦, $n = 20$). (*G* and *H*) Frequency of $CD4^+CD25^+$ (*G*) and $CD4^+CD25^+FoxP3^+$ (*H*) cells expressing CD39 in healthy donors (○, $n = 22$), R-MTX (●, $n = 27$), or UR-MTX (♦, $n = 30$). (*I*) Frequency of $CD4^+CD25^-$ cells expressing CD39 in healthy donors (○, $n = 16$), R-MTX (●, $n = 13$), or UR-MTX (♦, $n = 20$). Horizontal bars = mean; vertical bars = SEM. $*P < 0.05$.

assay. Consistent with results from the ADO assay, the Pi concentration in the supernatants of $CD4^+CD25^+$ cells from UR-MTX was significantly lower compared with those of R-MTX or healthy controls (Fig. 2*E*). The Pi concentrations in the supernatant of $CD4^+CD25^-$ cells remained at the background level for all of the three groups.

We then investigated the relative suppressive activity of the Tregs from the RA patients. $CD4^+CD25^+$ T cells were purified from RA patients and healthy donors and cultured with $CD4^+CD25^-$ T cells at a graded ratio of Teff: Treg. The proliferation of Teff cells against polyclonal activation (anti-CD3/CD28) was then tracked by Dye Eflour 670. Tregs from UR-MTX show significantly lower potency in suppressing Teff cells compared with those from R-MTX or healthy controls at a 1:1 and 1:0.5 Teff: Treg ratio (Fig. 2 *F* and *G*).

An additional potential mechanism of MTX unresponsiveness in RA patients might involve the reduction of adenosine receptors expression on the surface of leukocytes, especially A2aR and A2bR, which mediates anti-inflammatory response of ADO (21). However, there was no difference in the level of A2aR and A2bR mRNA expression in the PBMC cells from the three groups analyzed (Fig. S6).

Together, these data demonstrate that the reduced CD39 expression observed on Tregs from UR-MTX patients leads to decreased production of extracellular ADO, with subsequent diminished Treg suppressive potential.

CD39 Expression on Tregs Before and After MTX Treatment in RA Patients. We next investigated whether the increase in the frequency of Tregs and $CD39^+$ Tregs in R-MTX patients is a consequence of MTX treatment or is it intrinsic to these patients. To address this question, we determined the frequency of Tregs and $CD39^+$ Tregs, as well as the density of CD39 expression on Tregs in RA patients before and after MTX treatment (at least 3 mo of MTX administration at doses ≥15 mg/wk). First, we demonstrated that the MTX resistance is not a consequence of a low availability of intracellular active MTX (22, 23). We measured total MTX polyglutamate metabolites levels in erythrocytes from RA patients treated at least 3 mo with MTX. The concentrations of MTX polyglutamate are at reported saturation level and similar in R-MTX and UR-MTX groups (Fig. S7).

There was no difference in the frequency and absolute number of Tregs between R-MTX and UR-MTX patients before MTX treatment (Fig. S8 *A* and *B*). However, R-MTX showed a significant increase in frequency and number of circulating Tregs after MTX treatment, whereas the Tregs of UR-MTX remained unchanged. We also examined the frequency of $CD39^+$ Tregs before and after MTX treatment. There was no difference in the percentage of peripheral $CD39^+CD4^+CD25^+FoxP3^+$ T cells among the two groups before MTX treatment. However, the percentage of these cells markedly increased following treatment in the R-MTX but not in the UR-MTX (Fig. S8*C*). These data are consistent with the notion that MTX responsiveness is directly related to the expansion of $CD39^+$ Tregs after MTX treatment in R-MTX but not in UR-MTX, likely via the ADO/A2aR pathway.

Tregs of UR-MTX Patients Express Low Density of CD39 Before and After MTX Treatment. We then examined the density of CD39 expression on Tregs from the RA patients before and after MTX treatment. The MFI of CD39 on $CD39^+CD4^+CD25^+FoxP3^+$ T cells from R-MTX was significantly higher than those from the UR-MTX (Fig. 3 *A* and *B*). Importantly, the CD39 MFI of UR-MTX was significantly lower than those of the R-MTX and healthy controls before and after MTX treatment (Fig. 3*C*; $P < 0.01$). Strikingly, the MFI of CD39 expression was not altered following MTX treatment in all RA patients. Fig. 3*D* shows the combined data of CD39 density on Tregs from RA patients after MTX treatment. These results show that reduced density of CD39 on Tregs is strongly associated with the failure to responsive to MTX. Crucially, the low level of CD39 expression before MTX treatment could be used to predict MTX unresponsiveness, with >99% confidence.

Effect of MTX on the Development of Experimental Arthritis. To examine the relevance of our finding in vivo, we used the murine antigen-induced arthritis (AIA) model. C57BL/6 FoxP3-GFP

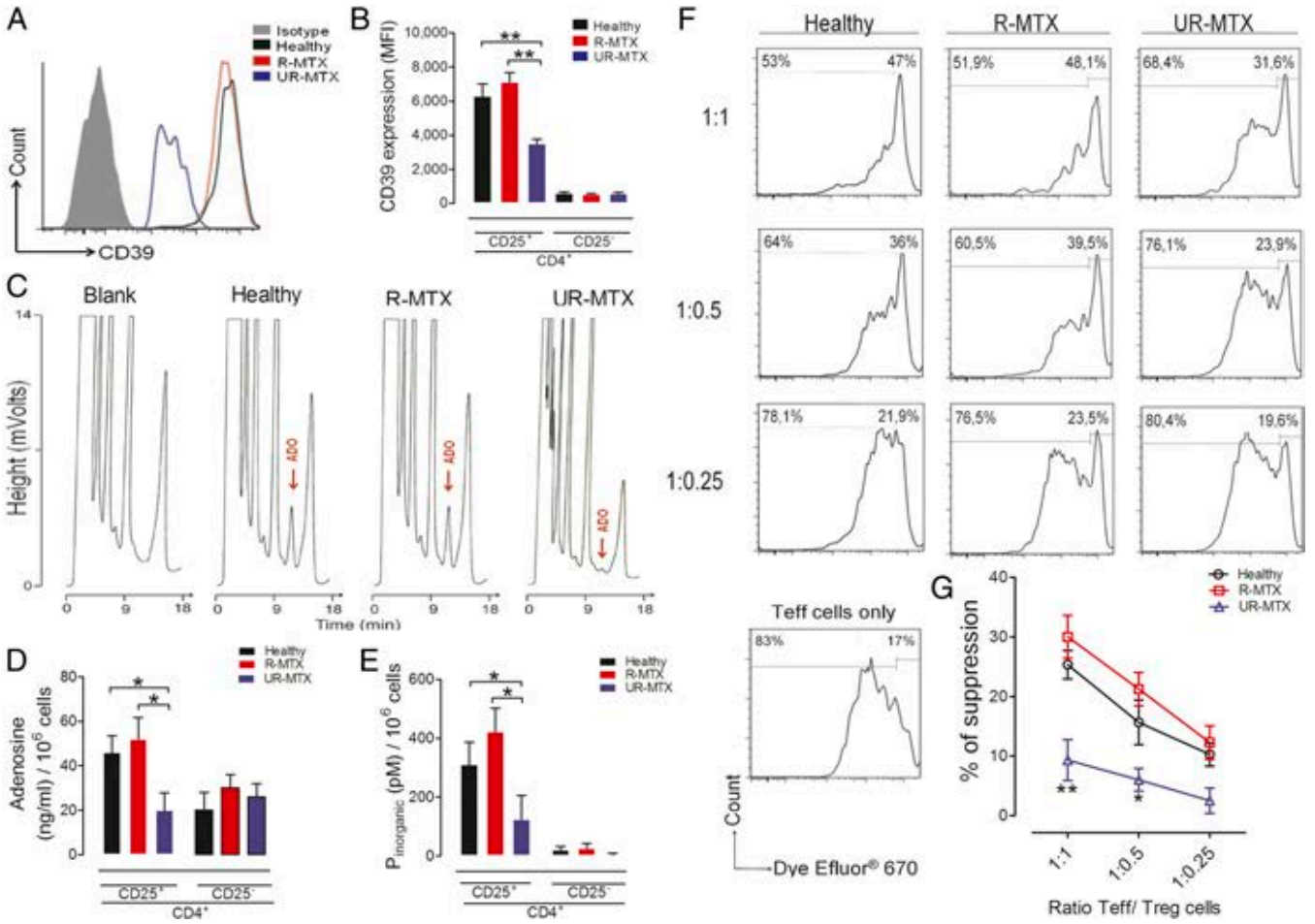

Fig. 2. Low CD39 expression, impaired ADO production, and reduced suppressive function of Tregs from UR-MTX patients. (*A*) Representative histogram of CD39 expression on $CD4^+CD25^+$ cells from healthy individuals, R-MTX, and UR-MTX patients. (*B*) Mean fluorescence intensity (MFI) of CD39 on $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from healthy donors ($n = 10$), R-MTX ($n = 13$), and UR-MTX ($n = 20$) 3 mo after MTX treatment. $^{**}P < 0.01$. (*C*) Representative chromatograms of ADO levels in culture supernatant of Tregs from healthy donors, R-MTX, and UR-MTX patients incubated with ADP. Tregs from healthy donors cultured without ADP serve as control (Blank). (*D*) Concentration of ADO in the supernatant of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells from healthy individuals ($n = 5$), R-MTX ($n = 6$), and UR-MTX patients ($n = 6$). $^*P < 0.05$. (*E*) Concentration of Pi in the supernatant of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells of healthy controls ($n = 5$), R-MTX ($n = 6$), and UR-MTX ($n = 6$) patients. $^*P < 0.05$. (*F*) Representatives histograms of the percentages of proliferation of Teff cells stained with Dye Efluor 670 and incubated with Treg cells from healthy or MTX-treated RA donors at the indicated Treg:Teff ratio. (*G*) Percentage of suppression of Teff by Tregs from healthy ($n = 5$), R-MTX ($n = 5$), and UR-MTX ($n = 4$) donors, using the formula [Teff only – (Treg + Teff)/Teff only] × 100. $^*P < 0.05$ and $^{**}P < 0.01$ compared with healthy controls. All data are mean ± SEM.

mice were immunized with methylated BSA (mBSA) in Freund's complete adjuvant and challenged in the joint with soluble mBSA after two booster injections. MTX was administered orally (2 mg/kg) once a week for 5 wk during the immunization protocol. MTX markedly reduced arthritis development as evidenced by a decrease of neutrophil migration into the joint and attenuation of mechanical articular hyperalgesia (Fig. 4 *A* and *B*). The effect of MTX treatment was not due to impaired mBSA priming, because the plasma titers of specific anti-mBSA IgG were similar between the MTX- and PBS-treated groups (Fig. 4*C*). To address the possibility that MTX treatment could induce apoptosis of activated T cells (24, 25), we assess the level of apoptotic $CD4^+$ T cells in the spleen and draining lymph nodes of MTX-treated mice. Under our experimental conditions, the apoptosis rate of activated ($CD44^+$) and naive ($CD44^-$) $CD4^+$ cells were similar in naïve, immunized, and MTX-treated and untreated mice (Fig. S9 *A* and *B*). Similar to that seen in the RA patients, MTX treatment significantly increased the Treg ($CD4^+FoxP3^+$) population in the spleen of arthritic mice (Fig. 4*D*).

To determine whether the effectiveness of MTX therapy is dependent on Treg expansion, mice were treated with α-CD25 antibody during MTX treatment. In our model, α-CD25 antibody had only a modest effect on arthritis development in mice not treated with MTX, but completely reversed the antiarthritic effect of MTX (Fig. 4 *E* and *F*). Naive mice treated with vehicle, anti-CD25, or MTX did not induce signs of hyperalgesia or neutrophil infiltration. These results show that the antiarthritic effect of MTX is likely Treg dependent.

To assess the role of CD39 in the antiarthritic effect of MTX in vivo, mice were treated with a selective CD39 inhibitor (CD39i, ARL67156, 1 mg/kg) (26) during MTX treatment. CD39i increased the arthritic severity in immunized mice not treated with MTX and completely abolished the antiarthritic effect of MTX as measured by neutrophil infiltration and hyperalgesia (Fig. 4 *G* and *H*). Consistent with this observation, the increased frequency of Tregs induced by MTX was also abrogated by CD39i (Fig. 4*I*). This finding was confirmed by histological analysis. The joints of immunized mice 14 d after challenge developed severe synovitis (inflammatory cell influx)

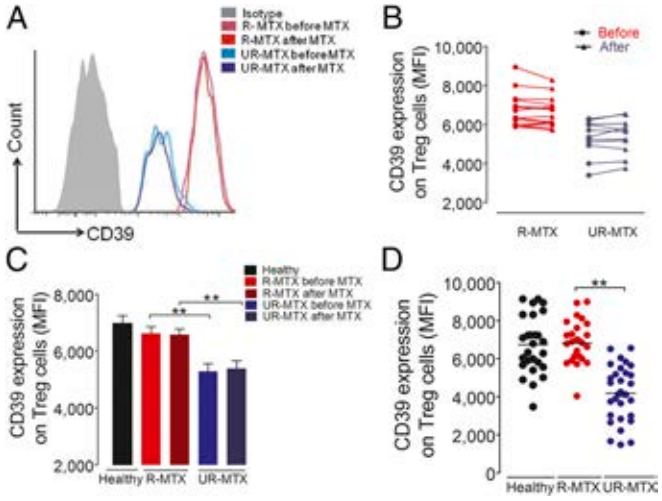

Fig. 3. Low CD39 expression density on Tregs in UR-MTX patients before and after MTX treatment. (*A*) Representative histogram of CD39 expression on the Tregs from R-MTX and UR-MTX patients before and after MTX treatment. (*B*) MFI of CD39 on $CD4^+CD25^+FoxP3^+$ Tregs from R-MTX ($n = 15$) and UR-MTX ($n = 11$) before (●) and after (▲) MTX treatment. (*C*) MFI of CD39 on the Tregs from R-MTX ($n = 15$) and UR-MTX ($n = 11$) before and after MTX treatment and healthy controls ($n = 16$). $^{**}P < 0.01$. (*D*) MFI of CD39 on $CD4^+CD25^+$ cells from healthy donors ($n = 26$), R-MTX ($n = 28$), and UR-MTX ($n = 31$) after MTX treatment. $^{**}P < 0.01$.

and tissue destruction compared with that of the naïve mice (Fig. S10 *A* and *B*). The inflammation and cartilage destruction in the joints of the immunized mice was not affected by the administration of CD39i. The joints of immunized mice treated with MTX show minimal cellular infiltration and tissue destruction, clearly demonstrating the protective effect of MTX in this model of arthritis. The joint-protective effect of MTX was completely abolished by CD39i (Fig. S10*C*). Together, these data show that CD39 is strongly associated with the antiarthritic effect of MTX and is key to the in vivo expansion of the MTX-induced Treg population.

Discussion

A number of studies reported potential genetic and epigenetic biomarkers predicting therapeutic efficacy to MTX. Polymorphisms within the *MTHFR* (methylenetetrahydrofolate reductase) gene, which encodes methylene-tetrahydrofolate reductase, an essential enzyme in the folate pathway, were associated with MTX response (27). Polymorphisms in the *SCL19A1* (solute carrier family 19, member 1) gene encoding the protein folate transporter 1 have also been described to implicate the therapeutic efficacy of the MTX (28). However, subsequent investigations produced conflicting results (29, 30). Thus, the mechanisms of, and functional biomarker for, the development of MTX resistance remains poorly understood (31).

We demonstrate here that failure of MTX treatment in RA patients is closely associated with a low expression density (MFI) of CD39 on Tregs. This phenomenon is observed on Tregs and is related to impaired production of ADO and reduced suppressive activity of these cells. Furthermore, MTX treatment increases the frequency of circulating CD39-expressing Tregs in R-MTX patients but not in UR-MTX patients. In a prospective study, before MTX treatment, UR-MTX patients presented lower density of CD39 expression on Tregs compared with that of R-MTX patients or healthy controls. However, the density of CD39 on the Tregs was not affected by MTX treatment. These data therefore strongly indicate that low expression density of CD39 on Tregs could be a biomarker for predicting unresponsiveness to MTX in RA patients. Whether a similar mechanism also applies to other disease-modifying antirheumatic drugs (DMARDs) is currently under investigation.

MTX was developed as an analog of folic acid. Thus, the mechanism by which MTX affects cellular functions is expected to be similar to those involved in folate metabolism. The rationale for using MTX to treat RA was based on the assumption that by inhibiting purine and pyrimidine synthesis required for cellular proliferation, MTX would prevent the expansion of the most rapidly dividing lymphocytes or other cells responsible for synovial inflammation. However, neither folic acid nor folinic acid can reverse the anti-inflammatory effects of MTX in RA (4). Thus, it is likely that a mechanism other than the folic acid pathway is responsible for the antiarthritic effect of low-dose MTX treatment.

Recent studies strongly suggest that MTX mediates its anti-inflammatory role in RA via the AICAR pathway, inhibiting the enzyme AICAR transformilase. A consequence of the inhibition of this enzyme is the increase in ADO concentration (Fig. S11). Our results provide clinical and experimental evidence for the mechanism by which MTX attenuates RA via CD39 on Tregs, leading to the increased production of ADO, which is not only directly anti-inflammatory but can also induce more Tregs (iTregs) in a feedback

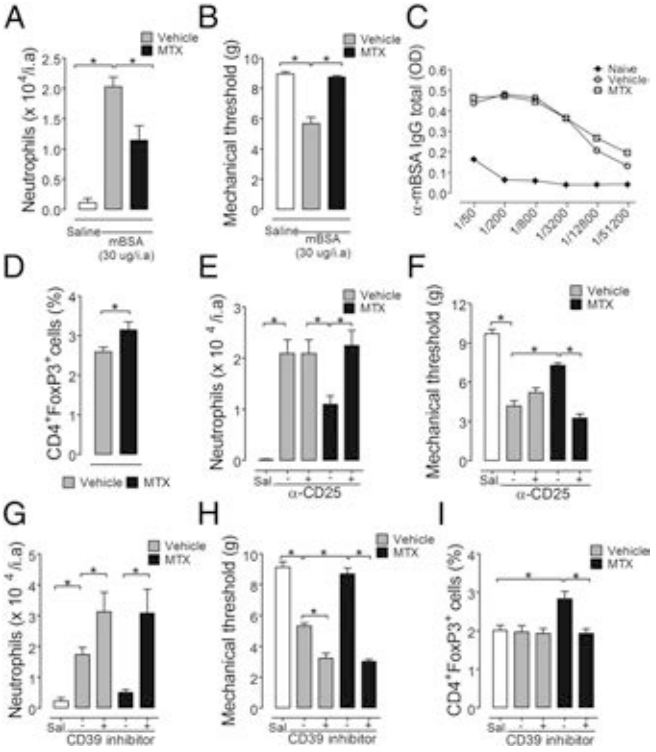

Fig. 4. Effect of MTX on AIA. C57BL/6 FoxP3-GFP mice immunized and boosted with mBSA and treated with MTX or vehicle were challenged intraarticular (i.a.) with mBSA or saline. Neutrophils in the joints (*A*) and intraarticular mechanical hyperalgesia (*B*) were determined. mBSA-specific IgG concentration in the serum was determined by ELISA (*C*). Frequency of $CD4^+FoxP3^+$ cells in the spleen was determined by FACS (*D*). Immunized mice pretreated with MTX or vehicle were injected with anti-CD25 or normal IgG (–) and challenged i.a. with mBSA or saline (Sal). Neutrophils in the joints (*E*) and mechanical hyperalgesia (*F*) were determined. Immunized mice pretreated with MTX or vehicle and injected with a CD39 inhibitor (CD39i, ARL67156) or not (–) were challenged i.a. with mBSA or saline. Neutrophils in the joints (*G*) and mechanical hyperalgesia (*H*) were determined. Frequency of $CD4^+FoxP3^+$ cells in the spleen was determined by FACS (*I*). Data represent mean ± SEM ($n = 5$), representative of two experiments. $^{*}P < 0.05$.

amplification manner. This may partly explain the markedly elevated Treg population in the R-MTX patients. Importantly, we demonstrate that the relative level of CD39 on Tregs plays a central role in determining the responsiveness to MTX treatment in vivo in a mouse model of arthritis.

The basis for the low CD39 expression on Tregs in UR-MTX patients is not immediately apparent. Association of genetic polymorphisms in UR-MTX patients with low CD39 expression may be involved. Genetic polymorphisms in the *ETNP-1* (ectonucleoside triphosphate diphosphohydrolase 1) gene (encoding CD39) that confer a lower CD39 expression have been associated with increased susceptibility to type 2 diabetes (32, 33). Cell signaling pathways that regulate CD39 expression on Tregs may also be involved such as the activation of cAMP-induced increase in CD39 mRNA (34). These possibilities are currently being investigated.

Due to its clinical importance, numerous efforts have been made to identify a biomarker for predicting MTX unresponsiveness. Thus far, pharmacogenetic approaches have produced equivocal results. Here, using an immunological approach, we provide evidence that low expression of CD39 density on Tregs is a potential biomarker for identifying RA patients who would be refractory to MTX treatment. The identification of low CD39 MFI on Tregs by FACS on a small sample of whole peripheral blood represents a noninvasive, rapid, and convenient procedure in predicting MTX unresponsiveness of RA patients with >99% confidence and would thus provide a valuable option for RA therapy.

Materials and Methods

Patients and Healthy Donors. We recruited 122 RA patients who fulfilled the 1987 revised American College of Rheumatology Criteria for RA classification. All patients received MTX monotherapy (15–20 mg/wk) for at least 4 wk before blood collection. Disease activity was measured by DAS28 (Disease Activity Score, including a 28-joint count). RA patients were stratified according to their response to MTX: (*i*) unresponsive RA patients (UR-MTX, $n = 69$), who received MTX doses ≥15 mg/wk for at least 3 mo and still presented DAS28 >4.0; and (*ii*) responsive RA patients (R-MTX, $n = 53$), who received MTX for >3 mo and presented DAS28 < 3.0. No other drugs such as leflunomide, sulfasalazine, cyclosporine, and biologic agents (TNF-α blockers, anti-CD20, and anti-IL-6) were in use at the time of sample collection. The clinical features of RA patients groups are shown on Table S1. Peripheral blood samples of healthy donors ($n = 33$), paired by sex and age, were also collected. All donors provided informed consent to participate in the study, approved by the Local Ethics Committee (Protocol 2981/2009). Subjects presenting other autoimmune or rheumatic diseases and infectious disorders or were serologic positive for Chagas disease, hepatitis B and C, or HIV were excluded. All laboratory analyses of the samples were performed blind to the donor status. Others information regarding the materials and methods are available in *SI Materials and Methods*.

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