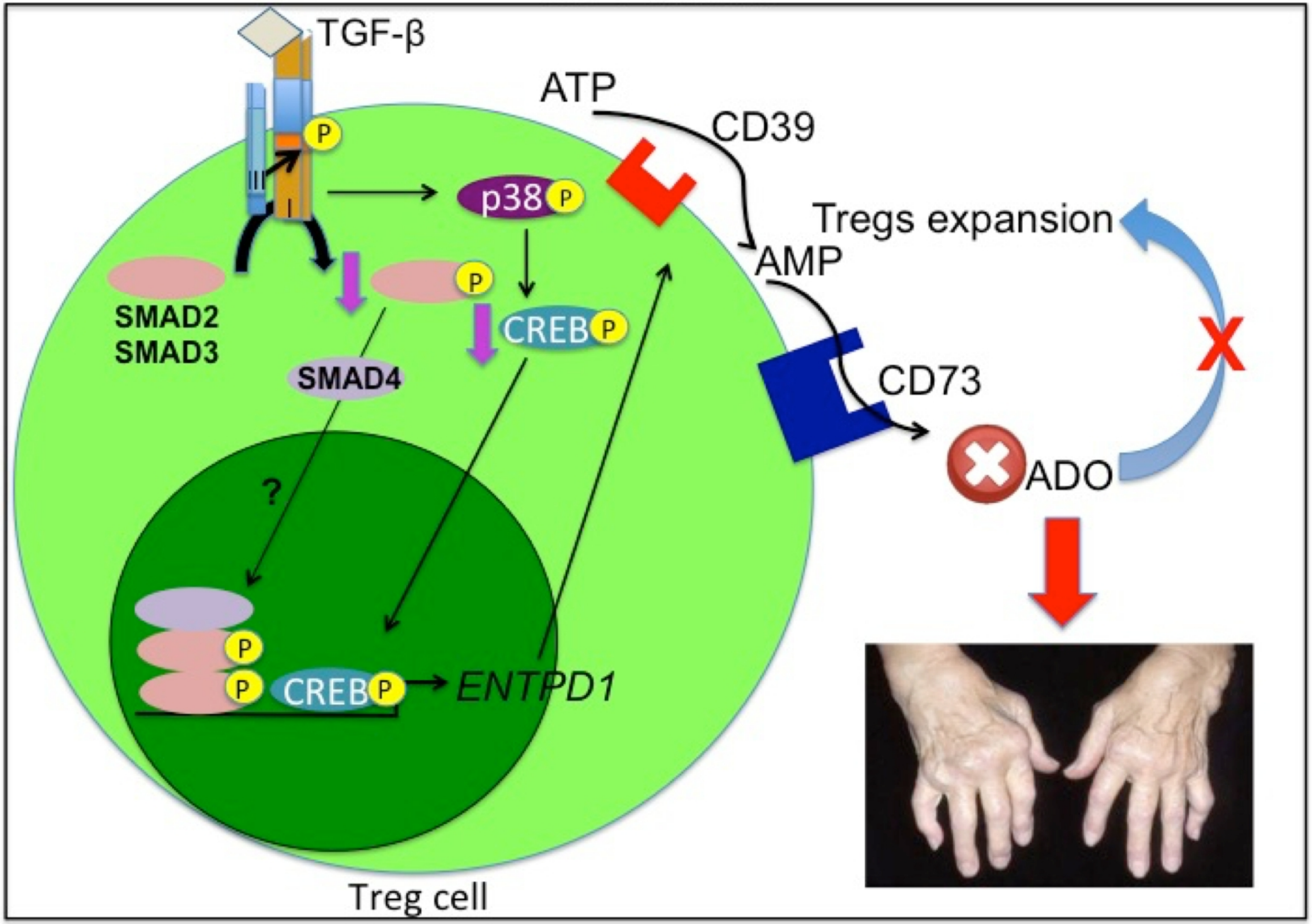
Highlights

- Low CD39 expression on Tregs is a potential biomarker for MTX resistance in RA
- We show here that impairment of TGF-β signalling reduces CD39 expression
- TGF-β-induced CD39 expression is triggered via TGFBRII/TGFBRI, SMAD2 and CREB
- MTX resistance is associated with SNP rs1431131 in the *TGFBR2* gene

UR-MTX


TGF-β
ATP
CD39
P
II
I
p38 P
Tregs expansion
AMP
P
SMAD2
SMAD3
CREB P
SMAD4
CD73
X
ADO
?
P
P
CREB P
ENTPD1
Treg cell

TGF-β signalling defect is linked to low CD39 expression on regulatory T cells and methotrexate resistance in rheumatoid arthritis

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Short title: Association of TGF-β signalling with methotrexate resistance

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ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune arthropathy characterized by chronic articular inflammation. Methotrexate (MTX) remains the first-line therapy for RA and its anti-inflammatory effect is associated with the maintenance of high levels of extracellular adenosine (ADO). Nonetheless, up to 40% of RA patients are resistant to MTX treatment and this is linked to a reduction of CD39 expression, an ectoenzyme involved in the generation of extracellular ADO by ATP metabolism, on circulating regulatory T cells (Tregs). However, the mechanism mediating the reduction of CD39 expression on Tregs is unknown. Here we demonstrated that the impairment in TGF-β signalling lead to the reduction of CD39 expression on Tregs that accounts for MTX resistance. TGF-β increases CD39 expression on Tregs via the activation of TGFBRII/TGFBRI, SMAD2 and the transcription factor CREB, which is activated in a p38-dependent manner and induces CD39 expression by promoting *ENTPD1* gene transcription. Importantly, unresponsive patients to MTX (UR-MTX) show reduced expression of *TGFBR2* and *CREB1* and decreased levels of p-SMAD2 and p-CREB in Tregs compared to MTX-responsive patients (R-MTX). Furthermore, RA patients carrying at least one mutant allele for rs1431131 (*AT or AA*) of the *TGFBR2* gene are significantly ($p = 0.0006$) associated with UR-MTX. Therefore, we have uncovered a molecular mechanism for the reduced CD39 expression on Tregs, and revealed potential targets for therapeutic intervention for MTX resistance.

Key Words: Regulatory T cells; CD39; Methotrexate; TGF-β signalling; Rheumatoid Arthritis.

1. Introduction

Despite advances in the treatment of RA the standard first-line therapeutic strategy for RA comprises the use of low-dose methotrexate (MTX) [1-4]. In addition to its anti-folate effects, MTX-mediated anti-inflammatory effects by maintaining high levels of extracellular adenosine (ADO) through activity of the ectonucleoside triphosphate diphosphohydrolase-1 (CD39/ENTDP1), which generates extracellular ADO by ATP metabolism [1,3–5]. However, around 40% of RA patients are resistant to MTX and unresponsive patients (UR-MTX) are only introduced to alternative therapy after a prolonged treatment of MTX, during which the disease has aggravated [6]. Thus, understanding MTX resistance is important, and a biomarker identifying MTX unresponsive RA patients would be valuable to start alternative effective therapies without undue delay [7]. We have recently demonstrated that UR-MTX patients show a reduction of CD39 expression on circulating regulatory T cells (Tregs), which culminates in an impairment of their capacity to generate extracellular ADO, leading to inability to dampen inflammation [8]. Moreover, we found that UR-MTX patients had lower CD39 expression on Tregs before MTX treatment, suggesting that the evaluation of CD39 expression on Tregs represents a potential non-invasive biomarker predicting MTX resistance in RA [8]. However, the molecular mechanism that contributes to the reduced CD39 expression on Tregs is unknown.

TGF-β is a key inducer of Tregs [9]. TGF-β interacts with both type I (TGFBRI) and type II (TGFBRII) receptors, forming a hetero-tetrameric complex [10], leading to the phosphorylation of SMADs (Mothers against decapentaplegic homologs) [10,11], which translocate to the nucleus and interact with other transcription factors, including CREB (cAMP response element binding) [12–15]. It has been reported that TGF-β is able to induce CREB activation on Tregs [16,17] and that activation of CREB induces CD39 expression on macrophages [18]. Furthermore, TGF-β down-regulates the transcription factor GFI-1 (zinc finger protein growth factor independent-1) that acts as a repressor of the *ENTPD1* (ectonucleoside triphosphate diphosphohydrolase-1) transcription [19].

We therefore hypothesised that TGF-β signalling influences the induction of CD39 on Tregs and its impairment is associated with the reduction of CD39 expression in UR-MTX.

2. Methods

2.1. Reagents

The following materials were used: agonist of the receptor A2a, NECA (5'-N-Ethylcarboxamidoadenosine, Tocris Bioscience), CD39 inhibitor (CD39i, ARL67156, Sigma-Aldrich), Apyrase from potato (Sigma-Aldrich); 8-(3-chloro-styryl)-caffeine (CSC, Sigma-Aldrich), CREB inhibitor (CREBi, Naphthol AS-E phosphate, Sigma-Aldrich); PKA inhibitor (H89, Sigma-Aldrich), TGFBRI inhibitor (SB431542, Sigma-Aldrich), p38 inhibitor (SB 203580, Calbiochem), AKT inhibitor (AKT inhibitor IV, Calbiochem), anti-CD3 and anti-CD28 (BD Biosciences) and RPMI 1640 (Sigma-Aldrich).

2.2. Subjects

For the study of molecular mechanism, we recruited 88 RA patients who fulfilled the 2010 American College of Rheumatology and European League against Rheumatism criteria for RA classification [20]. All patients received MTX monotherapy (15–20 mg/wk) for at least 4 week before blood collection. Disease activity was measured by DAS28 (Disease Activity Score, including a 28-joint count). RA patients were stratified according to their MTX response based on EULAR response criteria [21]: (i) unresponsive RA patients (UR-MTX, n = 38), who received MTX doses 15-20 mg/week for at least 3 months and did not present a change of >0.6 in DAS28, or presented a DAS28 change between 0.6-1.2, but still exhibited DAS28 >5.1; and (ii) responsive RA patients (R-MTX, n = 50), who received MTX for >3 months and presented a minimum change of 1.2 in DAS28 and also reached DAS28 <3.2. To minimize overlaps, we did not included patients who presented moderate response (Supplementary Table 1). 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 Supplementary Table

2. Peripheral blood samples of healthy donors (n = 30), paired by sex and age, were also collected. Healthy individuals with high or low expression of CD39 on circulating Tregs cells were also analyzed. Individuals were stratified as high (mean fluorescence intensity, MFI >7500) or low (<5000) expression of CD39 according to our previous study [8]. The percent of individuals with low CD39 expression from healthy population was 39.4%. All donors provided informed consent to participate in the study, approved by the Local Ethics Committee (Protocol 4191/2015). 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.

2.3. Genetic Association Analysis

RA patients (n = 257) were recruited for collection of genomic DNA at the Department of Rheumatology, Ribeirao Preto Medical School Hospital (University of Sao Paulo, Ribeirao Preto/Brazil). RA was diagnosed by board-certified rheumatologists and who fulfilled the revised 2010 American College of Rheumatology and European League against Rheumatism criteria for RA classification [20]. Disease activity was measured by DAS28. RA patients were stratified according to their MTX response based on EULAR response criteria [21] as described in *section 2.2* above. For genetic association analysis, the use of other drugs, such as leflunomide, sulfasalazine, cyclosporine and biologic agents (TNF-α blockers, anti-CD20, and anti-IL-6) was allowed at the time of sample collection. The clinical features of RA patients for this study are shown on Supplementary Table 3. All subjects enrolled in the study were Brazilians from the same geographically defined region of Ribeirao Preto, Sao Paulo, Brazil. All RA patients were submitted to medical examinations and none showed evidence of other underlying diseases and were serologically negative for Chagas' disease, hepatitis B and C, and HIV. All volunteers provided informed consent to participate in the study approved by the ethical committee of HC-FMRP (nº4191/2015).

2.4. Leukocyte Separation from Peripheral Blood

Peripheral blood (20 mL) was collected by venipuncture, and PBMCs were isolated by percoll gradient (Sigma-Aldrich), washed with PBS, resuspended in RPMI-1640 containing 10% (vol/vol) heat-inactivated FCS (bovine fetal calf serum), and counted in a Neubauer chamber.

2.5. Tregs and naïve CD4$^+$ cells purification

PBMC from R-MTX and UR-MTX patients and healthy individuals were used. For purification of Tregs and Teff cells, CD4$^+$CD25$^+$ Regulatory T Cell Isolation Kit human (Miltenyi Biotec), and for isolation of naïve CD4$^+$ cells (CD4$^+$CD45RA$^+$), Naive CD4$^+$ T Cell Isolation II (Miltenyi Biotec) were used according to the manufacturer's recommendations. Purity of the cell populations was (>95%) according to flow cytometric analysis.

2.6. Tregs polarization

Purified naïve CD4$^+$ (CD4$^+$CD45RA$^+$) cells (1 x 10^5) from healthy individuals were incubated for 4 days in 96 wells plates U-bottom (Corning) with RPMI-1640 + 10% FCS in the presence of soluble anti-CD3 (3 μg/ml), anti-CD28 (1.5 μg/ml) and a cocktail polarizing reagent containing TGF-β (3, 10 or 30 ng/ml) and IL-2 (10 ng/ml). In some experiments, cells were differentiated simultaneously in the presence of the CD39 inhibitor (ARL67156, 10 μM), Apyrase from potato (10 U), A2a receptor agonist (NECA, 10, 30 or 100 μM) or A2a receptor antagonist (CSC, 10 or 30 μM).

2.7. Cell culture

Purified Tregs (1 x 10^5) from healthy subjects were incubated at different times with graded concentrations of TGF-β in the presence of soluble anti-CD3 (3 μg/ml) and anti-CD28 (1.5 μg/ml). In some experiments, Tregs were co-incubated with TGFBRI inhibitor (SB431542, 10 μM), PKA inhibitor (H89, 10 μM), CREB inhibitor (Naphthol AS-E phosphate, 3, 10 or 30 μM), p38 inhibitor (SB203580, 10 μM) or AKT inhibitor (AKT inhibitor IV, 100 ηM).

2.8. Flow cytometry analysis

Cells were washed and re-suspended in 100 μL of PBS and incubated with 1.5 μg of monoclonal antibodies specific for CD4 (clone SK3), CD25 (clone M-A251), CD39 (clone TU66) or FoxP3 (clone PCH101) and were conjugated with FITC, PE, PE-Cy7 or APC (all from BD Biosciences). For intracellular FoxP3 staining, the Human FoxP3 Buffer kit (BD Biosciences) was used. Cells were then analyzed by FACSCanto II (BD Biosciences) or FACSVerse (BD Biosciences), using the software FACSdiva (BD Biosciences), FACSsuite (BD Biosciences) and Flow Jo (TreeStar). The quantification of CD39 expression was carried out using mean fluorescence intensity (MFI) gated on $CD39^+$ Tregs. For consistency, CD39 expression was determined throughout the experiments by adjusting the voltages of the PE channel through a standard control (cells from a same healthy donor).

2.9. TGF-β quantification

The levels of TGF-β in the plasma were determined using ELISA kits (R&D Systems), according to the manufacturer's recommendations. The results were expressed as pg/ml.

2.10. Real time PCR

Total RNA was extracted from isolated CD4$^+$ cells of R-MTX and UR-MTX patients and healthy individuals using PureLinkTM RNA Mini Kit (Ambion Life Technologies) according to the manufacturer's instructions. After extraction, 500 ng of the RNA was used for conversion into cDNA using High Capacity kit (Life Technologies). Quantification of the expression of the target genes was carried out by real time PCR using Syber Green Master Mix (Thermo Fisher Scientific) as amplification reporter. Quantitative real-time reaction was performed in ViiaTM 7 Real-Time PCR System (Applied Biosystems). Results were analyzed using the $2^{-\Delta\Delta CT}$. The primers pair used are listed below:

Gene	**Sense (5'-3')**	**Anti-sense (5'-3')**
HPRT1	GCAGTACAGCCCCAAAATGGC	GGTCCTTTTCACCAGCAAGCT
TGFBR2	AGGGGTCCGGGAAGGC	CTGGGCCTCCATTTCCACAT
TGFBR1	TGCTCGACGATGTTCCATTG	CTCTCAAGGCTTCACAGCTC
ENTPD1	GTGGGGTTGACCCAGAACAA	GTGAGAAGAACCCGCATCCA
GFI1	CAATGCCTGTGGACAAGATG	GCAGTCAGGGGTTGTGACTT
SMAD7	CCTCCTTACTCCAGATACCCGAT	AGCTGACTCTTGTTGTCCGAATT
CREB1	TGAAGAAGCAGCACGAAAGA	TTTCAAGCACTGCCACTCTG
SMAD2	ACCGAAATGCCACGGTAGAA	TGGGGCTCTGCACAAAGAT
P38	ACTCAGATGCCGAAGATGAAC	GTGCTCAGGACTCCATCTCT

2.11. Western Blot analysis

Tregs (1 x 10^5) were lysed in RIPA buffer (Sigma-Aldrich). Equal amounts (30 µg) of total cell lysate proteins were separated in Mini-Protean TGX 4–20% gradient SDS-PAGE gels (BioRad). After proteins were transferred into nitrocellulose membrane, target proteins were detected by immunoblotting by specific antibodies. The Western blot was read using a kit containing chemiluminescence (ECL) reagent (GE Healthcare) in a Chemidoc XRS device (Biorad). Proteins were detected using antibodies against CREB (cat. ab7540, Abcam, 1:1000; cat. 48H2, Cell

Signalling Technology, 1:1000), p-CREB (cat. ab10564, Abcam, 1:500; cat. 87G3, Cell Signalling Technology, 1:500), SMAD2 (cat. 86F7, Cell Signalling Technology, 1:250), p-SMAD2 (cat. 3101S, Cell Signalling Technology, 1:500) and CD39 (cat. ab189258, Abcam, 1:500). This was followed by incubation with HRP-conjugated goat anti-rabbit antibody (cat. 31460, Thermo Fisher Scientific, 1:5000) or HRP-conjugated goat anti-mouse antibody (cat. 31430, Thermo Fisher Scientific, 1:3000).

2.12. SNPs and genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMC) using a DNA extraction kit (QIAGEN) according to the manufacturer's instruction. The SNPs were genotyped using TaqMan genotyping assays and probes from Thermo Fisher Scientific: rs3773649 (C_27514584_10), rs3773645 (C_11566045_20), rs3087465 (C_27491740_10), rs1155708 (C_8778211_10), rs1431131 (C_11565979_10), rs6785358 (C_1981957_10), rs7625858 (C_29354774_10). Briefly, TaqMan genotyping reactions were performed on ViiA 7 Real-Time PCR System (Thermo Fisher Scientific) using 15 ng of genomic DNA following the manufacturer's instructions. Genotypes were scored by analyzing data on both real time and allele discrimination assay platforms using Sequence Detection System Software 2.3 (SDS 2.3, Applied Biosystems). Genotyping success rate was >90%.

2.13. Statistical analysis

Statistical analyses were performed using analysis of variance one-way nonparametric (ANOVA) followed by Bonferroni's *t* test (for three or more groups) comparing all pairs of columns, or two-tailed Student's *t* test (for two groups). $P < 0.05$ was considered statistically significant. For the analysis of correlation, Spearman`s test was carried out. Statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. TGF-β stimulation promotes the induction of CD39 expression on Tregs

We first determined whether TGF-β stimulation could promote the induction of CD39 expression on Tregs. Tregs ($CD4^+CD25^+$) were purified from the peripheral blood of healthy donors and incubated with polyclonal stimuli (soluble anti-CD3 and anti-CD28) in the presence of graded concentrations of TGF-β. TGF-β induced CD39 expression on Tregs in a dose (3 - 30 ng/ml)- and time (6 – 72 h)-dependent manners (Fig. 1A and B, Supplementary Fig. 1). Intriguingly, TGF-β stimulation did not affect CD39 expression in other leukocytes such as effector T cells (Teff, $CD4^+CD25^-$), granulocytes and $CD11c^+$ cells (Supplementary Fig. 2).

Next we assessed whether TGF-β stimulation could affect CD39 expression during Tregs differentiation. Naïve $CD4^+$ ($CD4^+CD45RA^+$) cells were purified from the peripheral blood of healthy donors and incubated for 4 days with polyclonal stimuli in the presence of a cocktail of cytokines (TGF-β and IL-2, for Tregs polarization) and then graded concentrations of TGF-β during the Tregs differentiation. As expected, TGF-β promoted Tregs differentiation, evidenced by an increase of the frequency of $CD4^+FoxP3^+$ cells (Fig. 1C and D). TGF-β markedly enhanced CD39 expression in differentiating Tregs (Fig. 1E and F). These data were further confirmed in a second round of TGF-β stimulation in differentiated Tregs. Tregs were polarized with TGF-β for 4 days, the culture supernatant removed and then the cells were re-stimulated with graded concentrations of

TGF-β. TGF-β enhanced further CD39 expression on differentiated Tregs (Fig. 1G). These results demonstrate that TGF-β can induce CD39 expression on differentiating and differentiated Tregs.

We next investigated the molecular mechanism by which TGF-β increases CD39 expression on Tregs. Purified Tregs from healthy donors were incubated with TGF-β in the presence of a TGFBRI inhibitor (SB431542, 10 μM). Flow cytometry analysis shows that SB431542 markedly reversed the CD39 enhancing effects of TGF-β on Tregs in a dose-dependent manner (Fig. 2A). As TGFBRI triggering can activate a range of kinases, such as PKA, p38 and PI3K/AKT [15,22–27]**,** we tested the effect of specific kinase inhibitors. The PKA inhibitor (H89, 10 mM [26]) or the AKT inhibitor (AKT inhibitor IV, 100 nM [28]) did not affect the induction of CD39 expression on Tregs by TGF-β (Supplementary Figs. 3A-B). In contrast, an inhibitor of p38 activity (SB203580, 10 μM [27]) completely abolished the induction of CD39 expression (Fig. 2B), suggesting that p38 activity has a crucial role for CD39 induction by TGF-β on Tregs.

We then addressed whether activation of the SMADs and CREB on Tregs could be involved in the increase of CD39 expression after TGF-β stimulation. TGF-β increased the expression of activated SMAD2 (p-SMAD2) and CREB (p-CREB) on Tregs in a dose-dependent manner (Fig. 2C and D). CREB plays an important role in the induction of CD39 mRNA in macrophages [18] and TGF-β-activated kinases from non-SMAD signalling pathways can also phosphorylate CREB [23–25]. We therefore tested the role of CREB in the induction of CD39 on Tregs using a CREB activity inhibitor (iCREB, Naphthol AS-E phosphate). iCREB completely abolished the CD39 enhancing effect of TGF-β on Tregs (Fig. 2E). Collectively, our findings suggest that the TGF-β-induced CD39 expression on Tregs is associated with the activation of TGFBRII/TGFBRI, SMAD2 and CREB, whose activation is dependent of p38 activity.

3.2. CD39 expression modulates Tregs polarization

Since CD39 levels on Tregs are variable even in healthy individuals [8,29], we tested whether this variability could influence Tregs differentiation. TGF-β could polarize Tregs ($CD4^+FoxP3^+$) from naïve $CD4^+$ cells of both high and low CD39 expression individuals (Fig. 3A-B). However, cells from individuals with high CD39 expression polarized to Tregs in significantly higher frequency than those from subjects with lower CD39 expression. The enhanced ability of TGF-β to induce Tregs expressing high levels of CD39 was blocked by a CD39 inhibitor (iCD39, ARL67156) (Fig. 3C). The iCD39 had little or no effect on Tregs from individuals with low levels of CD39. We explored further the relationship between CREB and CD39. $CD4^+$ T cells from individuals with high or low CD39 expression were polarized to Tregs in the presence of TGF-β with or without a TGFBRI inhibitor (SB431542) and the levels of CREB and p-CREB analysed by Western blot. Tregs from individuals with low CD39 expression had lower amounts of CREB and p-CREB compared to those of the cells from subjects with high CD39 expression. Tregs from both groups exhibited higher levels of p-CREB in response to TGF-β activation, though significantly higher level of p-CREB was detected in the high CD39 expresser group (Fig. 3D and E). These results demonstrate the signalling cascade of TGF-β/TGFBR→CREB→CD39→Tregs in the induction of Tregs.

We next investigated how CD39 promoted Tregs differentiation. It may be that elevated CD39 expression provides high levels of extracellular ADO, which, through an autocrine/paracrine mechanism, activates its receptor A2a resulting in an enhanced Tregs differentiation [30]. $CD4^+$ T cells were purified from individuals with high or low CD39 expression and polarized to Tregs *in vitro* in the presence of Apyrase (an analog of CD39)[31], NECA (5'-N-Ethylcarboxamidoadenosine, an A2a receptor agonist)[30], or CSC [8-(3-chloro-styryl)-caffeine, an A2a receptor antagonist][32]. Apyrase effectively increased the level of Tregs polarization of cells from individuals with low CD39 expression to the same level as that of subjects with high CD39 expression (Fig. 3F). Apyrase, however, had no effect on Tregs polarization in cells from individuals already with high CD39 expression. NECA also promoted Tregs polarization in cells from both groups of individuals, and restored Tregs differentiation from individuals with low CD39 expression (Fig. 3G). Concordantly, CSC markedly reduced the polarization of Tregs from donors of high CD39 expression but slightly increased Tregs polarization from subjects with low CD39 expression (Fig. 3H). Collectively, these data demonstrate that CD39 promotes extracellular ADO generation, which in turn acts via A2a to enhance Tregs polarization.

We next determined whether the failure of Tregs polarization from cells of individuals with low CD39 expression by TGF-β was intrinsic to TGF signalling. $CD4^+$ T cells from healthy subjects with high or low CD39 expression were polarized to Tregs in the presence of graded concentrations of TGF-β. While differentiated Tregs from individuals with high CD39 showed a further increase of CD39 expression, Tregs from subjects with low CD39 were unable to induce CD39 expression following TGF-β stimulation (Fig. 3I and J). Together, these results indicate an intrinsic defect in the TGF-β signalling pathway in individuals with low expression of CD39 on Tregs.

3.3. UR-MTX patients have intrinsic impairment of TGF-β signalling

We then investigated whether UR-MTX RA patients could have altered expression and activation of the molecules that participate in the TGF-β signalling. There was no difference in the plasma TGF-β level between R-MTX and UR-MTX (Supplementary Fig. 4A). We then purified $CD4^+$ T cells from the peripheral blood of R-MTX and UR-MTX patients and analyzed the expression of the genes associated with TGF-β signalling in these cells by qPCR. There was also no difference in the expression of *SMAD2*, *GFI1* (growth factor independent-1), *P38* and *TGFBR1* between the two groups of RA patients (Supplementary Figs. 4B-E). By contrast, cells from R-MTX patients expressed significantly higher levels of CREB1, *SMAD7* (a regulator of SMAD2 activity) and *TGFBR2* mRNA than those of cells from UR-MTX patients (Fig. 4A-C). Furthermore, there was a close correlation between *TGFBR2* and *CREB1* expression and between *TGFBR2* and *ENTPD1* expression (Fig. 4D). Importantly, there was a well-defined clustering of the *TGFBR2/CREB1* and *TGFBR2/ENTPD1* gene expression and MTX clinical response. All the UR-MTX patients had low *TGFBR2/CREB1* score (Fig. 4D). This finding may allow further patient stratification for predicting MTX unresponsiveness in addition to the low CD39 expression on Tregs reported previously [8]. We also found a decrease of p-CREB and CREB levels in un-stimulated Tregs from UR-MTX patients compared to those from the R-MTX patients (Fig. 4E). Moreover, while there was no difference in SMAD2 levels, we observed a reduction of p-SMAD2 concentration in Tregs from UR-MTX patients (Fig. 4F) compared to those of R-MTX patients. Taken together, we demonstrated that UR-MTX patients exhibit an impairment of TGF-β signalling on their Tregs, evidenced by reduced mRNA levels of *TGFBR2* and *CREB1,* and that this could account for the reduction of CD39 expression on their Tregs.

3.4. MTX resistance is associated with a polymorphism in TGFBR2 gene

Finally, we investigated whether genetic polymorphisms in *TGFBR2* gene could affect TGFBR2 expression and be associated with MTX resistance. Of the 7 SNPs we analysed, rs1431131 exhibited a significant association with the development of MTX resistance ($p = 0.0006$; Table 1). Carriers of at least one mutant allele for rs1431131 (*AT or AA*) were more likely to be UR-MTX (Table 1). rs1431131 is an intronic SNP characterized by a transition substitution (Supplementary Fig. 5). We then determined if this SNP would have implications for *TGFBR2* expression. We found that RA patients carrying at least one mutant allele for rs1431131 (*AT or AA*) had significantly ($P<0.001$) lower levels of *TGFBR2* mRNA compared to TT carriers, suggesting that this SNP affects *TGFBR2* function (Fig. 5A). Interestingly, the relative risk (RR) for carriers of at least one mutant allele for rs1431131 (*AT or AA*) was 1.704 compared to non-carriers, with 95% Confidence Interval: 1.206 to 2.407 (using the approximation of Katz.). Consistent with this observation, carriers of *AT or AA* alleles for *TGFBR2* showed a significant (P<0.01) reduction of *ENTPD1* mRNA levels in CD4$^+$ cells (Fig. 5B), indicating that the levels of *TGFBR2* expression affect the *ENTPD1* expression.

4. Discussion

Understanding the mechanisms involved in MTX resistance is crucially important to identify novel predictive biomarkers [9]. We have recently demonstrated that low CD39 expression on circulating Tregs is a potential biomarker for predicting resistance to MTX in RA [8]. In the present study, we identified the molecular mechanism that mediates lower CD39 expression on Tregs cells in UR-MTX.

Firstly, we found that TGF-β stimulation induces CD39 expression on Tregs cells through the activation of TGFBRII, TGFBRI, SMAD2 and CREB, which in turn is activated in a p38 activity-dependent manner. Interestingly, this phenomenon appears to be Tregs-specific, since other cell types such as effector T cells (CD4$^+$CD25$^-$), granulocytes and CD11b$^+$ cells were not affected. This specificity may be due to the higher expression of TGF-β receptors on Tregs compared to other leucocytes [33].

We also explored the effects of CD39 on Tregs differentiation. We found that CD39 was a booster of Tregs differentiation, since individuals with higher CD39 expression exhibited an increased capacity to polarize Tregs. Moreover, adding a CD39 analog (Apyrase) or A2a receptor agonist (NECA) during Tregs differentiation restored the ability of individuals with lower CD39 expression to promote Tregs polarization. By contrast, individuals with higher CD39 expression lost their capacity to polarize more Tregs when CD39 activity was abrogated using a CD39 inhibitor (ARL67156). These findings show that ADO produced by CD39 activity through extracellular ATP metabolism potentiates Tregs differentiation. Thus, during Tregs differentiation process, anti-CD3/CD28 stimulation triggers a rapid release of ATP [34], which is metabolized by CD39/CD73 into ADO. Once differentiated to Tregs by additional TGF-β stimulation, Tregs begin to express

FOXP3, the transcription factor that characterize them and drives their suppressive function [35,36]. A2aR activation by ADO could affect the stability of FOXP3 transcription, since it has been suggested that A2aR stimulation prevents FOXP3 degradation [30]. In this context, given that lower levels of CD39 expression on Tregs mediate a reduced ability to generate ADO from UR-MTX patients during MTX treatment [8], these findings suggest an immunological mechanism by which UR-MTX are unable to promote Treg expansion after MTX treatment (Fig. 5C).

Focusing our findings on RA patients, we found that UR-MTX patients and healthy individuals with lower expression of CD39 on Tregs cells have an intrinsic reduction in the expression and activation of TGFBRII and CREB, resulting in a compromised ability to induce CD39 expression. Genetic analysis showed an association between *TGFBR2* and *CREB1* mRNA levels with *ENTPD1* expression on circulating Tregs from RA patients. In genetic polymorphism analysis, we observed that RA patients carrying at least one mutant allele for rs1431131 are significantly associated with UR-MTX. Moreover, we demonstrated that this mutation affects mRNA levels of *TGFBR2*. Our data therefore indicate that resistance to MTX of RA patients is likely determined by a mutation of the *TGFBR2* gene, suggesting that genotyping RA patients for rs1431131, in addition to CD39 expression on Tregs, could also predict therapeutic response to MTX.

5. Conclusions

Data presented here show that impairment in TGF-β signaling attenuates the induction of CD39 expression by TGF-β leading to the reduction of CD39 expression on Tregs, which is a potential biomarker for UR-MTX RA patients. Our finding therefore not only unravels the mechanism of this intriguing fundamental biological pathway, but also reveals potential therapeutic intervention of MTX unresponsiveness that affects up to 40% of RA patients. Given the pervasive role of Tregs in immune homeostasis, our finding may also have broader implications beyond RA.

Authors' contributions

R.S.P., V.C., P.L-J., F.Y.L., F.Q.C. designed the study. R.S.P., H.I.N., T.M.C., J.C.A-F, P.L-J., F.Y.L., F.Q.C. planned experiments and analysed data. R.S.P., P.B.D., J.T., N.T.C., K.W.A.L., R.D.O. performed the experiments. P.R., C.C.M. helped with the recruitment of patients and data analysis. F.Y.L., T.M.C. and J.C.A-F provided essential materials and performed important hypothesis discussion. R.S.P., F.Y.L., P.L-J., F.Q.C. wrote the manuscript. F.Q.C. supervised the study. All authors had final approval of the version.

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Competing interests

The authors declare no competing interests.

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Figure legends

Fig. 1. TGF-β stimulation increases CD39 expression on naïve and differentiated Tregs. Tregs ($CD4^+CD25^+$) were purified from healthy donors (n = 5) and incubated with polyclonal stimuli (anti-CD3 plus anti-CD28) and TGF-β for 48 h. (**A**) Representative histogram of CD39 expression. (**B**) Statistical analysis of CD39 expression (MFI). (**C-F**) Naive $CD4^+$ ($CD4^+CD45RA^+$) cells from healthy donors (n = 4) were incubated with polyclonal stimuli and graded concentrations of TGF-β and IL-2 (10 ng/ml) for 4 days. (**C**) Representative dot plot of % differentiated Tregs ($CD4^+FoxP3^+$). (D) Frequency of differentiated Tregs. (E) Expression of CD39 (MFI) on Tregs ($CD4^+FoxP3^+$). (**F**) Western Blot analysis of CD39 protein concentration shown in duplicate in each group. (**G**) Naive $CD4^+$ ($CD4^+CD45RA^+$) cells from healthy donors (n = 5) were incubated with TGF-β (10 ng/ml) and IL-2 (10 ng/ml) plus polyclonal stimuli for 4 days. Differentiated Tregs were washed and re-stimulated with graded concentrations of TGF-β and polyclonal stimuli. Expression of CD39 (MFI) on Tregs ($CD4^+FoxP3^+$) is shown. All data are mean ± SEM, representative of two experiments with similar results. *P <0.05, **P <0.01 by one-way ANOVA result followed by Bonferroni's *t* test.

Fig. 2. Induction of CD39 on Tregs is dependent on the activation of the molecular cascade TGFBRII/TGFBRI/p38/SMAD2/CREB by TGF-β stimulation. (**A-B**) CD39 expression (MFI) on Tregs of healthy donors (n = 4) cultured for 48 h with anti-CD3 (3 μg/ml), anti-CD28 (1.5 μg/ml) and TGF-β (10 ng/ml) with (**A**) TGFBRI inhibitor (SB431542) or (**B**) p38 inhibitor (SB203580). (**C-D**) Western blot analysis of (**C**) p-SMAD2 and SMAD2 and (**D**) p-CREB and CREB in Tregs from healthy donors stimulated with graded concentrations of TGF-β, with duplicates for each concentration. (**E**) CD39 expression (MFI) on Tregs of healthy donors (n = 5) cultured for 48 h with anti-CD3, anti-CD28 and TGF-β with CREB inhibitor (iCREB, Naphthol AS-E phosphate). Data are

mean ± SEM, representative of two (**A**, **B**, **D**, **E**) or three (**C**) independent experiments with similar results. *P <0.05, **P <0.01 by one-way ANOVA result followed by Bonferroni's *t* test.

Fig. 3. TGF-β enhances CD39 expression on Tregs from individuals with high CD39 expression. Naive $CD4^+$ ($CD4^+CD45RA^+$) cells from PBMC of healthy donors with high or low CD39 expression on circulating Tregs were incubated for 4 days with anti-CD3 (3 µg/ml), anti-CD28 (1.5 µg/ml), TGF-β and IL-2 (10 ng/ml). Representative dot plots showing % differentiated Tregs ($CD4^+FoxP3^+$) from an individual with high and an individual with low CD39 expression (**A**). (**B**) Frequency of differentiated Treg cells from healthy subjects with high or low CD39 expression (n = 4 per group). (**C**) Frequency of Tregs differentiated in the presence of CD39 inhibitor (iCD39, 10 µM) from healthy subjects with high or low CD39 expression (n = 4 per group). (**D-E**) Tregs were isolated from PBMC of healthy donors with high or low CD39 expression (n = 4 per group) and incubated for 48 h with anti-CD3, anti-CD28, TGF-β and TGFBRI inhibitor (SB431542, 10 µM). Western blot (**D**) and statistical analysis (**E**) of p-CREB and CREB in Tregs. (**F-H**) Frequency of Tregs from healthy subjects with high or low CD39 expression (n = 4 per group) differentiated in the presence of (**F**) Apyrase (10 U), (**G**) A2a receptor agonist (NECA) or (**H**) A2a receptor antagonist (CSC). (**I**) Representative histogram of CD39 expression (MFI) on Tregs from healthy subjects with high or low CD39 expression differentiated with TGF-β. (**J**) Expression of CD39 (MFI) on differentiated Tregs from healthy subjects with high or low CD39 expression (n = 5 per group). Data are mean ± SEM of one experiment (**G**, **H**); or representative of two (**C**, **E**, **F**) or three (**B**, **J**) independent experiments. *P <0.05, **P <0.01, ***P <0.001 by one-way ANOVA result followed by Bonferroni's *t* test.

Fig. 4. Reduction of CD39 expression on Tregs is associated with impairment in TGF-β signalling. (**A-C**) Quantification of mRNA for (**A**) *CREB1*, (**B**) *SMAD7* and (**C**) *TGFBR2* from

isolated CD4$^+$ cells of R-MTX (n = 23) or UR-MTX (n = 17) RA patients. (**D**) Correlation analysis between TGFBR2 and CREB1 (left panel) and *TGFBR2* and *ENTPD1* (right panel) on CD4$^+$ cells from R-MTX (n = 18) and UR-MTX (n = 13) patients. (**E-F**) Tregs from R-MTX (n = 7), and UR-MTX (n = 6) patients were analysed by Western blot for the expression of (**E**) p-CREB / CREB and (**F**) pSMAD2 / SMAD2. Data are mean ± SEM, *P <0.05, **P <0.01, ***P <0.001 by two-tailed unpaired Student's t-test in A, B, C, E, F. Spearman correlation in D.

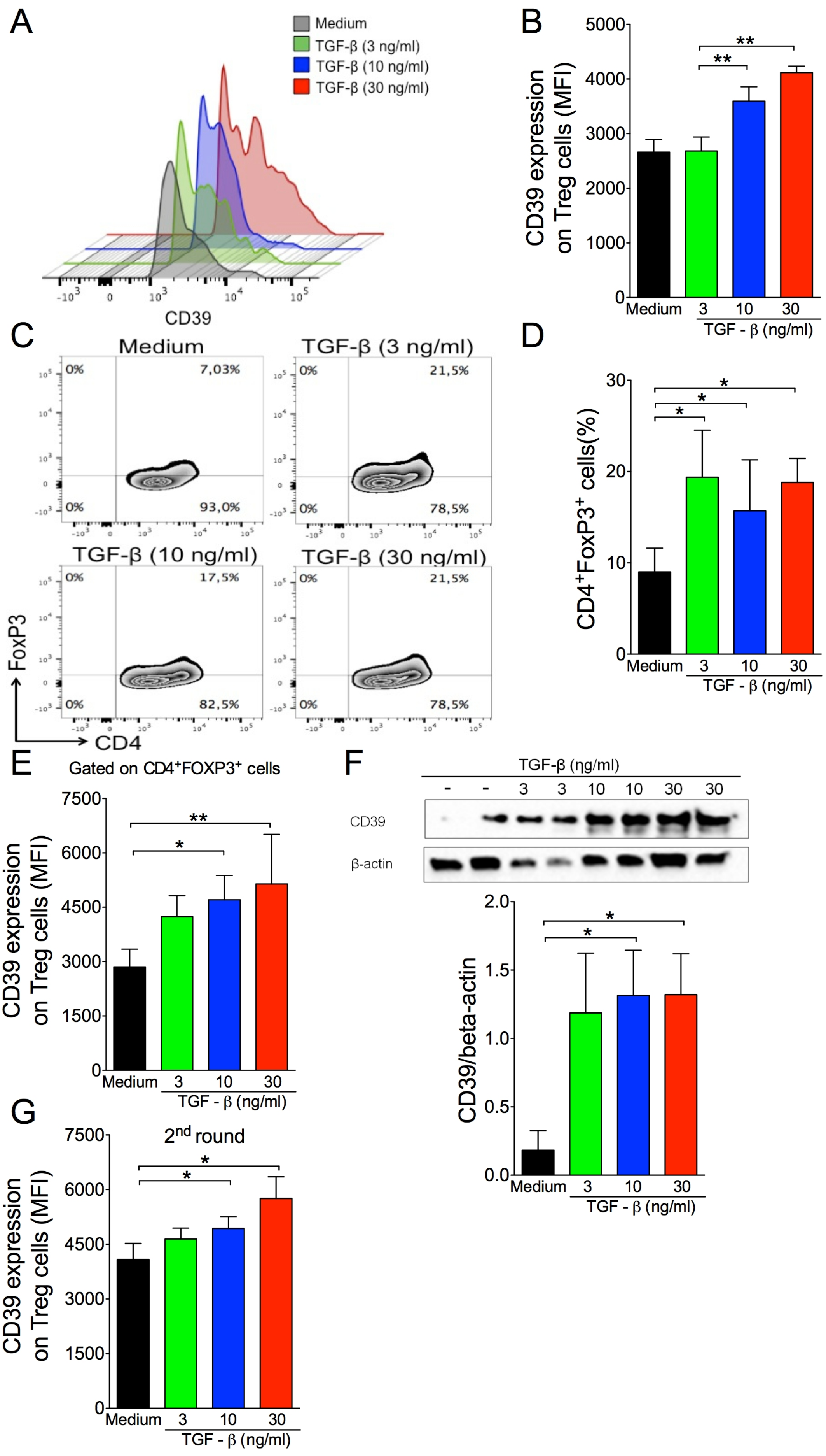
Fig. 5. *TGFBR2* and *ENTPD1* mRNA levels in genotyped RA patients for rs1431131 and schematic representation. mRNA of *TGFBR2* (**A**) and *ENTPD1* (**B**) in CD4$^+$ cells from RA patients stratified by the presence of *TT* and *AT/AA* alleles for rs1431131. Data are mean ± SEM, **P<0.01, ***P<0.001, by non-parametric two-tailed *t* test. n = 25. (**C**) Schematic representation of TGF-β signalling and responsiveness to MTX. TGF-β activates Tregs sequentially through TGFBRII/TGFBRI, p38 and the transcription factor CREB, which in turn induces CD39 expression by promoting *ENTPD1* gene transcription. CD39 expression on the surface of Tregs cells is essential for mediating extracellular ADO generation and consequently Tregs expansion under MTX treatment. UR-MTX patients display an intrinsic defect in TGFBRII expression, which compromises the downstream CD39 expression, and hence resistant to MTX treatment leading to disease progression.

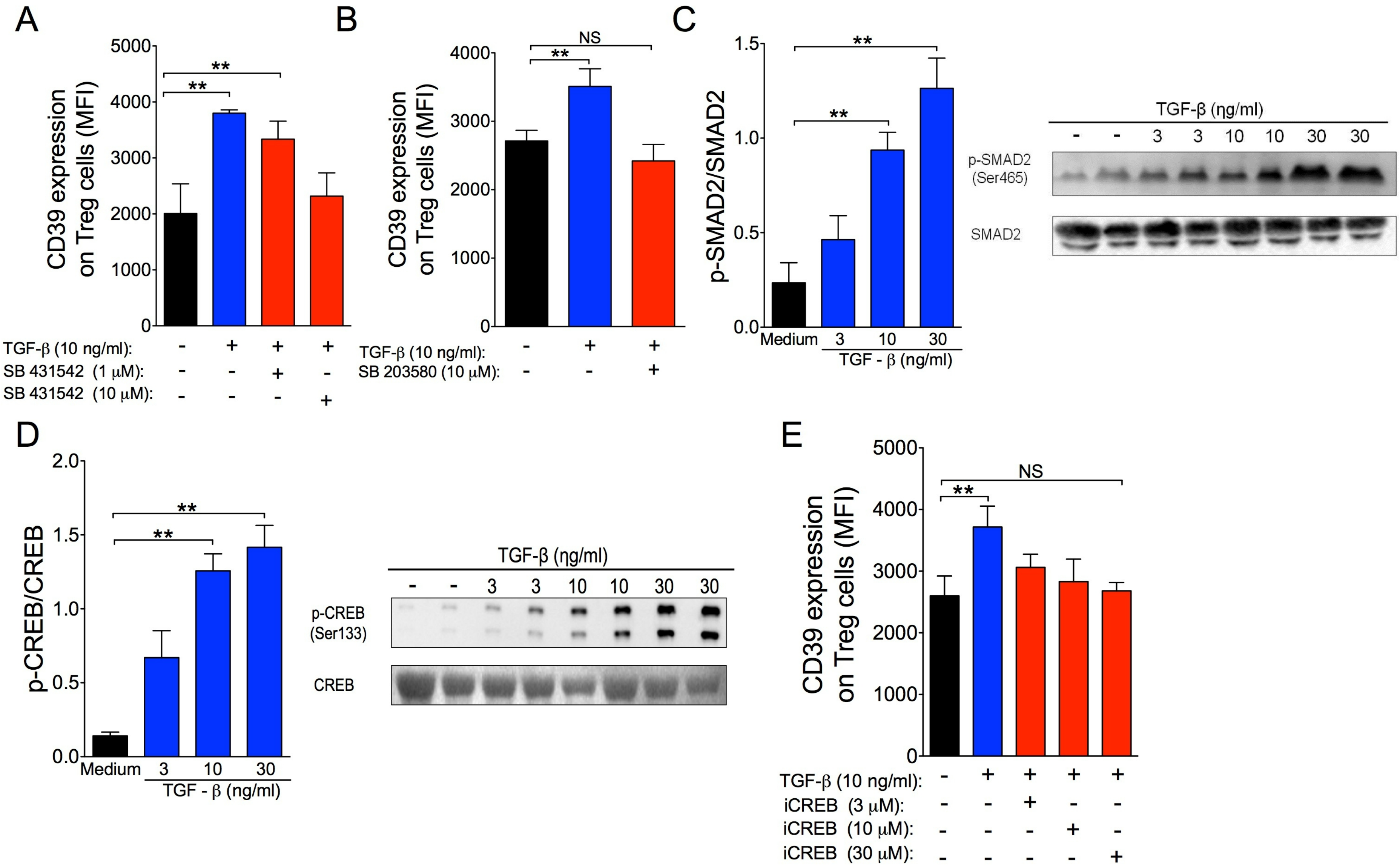
Table 1. Influence of polymorphisms in *TGFBR2* for the efficacy of MTX treatment in RA patients

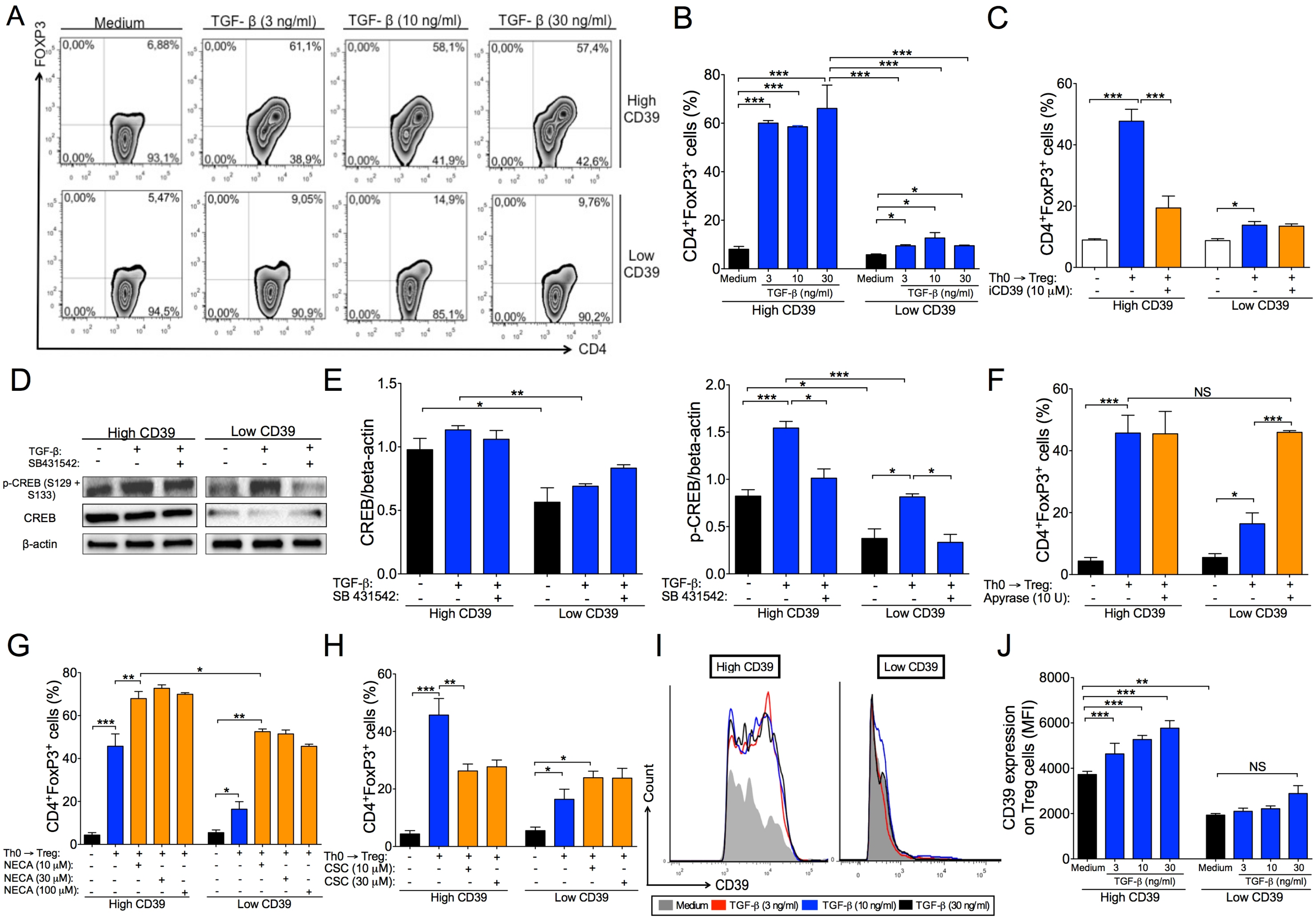
Genetic profile	R-MTX n (% of subjects in the group)	UR-MTX n (% of subjects in the group)	*P*
rs3773649			
GG	46 (48)	76 (52)	*0.671*
GA/AA	50 (52)	70 (48)	
rs3773645			
CC	44 (46)	78 (51)	*0.483*
CG/GG	52 (54)	77 (49)	
rs3087465			
GG	36 (36)	63 (40)	*0.662*
AG/AA	63 (64)	95 (60)	
rs1155708			
GG	48 (49)	58 (37)	*0.089*
AG/AA	49 (51)	99 (63)	
rs1431131			
TT	31 (37)	21 (15)	*0.0006****
AT/AA	53 (63)	117 (85)	
rs6785358			
AA	60 (67)	98 (75)	*0.275*
AG/GG	29 (33)	34 (25)	
rs7625858			
TT	39 (44)	67 (47)	*0.331*
CT/CC	50 (66)	76 (53)	

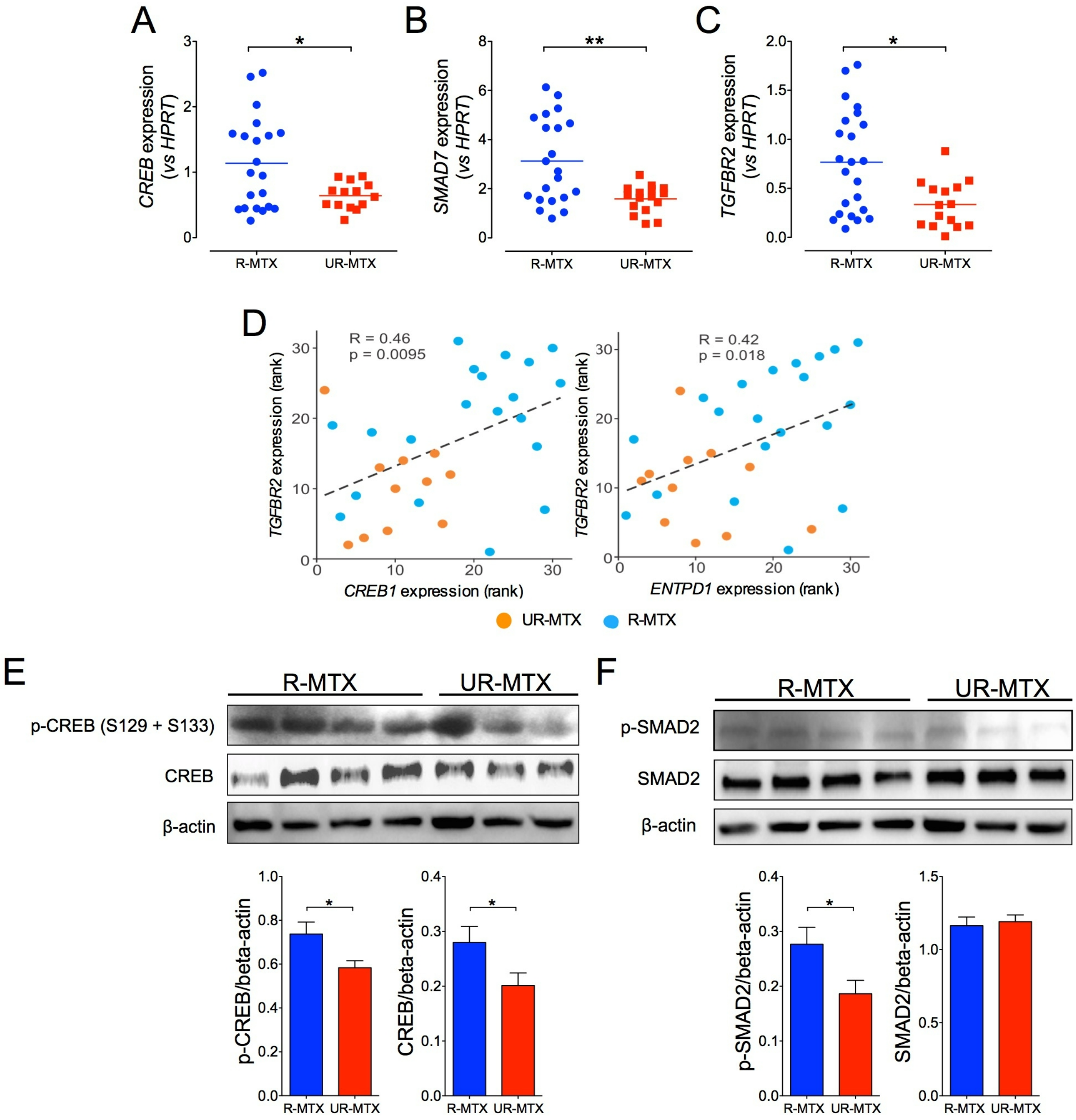
n, number of subjects. (%), frequency of subjects

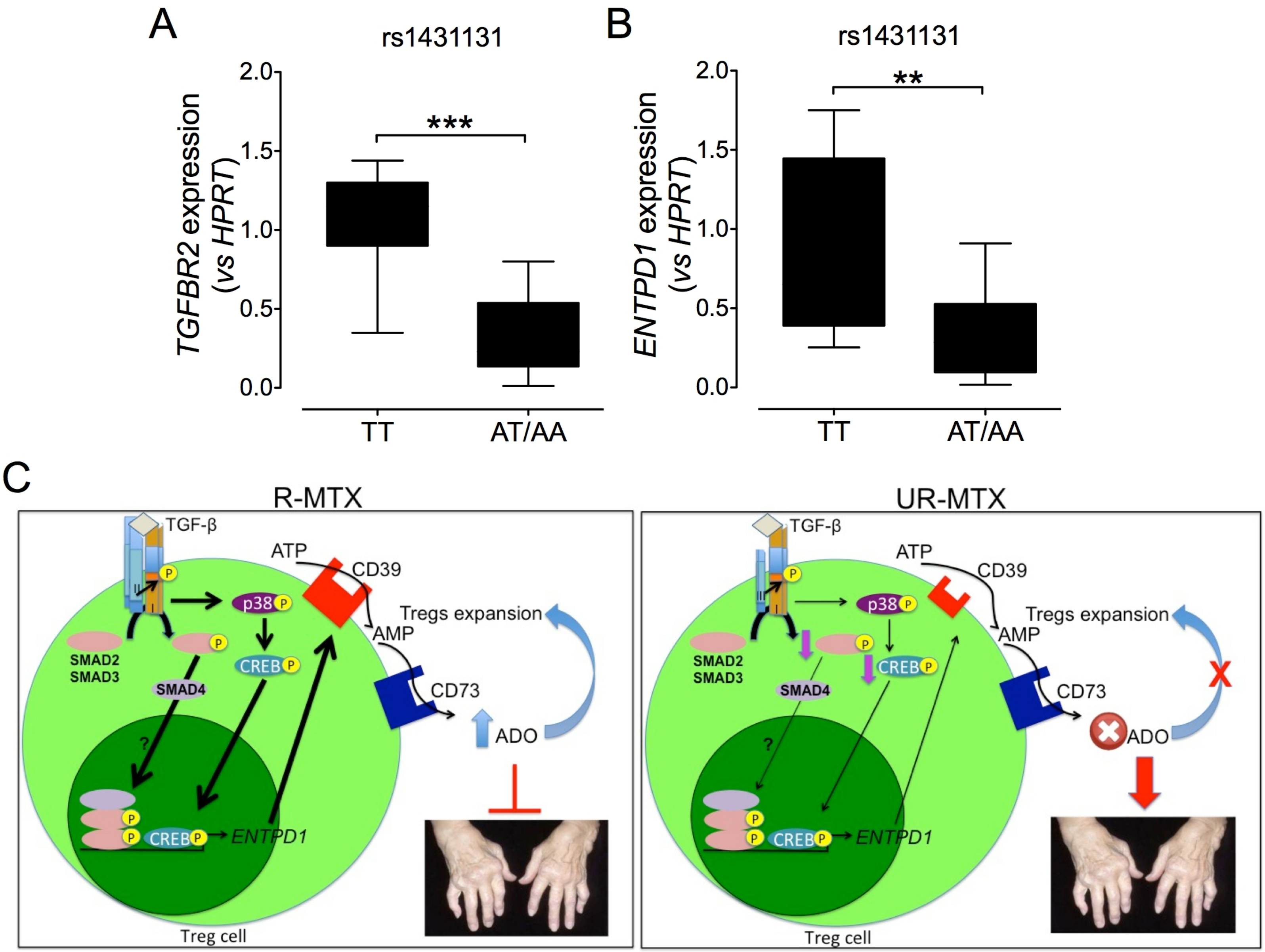
P calculated by Fisher's exact test. *** for P <0.001.


A
Medium
TGF-β (3 ng/ml)
TGF-β (10 ng/ml)
TGF-β (30 ng/ml)
-10³
0
10³
10⁴
10⁵
CD39
B
CD39 expression on Treg cells (MFI)
5000
4000
3000
2000
1000
0
**
**
Medium
3
10
30
TGF - β (ng/ml)
C
Medium
TGF-β (3 ng/ml)
TGF-β (10 ng/ml)
TGF-β (30 ng/ml)
0%
7,03%
0%
93,0%
0%
21,5%
0%
78,5%
0%
17,5%
0%
82,5%
0%
21,5%
0%
78,5%
FoxP3
CD4
D
CD4⁺FoxP3⁺ cells(%)
30
20
10
0
*
*
*
Medium
3
10
30
TGF - β (ng/ml)
E
Gated on CD4⁺FOXP3⁺ cells
CD39 expression on Treg cells (MFI)
7500
6000
4500
3000
1500
0
**
*
Medium
3
10
30
TGF - β (ng/ml)
F
TGF-β (ηg/ml)
-
-
3
3
10
10
30
30
CD39
β-actin
CD39/beta-actin
2.0
1.5
1.0
0.5
0.0
*
*
Medium
3
10
30
TGF - β (ng/ml)
G
2nd round
CD39 expression on Treg cells (MFI)
7500
6000
4500
3000
1500
0
*
*
Medium
3
10
30
TGF - β (ng/ml)


A
CD39 expression on Treg cells (MFI)
5000
4000
3000
2000
1000
0
**
**
TGF-β (10 ng/ml): - + + +
SB 431542 (1 μM): - - + -
SB 431542 (10 μM): - - - +
B
CD39 expression on Treg cells (MFI)
4000
3000
2000
1000
0
NS
**
TGF-β (10 ng/ml): - + +
SB 203580 (10 μM): - - +
C
p-SMAD2/SMAD2
1.5
1.0
0.5
0.0
**
**
Medium
3
10
30
TGF - β (ng/ml)
TGF-β (ηg/ml)
- - 3 3 10 10 30 30
p-SMAD2 (Ser465)
SMAD2
D
p-CREB/CREB
2.0
1.5
1.0
0.5
0.0
**
**
Medium
3
10
30
TGF - β (ng/ml)
TGF-β (ηg/ml)
- - 3 3 10 10 30 30
p-CREB (Ser133)
CREB
E
CD39 expression on Treg cells (MFI)
5000
4000
3000
2000
1000
0
NS
**
TGF-β (10 ng/ml): - + + + +
iCREB (3 μM): - - + - -
iCREB (10 μM): - - - + -
iCREB (30 μM): - - - - +


A
Medium
TGF- β (3 ng/ml)
TGF- β (10 ng/ml)
TGF- β (30 ng/ml)
FOXP3
CD4
High CD39
Low CD39
0,00% 6,88% 0,00% 93,1%
0,00% 61,1% 0,00% 38,9%
0,00% 58,1% 0,00% 41,9%
0,00% 57,4% 0,00% 42,6%
0,00% 5,47% 0,00% 94,5%
0,00% 9,05% 0,00% 90,9%
0,00% 14,9% 0,00% 85,1%
0,00% 9,76% 0,00% 90,2%
B
CD4$^+$FoxP3$^+$ cells (%)
Medium 3 10 30
TGF-β (ng/ml)
High CD39
Low CD39
***
*
C
CD4$^+$FoxP3$^+$ cells (%)
Th0 → Treg:
iCD39 (10 μM):
High CD39
Low CD39
D
High CD39
Low CD39
TGF-β:
SB431542:
p-CREB (S129 + S133)
CREB
β-actin
E
CREB/beta-actin
p-CREB/beta-actin
TGF-β:
SB 431542:
High CD39
Low CD39
**
F
CD4$^+$FoxP3$^+$ cells (%)
Th0 → Treg:
Apyrase (10 U):
NS
High CD39
Low CD39
G
CD4$^+$FoxP3$^+$ cells (%)
Th0 → Treg:
NECA (10 μM):
NECA (30 μM):
NECA (100 μM):
High CD39
Low CD39
H
CD4$^+$FoxP3$^+$ cells (%)
Th0 → Treg:
CSC (10 μM):
CSC (30 μM):
High CD39
Low CD39
I
High CD39
Low CD39
Count
CD39
Medium
TGF-β (3 ng/ml)
TGF-β (10 ng/ml)
TGF-β (30 ng/ml)
J
CD39 expression on Treg cells (MFI)
Medium 3 10 30
TGF-β (ng/ml)
NS
High CD39
Low CD39


A
CREB expression (vs HPRT)
*
R-MTX
UR-MTX
B
SMAD7 expression (vs HPRT)
**
R-MTX
UR-MTX
C
TGFBR2 expression (vs HPRT)
*
R-MTX
UR-MTX
D
R = 0.46
p = 0.0095
TGFBR2 expression (rank)
CREB1 expression (rank)
R = 0.42
p = 0.018
TGFBR2 expression (rank)
ENTPD1 expression (rank)
● UR-MTX ● R-MTX
E
R-MTX
UR-MTX
p-CREB (S129 + S133)
CREB
β-actin
p-CREB/beta-actin
*
CREB/beta-actin
*
R-MTX UR-MTX
F
R-MTX
UR-MTX
p-SMAD2
SMAD2
β-actin
p-SMAD2/beta-actin
*
SMAD2/beta-actin
R-MTX UR-MTX


A
rs1431131
TGFBR2 expression (vs HPRT)
2.0
1.5
1.0
0.5
0.0
***
TT
AT/AA
B
rs1431131
ENTPD1 expression (vs HPRT)
2.0
1.5
1.0
0.5
0.0
**
TT
AT/AA
C
R-MTX
TGF-β
P
ATP
CD39
p38
P
Tregs expansion
AMP
SMAD2
SMAD3
CREB
SMAD4
CD73
ADO
?
CREB
ENTPD1
Treg cell
UR-MTX
TGF-β
ATP
CD39
p38
Tregs expansion
AMP
SMAD2
SMAD3
CREB
SMAD4
CD73
X
ADO
?
CREB
ENTPD1
Treg cell

Supplementary Materials for

TGF-β signalling defect is linked to low CD39 expression on regulatory T cells and methotrexate resistance in rheumatoid arthritis

Raphael S. Peres, Paula B. Donate, Jhimmy Talbot, Nerry T. Cecilio, Patricia R. Lobo, Caio C. Machado, Kalil W. A. Lima, Rene D. Oliveira, Vanessa Carregaro, Helder I. Nakaya, Thiago M. Cunha, José Carlos Alves-Filho, Foo Y. Liew*, Paulo Louzada-Júnior*, Fernando Q. Cunha*

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Supplementary Table 1. EULAR response criteria for stratification of RA patients			
DAS28 improvement ➔			
Present DAS28 🡇	>1.2	>0.6 and ≤1.2	≤0.6
≤3.2	Good response	Moderate response	No response
>3.2 and ≤5.1	Moderate response	Moderate response	No response
>5.1	Moderate response	No response	No response

Supplementary Table 2. Clinical features of RA patients for Tregs analysis

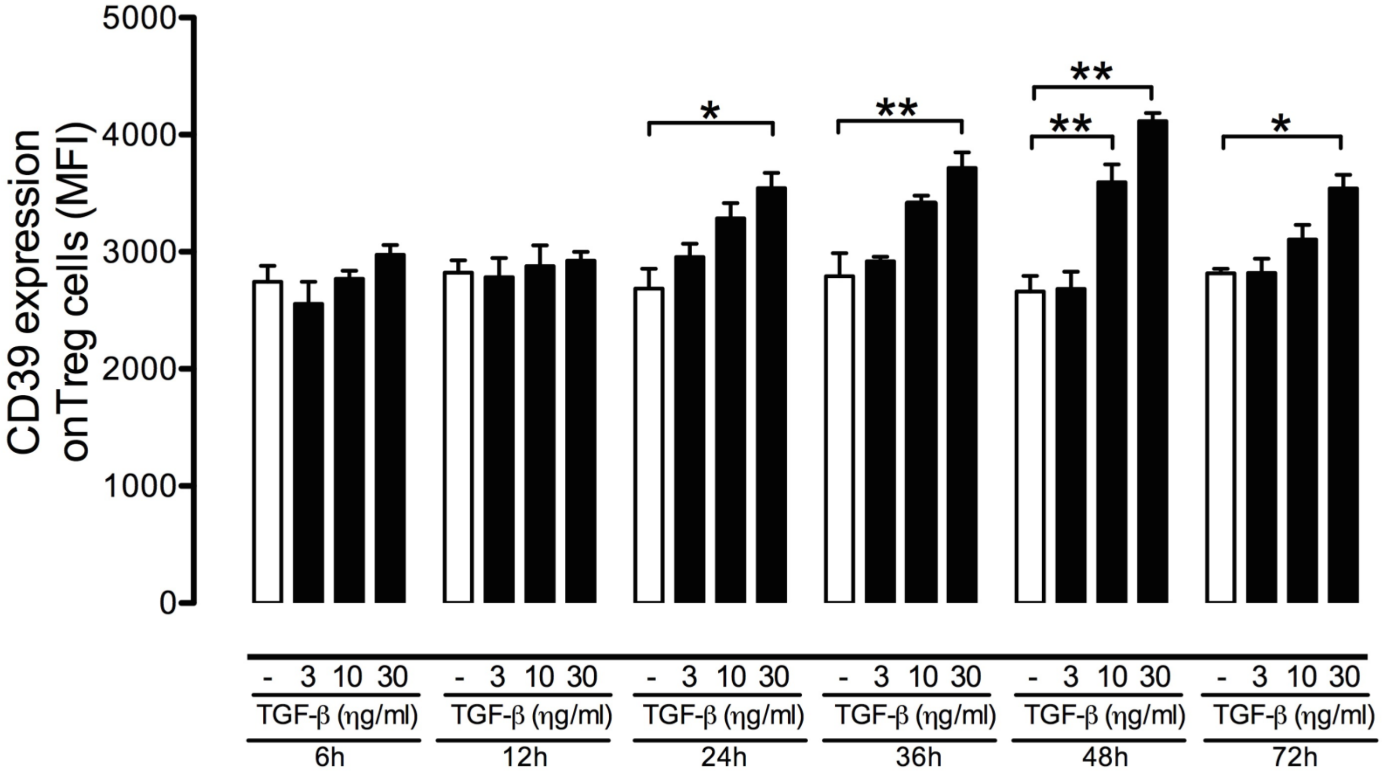
	RA patients			Healthy controls (n= 30)
	R-MTX (n = 50)	UR-MTX (n = 38)	P	
Mean of age (years)	56,0 ± 13,72	53,0 ± 16,38	NS[a]	37,6± 9,5
Woman (%)	88	80	NS[b]	66,7
Smoking (%)	30	44,7	NS[b]	23,3
Time of disease (months)	30,54 ± 23,42	32,29 ± 22,52	NS[a]	NA
FR-positive (%)	86	86,84	NS[b]	NA
ACPA-positive (%)	90	92,1	NS[b]	NA
DAS28 before MTX (mean ± SD)	5,78 ± 0,47	6,03 ±0,55	NS[a]	NA
DAS28 after MTX (mean ± SD)	2,31 ± 0,58	4,74 ± 1,32	$P < 0.01$[a]	NA
MTX (%)	100	100	NS[b]	NA

RF, rheumatoid factor; ACPA, anticitrullinated protein antibodies; DAS28, disease activity score; MTX, Methotrexate; R-MTX, responsive to MTX; UR-MTX, unresponsive to MTX; NA, not applicable; SD, standard deviation; NS, not significant; a, 2-sample exact test; b, Fisher's exact test

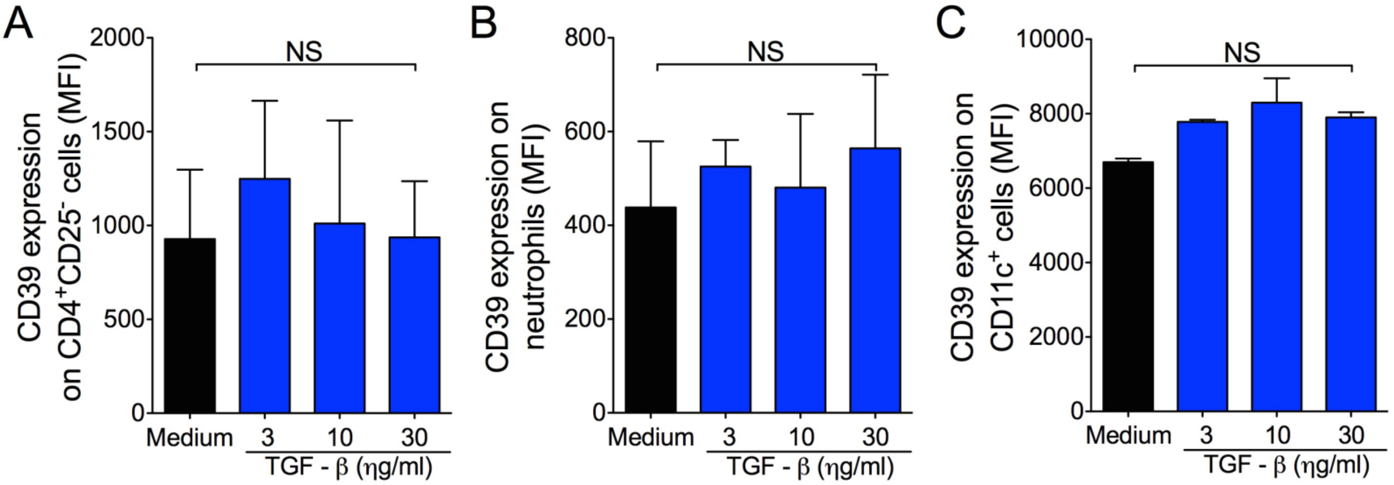
Supplementary Table 3. Clinical features of RA patients in genetic association analysis

	RA patients		
	R-MTX (n = 99)	UR-MTX (n = 158)	P
Mean of age (years)	61,5 ± 15,48	59,7 ± 12,44	NS[a]
Woman (%)	82,8	88,6	NS[b]
Smoking (%)	30,3	34,8	NS[b]
Time of disease (months)	113,35 ± 46,94	116, 36 ± 76,5	NS[a]
FR-positive (%)	65,6	69,6	NS[b]
ACPA-positive (%)	70,7	82,2	NS[b]
Biologic agents use (%)	0	49,4	NS[b]

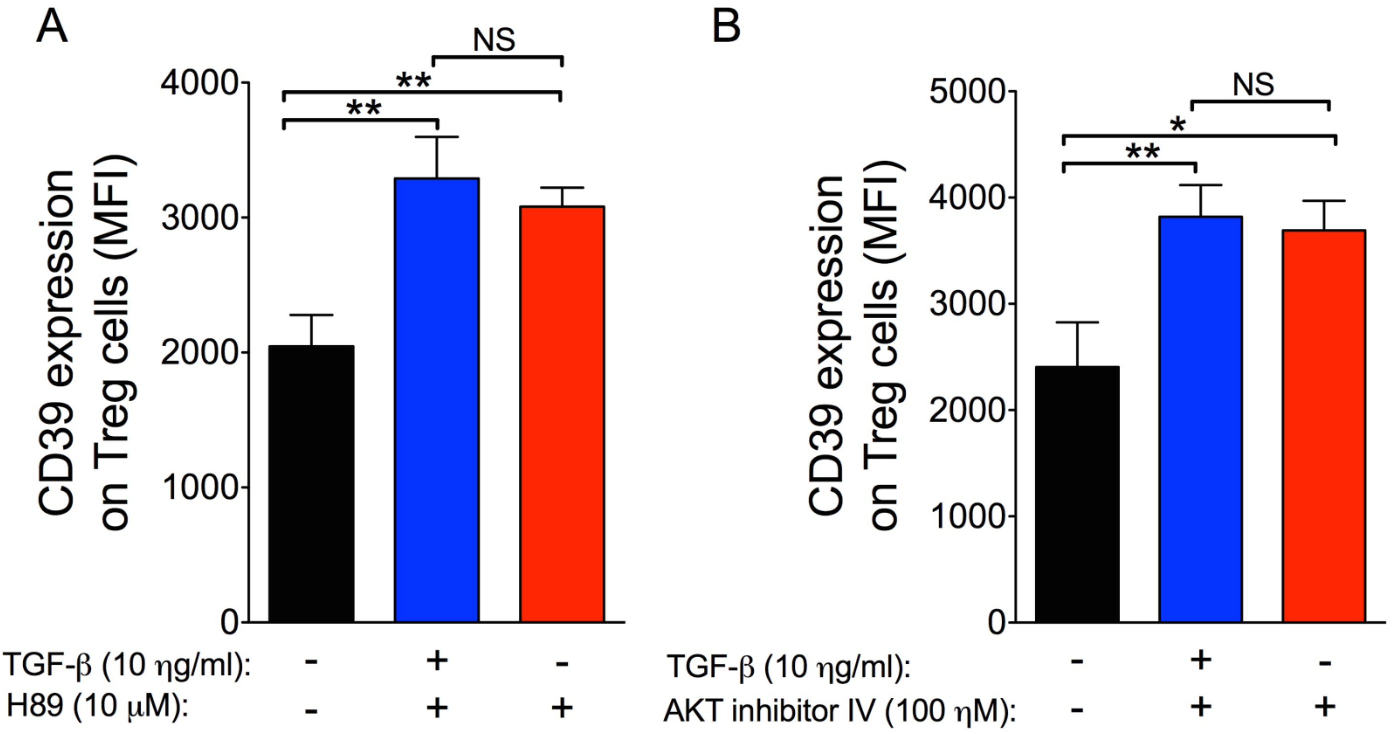
RF, rheumatoid factor; ACPA, anticitrullinated protein antibodies; DAS28, disease activity score; MTX, Methotrexate; R-MTX, responsive to MTX; UR-MTX, unresponsive to MTX; SD, standard deviation; NS, not significant; a, 2-sample exact test; b, Fisher's exact test



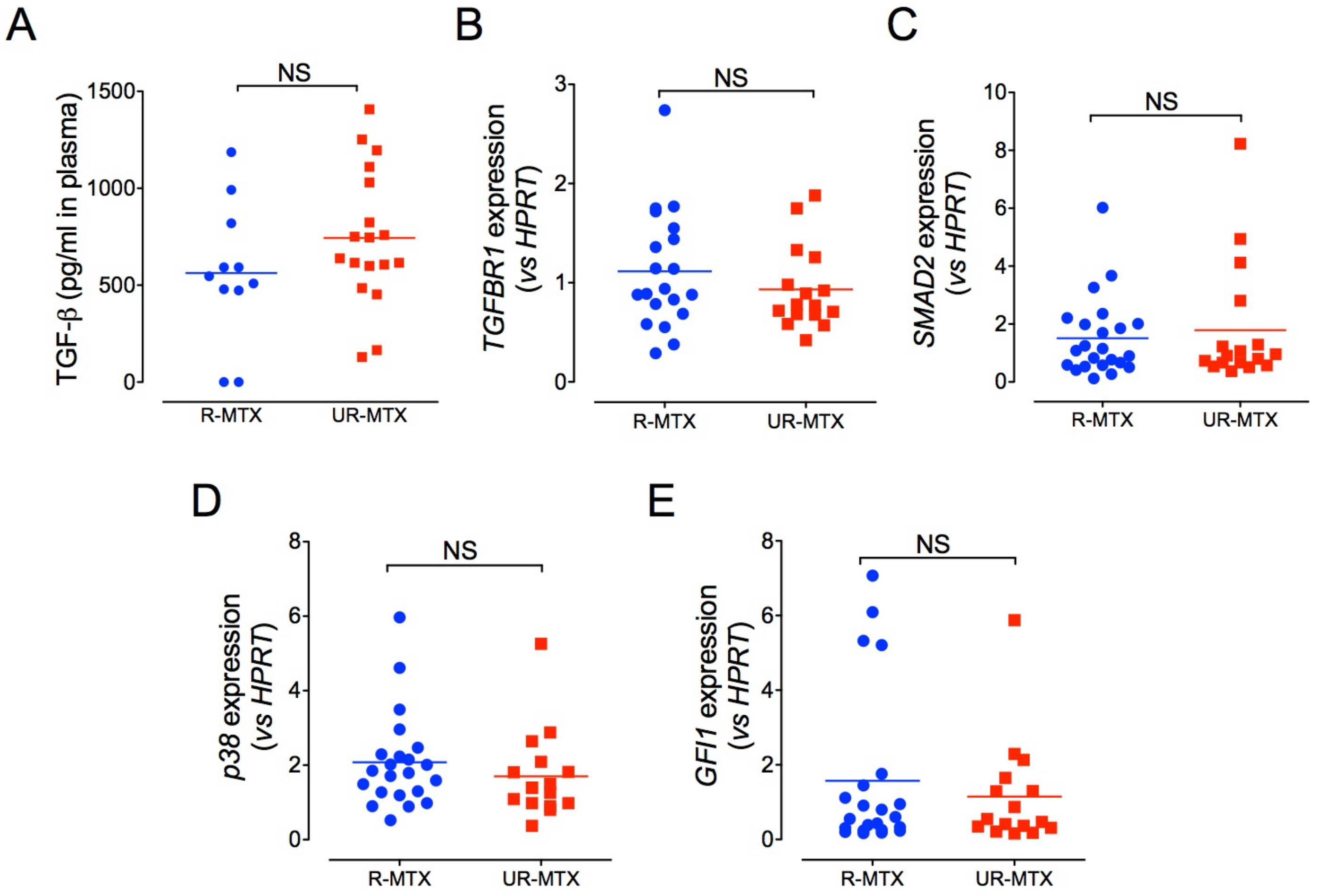
Supplementary Fig. 1. Time-course of induction of CD39 expression on Tregs by TGF-β. Tregs ($CD4^+CD25^+$) were purified from the PBMC of healthy donors (n = 5) and cultured for up to 72 h with anti-CD3 and anti-CD28 in the presence of graded concentrations of TGF-β. The graph shows CD39 expression (MFI) on Tregs. Data are mean ± SEM, *P <0.05, **P <0.01 by one-way ANOVA result followed by Bonferroni's t test.



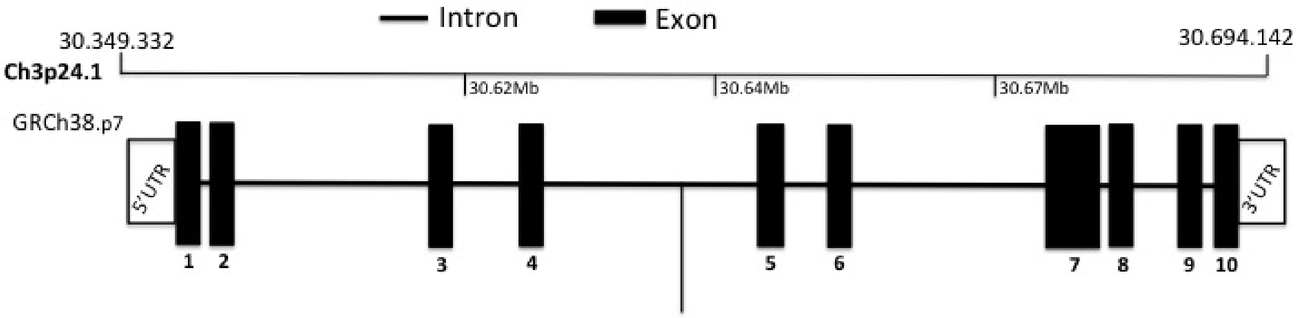
Supplementary Fig. 2. TGF-β does not affect CD39 expression on Teff, neutrophils or CD11c$^+$ cells. CD39 expression (MFI) on effector T cells (CD4$^+$CD25$^-$) cultured for 48 h (**A**), on granulocytes incubated for 12 h (**B**), on CD11c$^+$ cells cultured for 48 h (**C**) with graded concentrations of TGF-β. NS = Not significant, n=4.



Supplementary Fig. 3. Inhibitions of PKA or p38 activity do not affect the TGF-β-mediated CD39 expression on Tregs. CD39 expression (MFI) on Tregs from healthy donors (n = 4) were cultured for 48 h with anti-CD3, anti-CD28 and TGF-β with (**A**) PKA inhibitor (H89) or (**B**) AKT inhibitor IV. Data are mean ± SEM, *P<0.05, **P<0.01, NS = Not significant by one-way ANOVA result followed by Bonferroni's t test.



Supplementary Fig. 4. Quantification of the TGF-β signalling pathway in Tregs from R-MTX and UR-MTX patients. (**A**) Levels of TGF-β in plasma from R-MTX (n = 11) and UR-MTX (n = 18). (**B-E**) mRNA of *TGFBR1* (**B**), *SMAD2* (C), *p38* (**D**), *GFI-1* (**E**) in CD4$^+$ cells of R-MTX (n = 23) and UR-MTX (n = 17). NS = Not significant.



refSNP	**rs1431131**
Chr Pos	30.634.388
Ancestral/ Mutant	T/A
Type	Transition Substitution

Supplementary Fig. 5. Schematic representation of rs1431131 location in human *TGFBR2* gene (HGNC:11773; NC_000003.12). *rs1431131* is located in the intronic region between exons 4 and 5. Figure adapted from the Ensembl display:

http://www.ensembl.org/Homo_sapiens/Gene/Summary?g=ENSG00000163513;r=3:30606502-30694142