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Eltrombopag Preserves the Clonogenic Potential of Hematopoietic Stem Cells During Treatment With Antithymocyte Globulin in Patients With Aplastic Anemia

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ABSTRACT

Aplastic anemia (AA) is frequently caused by a T-cell mediated autoimmune depletion of the hematopoietic stem and progenitor cell (HSPC) compartment. Immunosuppressive therapy (IST) with antithymocyte globulin (ATG) and cyclosporine represents the first-line treatment of AA. One side effect of ATG therapy is the release of proinflammatory cytokines such as interferon-gamma (IFN-γ), which is considered a major factor in the pathogenic autoimmune depletion of HSPC. Recently, eltrombopag (EPAG) was introduced for therapy of refractory AA patients due to its ability to bypass IFN-γ-mediated HSPC inhibition among other mechanisms. Clinical trials have evidenced that EPAG started simultaneously with IST leads to a higher response rate compared with its later administration schedules. We hypothesize that EPAG might protect HSPC from negative effects of ATG-induced release of cytokines. We observed a significant decrease in colony numbers when both healthy peripheral blood (PB) CD34$^+$ cells and AA-derived bone marrow cells were cultured in the presence of serum from patients under ATG treatment, as compared with before treatment. Consistent with our hypothesis, this effect could be rescued by adding EPAG in vitro to both healthy and AA-derived cells. By employing an IFN-γ neutralizing antibody, we also demonstrated that the deleterious early ATG effects on the healthy PB CD34$^+$ compartment were mediated at least partially by IFN-γ. Hence, we provide evidence for the hitherto unexplained clinical observation that concomitant use of EPAG in addition to IST comprising ATG leads to improved response in patients with AA.

INTRODUCTION

Aplastic anemia (AA) is a rare life-threatening disease characterized by pancytopenia due to progressive bone marrow (BM) failure.[1,2] In the majority of the cases, AA is caused by an autoimmune T-cell-mediated attack against hematopoietic stem and progenitor cells (HSPC). Interferon-gamma (IFN-γ) is involved in the pathogenesis of AA[3–5] and has a negative effect on HSPC survival by preventing the engagement of the thrombopoietin (TPO) axis.[6]

The basic treatment principle in acquired AA is the termination of the T-cell-mediated attack against HSPC. Immunosuppressive therapy (IST) with antithymocyte globulin (ATG) and cyclosporine A (CSA) has become the standard first-line therapy and about two thirds of the patients respond to a first treatment cycle.[7] Despite being beneficial for the depletion of autoreactive T cells,[8] ATG administration also results in a massive release of inflammatory cytokines such as IFN-γ, tumor necrosis factor (TNF), and other interleukins.[9,10]

Eltrombopag (EPAG) induces downstream signaling of the TPO-R[11] and was recently introduced in the therapy of refractory AA due to its ability to improve hematopoiesis as monotherapy and in combination with IST.[12–14] A recent study has shown that EPAG enhances HSPC function by bypassing the IFN-γ-mediated perturbation of the TPO axis.[15]

When Townsley et al[16,17] published the results of a clinical trial combining horse ATG and CSA with EPAG, the authors showed that the concomitant treatment of EPAG right from the beginning of IST led to even higher rates of complete and overall response rates as compared with the application of EPAG only from day 14 post-ATG treatment (ATGt).

In our hypothesis, such differences in treatment response might be initiated by EPAG ability to rescue HSPCs from the early negative effects of ATG-related release of inflammatory cytokines as for example IFN-γ. Consequently, we studied HSPCs function in vitro after their incubation with EPAG and the sera from AA patients undergoing ATGt.

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PATIENTS AND METHODS

Patients

Peripheral blood (PB) for isolation of PB CD34+ cells was taken from 10 healthy donors (HD) undergoing granulocyte colony-stimulating factor (G-CSF) treatment for stem cell mobilization. Serum was collected from 5 untreated healthy individuals.

BM and sera from 11 patients with relapse/refractory, severe or moderate AA undergoing ATGt (horse or rabbit), and CSA were analyzed for this study. Two patients received EPAG during previous therapies, but in both patients EPAG administration was stopped at least 2 months before the ATGt. No patient showed signs of clonal evolution at the time-point of ATG administration. Detailed patient characteristics are shown in Table 1. All samples were taken after written consent and according to the approval by the local ethics committee (EK206/09 for healthy individuals; EK332/20 for AA patients, all enrolled in the AA-BMF Registry).

BM derived from severe/very severe AA patients does not give rise to sufficient colonies assayed in standard colony-forming unit (CFU) assay. Therefore, we preferentially employed samples derived from patients with relapsed or moderate AA in need of ATG therapy. Acquisition of BM mononuclear cells (BM-MNC) from AA patients was carried out 1–2 weeks before ATGt during routine diagnostic work-up.

Serum from the PB of patients undergoing IST was taken and frozen down the day before ATG administration and 1–2 days after the start of ATG administration.

CFU assay

PB from the healthy individuals and AA-derived BM samples were subjected to gradient centrifugation with Pancoll (Pan Biotech, Germany). Next, HD-derived mononuclear cells (MNC) were subjected to magnetic-activated cell sorting using CD34+ microbeads (Miltenyi Biotech, Germany) according to the manufacturer´s instructions, whereas AA-derived BM-MNC were not. A total of 100,000 HD-derived PB CD34+ cells or AA-derived BM-MNC were cultured in Stem Pro-34 SFM medium (Thermo Fisher Scientific, USA) without adding any cytokines but in the presence of 100 µL of the serum from patients with AA before treatment with ATG, 100 µL of serum from patients with AA after treatment with ATG or their combination with EPAG 5 µM (Selleckchem, Germany), romiplostim 500 ng/mL (Amgen Europe, Germany), or TPO 100 ng/mL (Immunotools, Germany). DMSO 0.005% and the serum of a healthy individual were added as a control condition. Cells were cultured with these conditions at a density of 65,000 cells/mL for 48 hours in order to appreciate the effects of cytokines and growth factors present in the patient sera without the confounding effect of additional added cytokines. Afterwards, to perform CFU assays, cells were centrifuged down and transferred on a semisolid medium at a density of 25,000 cells/mL in medium containing 80% methylcellulose (Stem Cell Technologies, Germany), 20% Iscove's Modified Dulbecco's Medium (Gibco Thermo Fisher, USA), in the presence of 50 ng/mL recombinant human stem cell factor, 10 ng/mL rhIL-3, 10 ng/mL rhGM-CSF, 3 U/mL rhEPO (all Immunotools, Germany), and 0.5% ciprofloxacin. To perform serial replating CFU experiments, cells derived from 3 patients were recovered from the CFU plates by adding PBS at 37°C in order to dissolve the methylcellulose. After centrifugation, 500,000 cells/mL per condition were then reseeded and a second CFU assay performed.

One milliliter of semisolid medium containing cells was seeded for both CFU and replating CFU experiments in 35-mm cell culture dishes (with grid, Thermo Fisher, USA), and 3 dishes were plated per condition and colonies were counted manually by 2 operators independently after 14 days. Representative images of the assay were taken with an automated, inverted imaging system (EVOS M700, Thermo Fisher, Germany) with a 10× magnification or with an inverted fluorescence microscope (EVOS FL, Thermo Fisher). In this case, 500 µL of semisolid medium was seeded in standard 24-well plates. A validation of our manual score of colonies was performed using ImageJ software. Briefly, images were converted to 8-bit and an intensity threshold was determined. Finally, a particle analysis was performed (Suppl. Figure S1A). Data obtained with this protocol are reported in Suppl. Figure S1B and manual counting of the same samples seeded in 35-mm cell culture dishes is shown in Suppl. Figure S1C.

Measurement of IFN-γ concentration

The measurement of the concentration of IFN-γ in the serum of patients with AA before and after treatment with ATG was performed using the Cytometric-Bead-Assay according to the manufacturer´s instruction.

IFN-γ depletion

The serum of patients with ATG was depleted of IFN-γ using a monoclonal antibody (Immunotools cat no 21853530, Germany). The serum was incubated for 2 hours at 4°C with the antibody at a concentration of 10 µg/mL and employed for further experiments.

Intracellular staining

HD-derived PB CD34+ cells were incubated for 3 hours with the serum of patients before and after ATGt, in combination with EPAG 5 µM or DMSO. Stimulation with 500 units IFN-γ was

Table 1

Main Characteristics of the Aplastic Anemia Patients Analyzed in This Study

UPN	Age (y)	Sex	AA Classification	Treatment at Our Department	Previous Treatments
1	57	F	Refractory mAA	hATG/CSA	CSA mono
2	71	F	Relapse sAA	rATG/CSA	hATG/CSA 2 y earlier, EPAG (stop 2 mo before rATG)
3	71	F	First Dx sAA	hATG/CSA	None
4	61	M	Relapse sAA	rATG/CSA	hATG/CSA 8 mo earlier
5	59	F	Relapse sAA	rATG/CSA	hATG/CSA 3 y ago, no treatment at Dx of relapse
6	68	M	Relapse sAA	hATG/CSA	CSA mono, rATG/CSA (6 y earlier)
7	28	M	First Dx sAA	hATG/CSA	None
8	55	M	Refractory mAA	hATG/CSA	CSA mono, EPAG for 6 mo (stop 2 mo before ATG treatment)
9	58	M	First Dx sAA	hATG/CSA	None
10	59	F	First Dx mAA	hATG/CSA	None
11	68	F	First Dx sAA	hATG/CSA	None

CSA = cyclosporine A; Dx = diagnosis; EPAG = eltrombopag; hAT = horse antithymocyte globulin; mAA = moderate aplastic anemia; rATG = rabbit antithymocyte globulin; sAA = severe aplastic anemia; UPN = unique patient number.

used as positive control. Afterwards, cells were fixed with paraformaldehyde (1.5%) and permeabilized with 80% ice-cold methanol and finally incubated with an anti-phospho-protein-directed monoclonal antibody (or isotype-matched IgG). Specifically, Alexa Fluor 647 Anti-Smad2 (pS465/pS467)/Smad3 (pS423/pS425) (Clone O72-670, BD International, Germany) was used. Cells were acquired on a BD Accuri flow cytometer (BD). Data were collected and analyzed using Flowjo (version 10; BD).

RNA isolation and quantitative PCR

HD-derived PB CD34+ cells and AA-derived BM-MNC were handled as described in the previous paragraph. Total RNA from MNCs was extracted using miRNeasy micro Kit plus (Qiagen, Germany) according to the manufacturer's instruction. cDNA was generated using random hexamers and the M-MLV Reverse Transcriptase (both Invitrogen, USA). Quantitative PCR (RT-qPCR) was performed with the SYBRGreen mix (Invitrogen, USA) and the ABI7500fast real-time PCR system (Applied Biosystems, USA) according to the standard PCR conditions. Primers used for RT-qPCR are the following: IRF1-F: GCCATTCACACAGGCCGATA; IRF1-R: GTGGAAGCATCCGGTACACT; MT-ATP6-F: CGT ACG CCT AAC CGC TAA CA; MT-ATP6-R: AGG CGA CAG CGA TTT CTA GG; STAT1-F: TGTATGCCATCCTCGAGAGC; STAT1-R: AGACATCCTGCCACCTTGTG. *MT-ATP6* was used as a housekeeping gene.

Statistical analysis

Statistical analysis was performed with GraphPad Prism (version 9.1.1, GraphPad Software, USA). One-way ANOVA was used, followed by Holm-Šídák's multiple comparisons test. In Suppl. Figure S2C, a paired *t* test was used to assess statistical significance. *P*-values <0.05*, <0.01**, <0.001***, and <0.0001**** were considered as statistically significant.

RESULTS

Patient serum under ATGt impairs the colony-forming potential of healthy PB CD34+ cells and AA-derived BM-MNC, an effect that can be partially reverted by EPAG

Incubation with serum collected under ATGt significantly impaired the clonogenic potential of healthy PB CD34+ cells when compared with the treatment with serum collected before ATGt (Figure 1A and 1B). The addition of EPAG was able to rescue this effect and reestablished the colony numbers to the levels of the pre-ATGt condition. Validation of manual colony scoring was performed by automated colony quantification (Suppl. Figure S1A-S1C). Interestingly, healthy PB CD34+ cells incubated with AA-derived serum before ATGt produced more colonies compared with cells incubated with HD-derived serum, likely because of an increased concentration of stimulatory cytokines, such as erythropoietin, TPO, or G-CSF in the serum of AA patients.[18–21] Treatment of healthy donor-derived PB CD34+ cells with serum pre- and post-ATG in combination with romiplostim, a fusion protein analog of TPO,[22] showed similar results, because romiplostim was also able to improve the clonogenic potential when added to the serum post-ATG (Suppl. Figure S2A). In contrast, treatment with TPO did not improve colony numbers when added to serum after ATGt (Suppl. Figure S2B).

Next, we analyzed the effect of ATG sera on the BM of the respective AA patients. Serum collected under ATGt caused a decrease in the number of colonies grown from AA-derived BM-MNC, compared with the colony-growing potential of serum before ATGt (Figure 1C). Again, addition of EPAG significantly increased colony growth similar to the numbers obtained with serum before ATGt. To assess the effect of the ATGt on more primitive HSPC, we performed serial replating using cells obtained from the assays depicted in Figure 1C, without any further treatment. We observed that initial treatment with the serum after ATGt had severely impaired the ability of cells to form colonies, and the addition of EPAG could rescue them, at least partially (Figure 1D).

Overall, the exposure of both healthy PB CD34+ cells and AA-derived BM-MNC to the serum of AA patients after ATGt impaired their colony-forming potential. Importantly, the addition of EPAG to the serum obtained under ATGt reverted this ATG-induced suppressive effect.

IFN-γ signaling is upregulated in cells exposed to serum after ATGt and antibody-mediated depletion of IFN-γ partially neutralized the negative effects of serum after ATGt

We studied the contribution of IFN-γ to the negative effects of ATGt on HSPC and whether EPAG could specifically interfere. First, analogous to previous studies,[23] we found increased levels of IFN-γ in the serum of AA patients following ATGt (Suppl. Figure S2C). Second, the mRNA levels of interferon regulatory factor 1 (*IRF1),* which are upregulated in cells after IFN-γ exposure,[24] were significantly increased in healthy PB CD34+ cells incubated with serum post-ATG compared with incubations with serum before ATGt. This effect was most striking after 2–3 hours of exposure (Suppl. Figure S2D). Similarly, levels of signal transducer and activator of transcription 1 (*STAT1*), another factor upregulated in response to IFN-γ,[25] were significantly increased over a time course of 6 hours of cultivation with serum after ATGt (Suppl. Figure S2E). The addition of EPAG lead to significantly lower levels of both *IRF1* and *STAT1* already after 3 hours of incubation, thereby showing an early beneficial priming effect of EPAG on CD34+ cells (Figure 2A; Suppl. Figure S2F). In line with the data of healthy PB CD34+ cells, the mRNA levels of *IRF1* and *STAT1* were also significantly increased in AA-derived BM-MNC incubated with serum post-ATG, but EPAG only partially decreased their levels (Figure 2B and 2C).

Third, we observed a decreased expression of phospho-SMAD2/3, which is indirectly caused by IFN-γ signaling phosphorylation,[26] in healthy PB CD34+ cells cultured in the presence of AA-derived serum after ATGt, when compared with their levels before ATG (Figure 2D). And again, addition of EPAG only partially increased the expression of phospho-SMAD2/3.

Finally, we incubated healthy PB CD34+ cells with AA patient-derived serum after ATG with an IFN-γ-depleting antibody and we observed a significant 2-fold increase of colony number as compared with the setting without antibody (Figure 2E).

In conclusion, we could detect IFN-γ activity in HSPC exposed to serum derived from patients after ATGt, which seems, at least in part, responsible for the significant impairment of colony growth observed. Of note, despite the fact that EPAG was successful in restoring the clonogenic capability of HSPC by mitigating the adverse effects of serum post-ATGt, EPAG only initiated a partial inhibition of the IFN-γ activity.

DISCUSSION

In this study, we hypothesized that during the standard treatment of AA with IST, the HSPC might be directly harmed in particular due to the ATG-mediated release of proinflammatory cytokines such as IFN-γ[9,10] and that concomitant EPAG treatment might overcome this early toxic effect on HSPC. EPAG has been previously described to be able to stimulate immature HSPC by numerous studies,[12,14,27] leading to an increased multilineage differentiation and an increase in the number of functional stem cells. Here, we show that ATG-related cytokines directly affect the colony-forming potential of HSPC derived from healthy individuals and patients with AA and that such effect can be overcome by the addition of EPAG to ATGt. We initially hypothesized that EPAG might protect HSPC from the toxic effects of ATGt

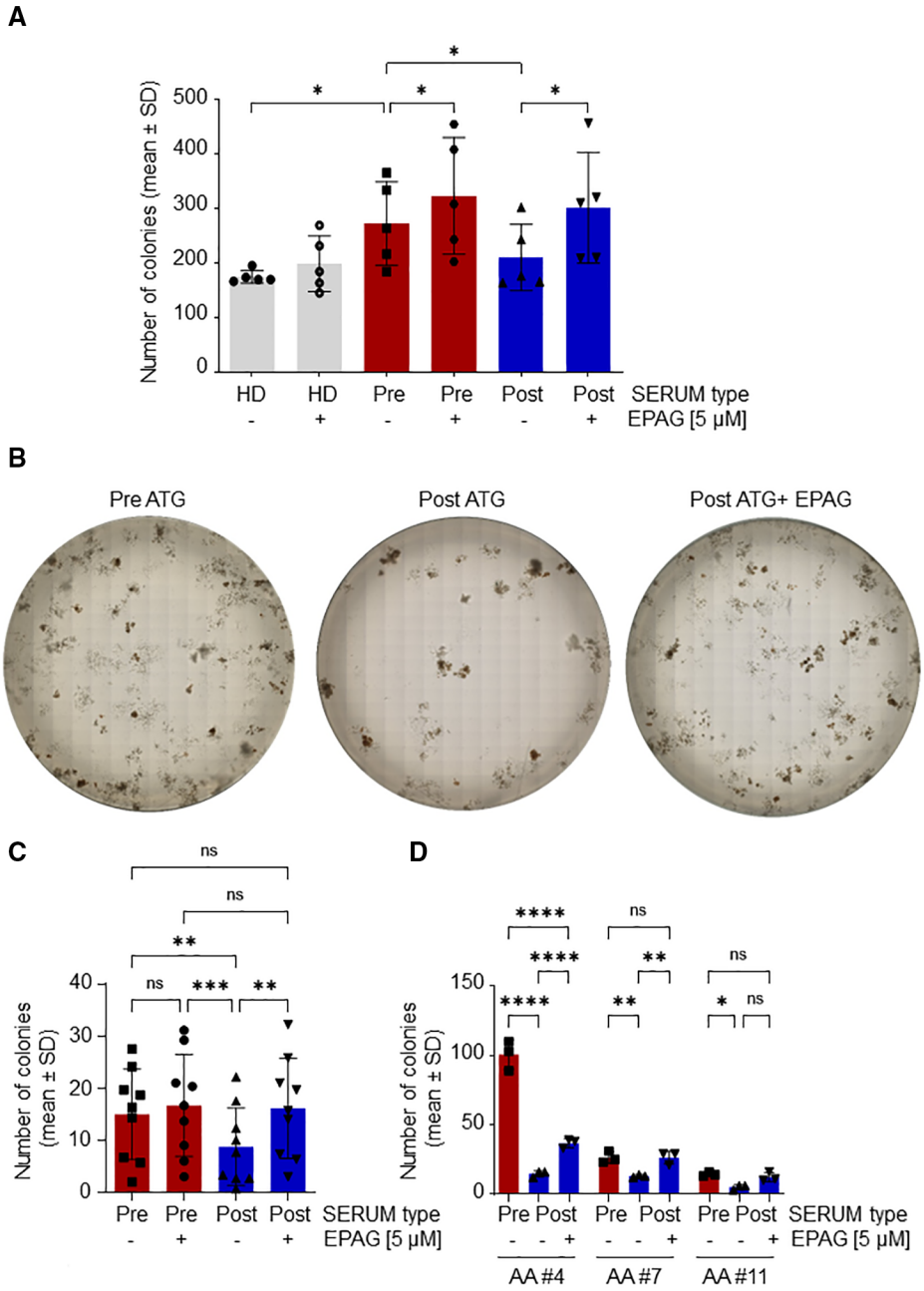

Figure 1. Effects of AA-derived sera post-ATG treatment and EPAG in healthy and diseased HSPCs. (A) CFU assay of MACS-sorted PB CD34⁺ cells isolated from 5 HD after 48 h incubation with serum from 4 patients with AA before and after treatment with ATG or their combination with EPAG 5 µM. DMSO 0.005% and the serum of a healthy controls was added to the untreated condition. Colonies were counted after 14 d. Five independent experiments were conducted. Triplicates of each condition were analyzed. (B) Representative pictures of a CFU assay performed with PB CD34⁺ cells isolated from HD treated with serum from a patient with AA before ATG, after treatment with ATG with or without EPAG 5 µM. (C) CFU assay of 9 AA-derived BM-MNC after 48 h incubation with matching serum before and after treatment with ATG or their combination with EPAG 5 µM. DMSO 0.005% was used as vehicle control. Triplicates of each condition were analyzed. (D) Replating of CFU assays of 3 AA-derived BM-MNC without any further treatment. Patient ID (AA #) is reported. Triplicates of each condition were analyzed. AA = aplastic anemia; ATG = antithymocyte globulin; BM-MNC = bone marrow mononuclear cells; CFU = colony-forming unit; EPAG = eltrombopag; HD = healthy donors; PB = peripheral blood.

by counteracting their IFN-γ-mediated damage, as previously described,[15] but we observed only a partial disabling of IFN-γ signaling upon EPAG addition. Interestingly, also the addition of romiplostim to the serum post-ATG lead to results comparable to EPAG, with a significant improvement of colony numbers. Romiplostim does not share the ability of EPAG to bind TPO-R receptor allosterically and therefore its ability to effectively stimulate the TPO-R receptor in a high-IFN-γ environment is not completely understood.[22,28] Overall, these results suggest that the counteraction of IFN-γ signaling is only partially responsible for the observed improved clonogenic potential and implicates the involvement of other additional factors.

Another important observation is that, based on our in vitro data, we cannot definitively conclude whether ATG-related IFN-γ release results in long-lasting, irreversibly impaired reconstitution of the HSPC pool in vivo. However, we see a positive effect of EPAG on more primitive HSPC in our replating assay. In addition, the positive effects of EPAG on HSPC might be still long-term,

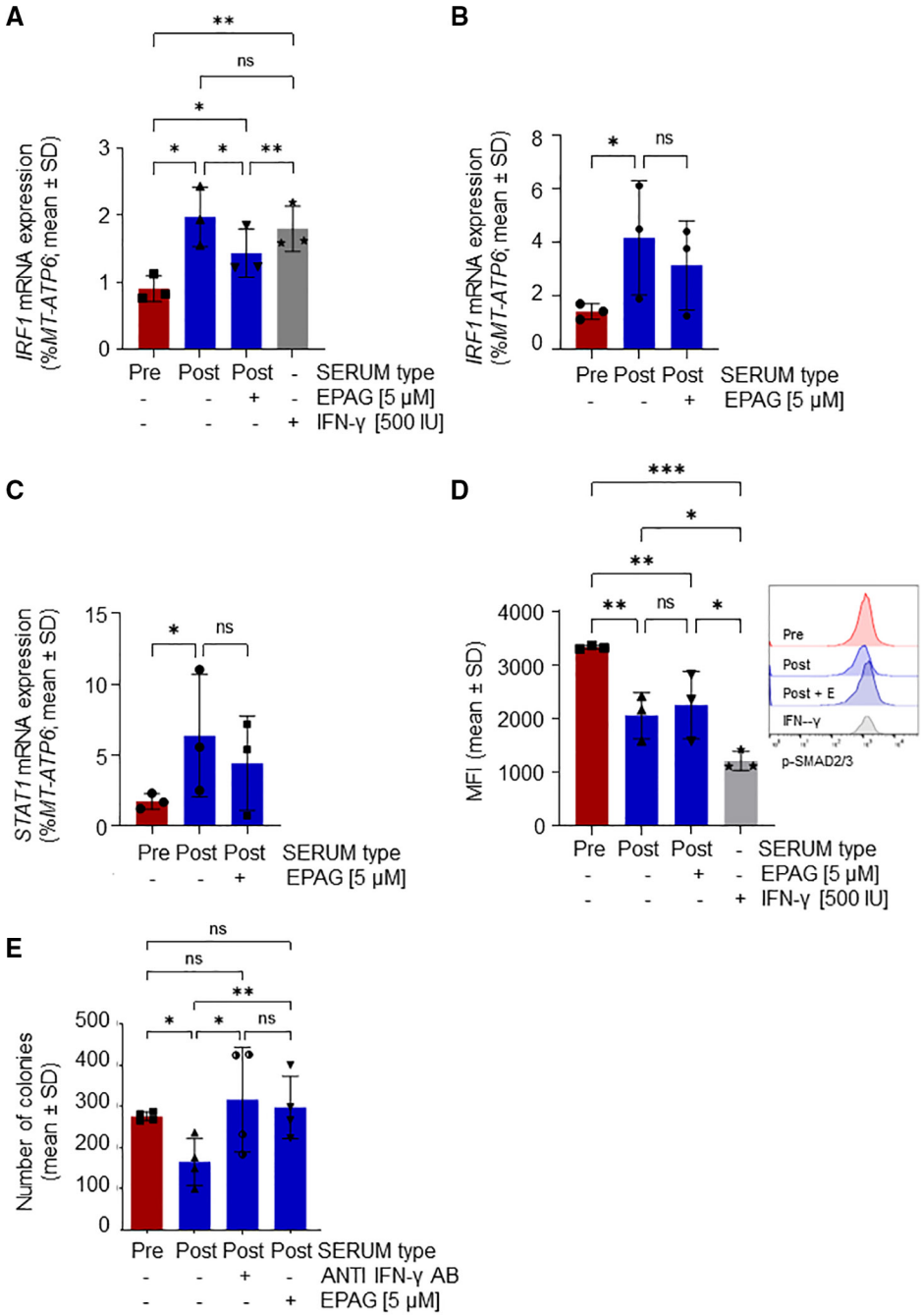

Figure 2. Interferon-γ signaling in AA-derived sera post-ATG and EPAG treatment. (A) mRNA expression analysis of *IRF-1* in 3 healthy PB CD34⁺ after 3 h of incubation with serum from 3 patients with AA before and after treatment with ATG, their combination with EPAG 5 µM or treated with IFN-γ 500 U. Triplicates of each condition were analyzed. mRNA expression analysis of *IRF-1* (B) and *STAT1* (C) in 3 AA-derived BM-MNC samples exposed to serum before ATGt, after ATGt, and after ATGt + EPAG 5 µM. Triplicates of each condition were analyzed. (D) Flow cytometry analysis of phospho-SMAD2/3 in 3 healthy PB CD34⁺ after 3 h of incubation with serum from 3 patients with AA before and after treatment with ATG, their combination with EPAG 5 µM or treated with IFN-γ 500 U. Three independent experiments were conducted. (E) CFU assay of 2 healthy PB CD34⁺ cells after 48 h of incubation with serum from 4 patients with AA after treatment with ATG and either vehicle control, IFN-γ depleting antibody or EPAG 5 µM. Four independent experiments in triplicates were performed. AA = aplastic anemia; ATG = antithymocyte globulin; ATGt = ATG treatment; BM-MNC = bone marrow mononuclear cells; CFU = colony-forming unit; EPAG = eltrombopag; PB = peripheral blood.

given recent findings showing that EPAG acts like a pan ten-eleven translocation dioxygenase (TET-dioxygenase) inhibitor and that TET2 inhibition could contribute to the observed prolonged clonogenic potential of healthy HSPCs observed in vivo.[27,29,30]

Our study justifies the question whether EPAG should be added concomitantly from treatment start instead of only from day +14 of conventional ATG/CSA-treatment observed by Townsley et al.[16] This finding is of high importance given that more recent confirmatory data obtained within the Randomized, Multicenter Trial Comparing Horse ATG plus Cyclosporine with or without Eltrombopag as First-Line trial established EPAG start at day +14 after ATG.[17] This strategy was used with the aim to avoid potential additional toxicity when EPAG is administered parallel to ATG/CSA. Our data, however, reinforce the idea to initiate concomitant EPAG right from the start of IST to salvage the HSPC pool from early cytokine-mediated damage.

The observation that an IFN-γ antibody resulted in similar effects as EPAG might provide a strong rational for follow-up clinical research in AA. Given that a fully human monoclonal antibody against IFN-γ, namely emapalumab, is already approved for the treatment of hemophagocytic lymphohistiocytosis, thus could provide a novel approach to be investigated in AA as well.[31]

One limitation of our study is that we could not yet identify additional factors contributing to the impaired clonogenic potential of HSPC after ATGt. TNF or interleukin-6 might be relevant players because they are also increased after ATGt.[9,23] Moreover, TNF is known to contribute to BM failure in AA.[32] Additional studies are needed to address this issue.

In summary, our data provide first evidence for the role of ATG-related cytokine release on the colony-forming potential of HSPC derived from HD and patients with AA. The improvement of efficacy by the addition of EPAG to ATGt only partially mediated via the bypassing of early HSPC-toxic effects of IFN-γ and concomitant treatment with EPAG and ATGt might result in improved long-term outcome in patients with AA.

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AUTHOR CONTRIBUTIONS

MV and BR contributed equally to this work. MV performed the experiments, analyzed and interpreted the data, and wrote the article. BR performed the experiments, interpreted the data and provided patient samples and clinical data, assisted and participated during article drafting, and edited the article. MC performed parts of the experiments. MSR, JP, SI, and THB analyzed and interpreted the data. FB conceived and planned the study design, provided patient samples, clinical data, interpreted the data, and wrote the article.

DISCLOSURES

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