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Research Article

Dynamics of Interaction Between Erythrocytes and Vascular Endothelium Under IFN-Alpha: An Optical Tweezers Study

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Abstract

Background: Interferon alpha (IFN-α) is an antiviral drug with widespread clinical use. Several studies report its effects on the endothelium, including the regulation of nitric oxide (NO) production. Such effects may cause adverse effects in patients with altered blood microrheology, as the interaction between red blood cells (RBCs) and the endothelium is a key factor governing blood microrheology. This study aimed to investigate the effect of IFN-α on the RBC–endothelium system *in vitro*. **Methods:** Human umbilical vein endothelial cells (HUVECs) were used as a model of vascular endothelium; human RBCs were obtained from healthy donors, and the forces of intercellular interactions were measured using optical tweezers. Statistical significance was assessed using the Mann–Whitney U test. **Results:** In the short-term exposure (15–60 minutes), the addition of IFN-α decreased RBC aggregation forces indirectly via the activation of the capacity of HUVECs to produce signaling molecules. Long-term (24 hours) HUVEC incubation with IFN-α had almost no effect, and long-term IFN-α incubation followed by short-term IFN-α reintroduction resulted in a weaker effect at higher concentrations. In the latter case, RBC–HUVEC adhesion decreased at certain concentrations. The minimal sample size comprised five measurements for each of the three donors at each IFN-α concentration. **Conclusions:** These findings reveal a complex interplay between interferon, endothelium, and RBCs, which should be considered in certain clinical applications of IFN-α. In particular, short-term endothelial exposure to IFN-α can decrease RBC aggregation, which may be helpful or harmful depending on the clinical condition of the patients. Long-term IFN-α treatment followed by short-term IFN-α exposure can decrease RBC–endothelium adhesion forces, altering blood microrheology.

Keywords: interferon-alpha; erythrocytes; endothelium; human umbilical vein endothelial cells; optical tweezers; hemorheology

1. Introduction

Interferon alpha (IFN-α) is a protein with an approximate molecular mass of 19 kDa and is widely used as an antiviral and antineoplastic agent [1]. This compound is used to treat hepatitis B and C, certain cancers, and some ophthalmic disorders [2,3,4,5]. Moreover, IFN-α has recently been used to treat patients with COVID-19; however, the effectiveness of this treatment remains uncertain [6]. The therapeutic effects of IFN-α are attributed to its immunomodulatory, antitumor, and antiangiogenic activities [1]. There are two commonly used subtypes of IFN-α, interferon alpha-2a (IFN-α2a) and alpha-2b (IFN-α2b).

IFN-α affects the endothelium in several ways, for example, by reducing its permeability and nitric oxide (NO) synthesis [7,8,9]. The vascular endothelium is the main source of NO in the circulation and produces it via endothelial nitric oxide synthase (eNOS), which uses the amino acid L-arginine as a substrate. Buie et al. [10] proposed a mechanism through which IFN-α reduces endothelium-derived NO in human umbilical vein endothelial cells (HUVECs) exposed to IFN-α2a. First, IFN-α2a interacts with its receptor on the endothelial membrane, triggering a cascade that suppresses eNOS expression. IFN-α also directly affects eNOS protein in the presence of insulin: IFN-α2a induces phosphorylation at Thr^{495}, which impairs NO production. The authors further showed that IFN-α treatment increased expression of vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemoattractant protein 1 (MCP-1 or CCL2), which are important for endothelial cell–neutrophil adhesion. These findings were supported by measurements of neutrophil adhesion to IFN-α2a-pretreated endothelium, which were significantly increased compared with the control.

Evidence also suggests a more complex effect of IFN-α on the endothelial NO-producing system, including decreased availability of eNOS cofactors and L-arginine [8]. IFN-α2a and IFN-α2b subtypes may exhibit biological activities that differ in magnitude despite their similarities [9]. Jia et al. [9] studied HUVECs *in vitro* and demonstrated an IFN-α-induced downregulation of urokinase-type plasminogen activator secretion. This proteolytic enzyme

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participates in fibrinolysis and may thereby affect the balance between fibrinolysis and coagulation. Prendergast et al. [11] demonstrated a proliferative effect of acute IFN-α treatment in the murine bone marrow endothelium under inflammatory stress conditions *in vivo*. *In vitro* treatment of HUVECs with IFN-α yields ambiguous results. Some authors report a proliferative effect [12], others report an antiproliferative effect [13,14], and some report no significant effect [9,15].

Meanwhile, Jia et al. [9] noted that these discrepancies may be explained by the use of different IFN-α subtypes (IFN-α2a or IFN-α2b) and other experimental conditions. Bauer et al. [16] demonstrated an antiproliferative effect of IFN-α treatment on the endothelium of rats and mice with experimentally induced pulmonary hypertension. Reynolds et al. [17] reported no significant effect in endothelial cell types other than HUVECs. Thus, depending on the endothelium type and the surrounding conditions, IFN-α treatment may result in either proliferative or antiproliferative effects. Some authors report possible toxic effects of cumulative IFN-α treatment on microvasculature and other tissues, which should be considered [18,19,20]. Collectively, IFN-α decreases endothelial permeability, has complex effects on the eNOS system, increases endothelium–neutrophil adhesion, and decreases expression of a plasma proteolytic enzyme that may impair blood coagulation. IFN-α may cause either proliferative or antiproliferative effects depending on the endothelium subtype and the surrounding conditions.

Red blood cell (RBC)–endothelium interactions occur throughout the vascular system, most intensely in terminal capillaries with diameters smaller than a single RBC. These interactions may be direct (i.e., cell adhesion) or indirect (i.e., extracellular signaling). Researchers have studied endothelium–RBC adhesion primarily in the context of several diseases, such as sickle cell anemia, diabetes mellitus, polycythemia vera, and retinal vein occlusion [21,22]. In these disorders, abnormal RBC adhesion to the endothelium disrupts blood flow and may even cause complete vessel occlusion (vaso-occlusion). The molecular basis of this interaction is well described; however, data on RBC–endothelium adhesion in health remain limited because it is often considered insignificant. This interaction could determine RBC clearance from circulation and influence RBC deformability, since RBCs pass through narrow capillaries in the spleen and liver. To our knowledge, no studies have examined RBC adhesive properties to the endothelium as a function of RBC biological age in healthy donors, except for Alshalani et al. [23], which found no significant differences between young and old RBC subgroups. However, data show an age-associated decrease in RBC surface charge, which theoretically favors cell adhesion [24]. In addition to RBC clearance, RBC–endothelium adhesion contributes to vascular flow resistance by affecting axial cell migration. This increases mechanical interaction between blood and the vessel wall, altering hemodynamics [25]. Indirect RBC–endothelium interaction occurs via the exchange of signaling molecules, which trigger intracellular cascades that alter cellular properties. For example, endothelium-derived NO increases RBC deformability and decreases RBC aggregation [26]. The observed complexity of this system increases substantially if one considers that (1) RBCs are capable of producing NO [27,28], (2) RBCs release ATP and, in some cases, free heme, which stimulate endothelium and lead to an increase in NO production [29,30], and (3) RBCs regulate NO bioavailability in the blood flow [31].

In light of the above, it is important to determine whether IFN-α alters the RBC–endothelium interplay. Previous studies of IFN-α interactions with red blood cells were mostly limited to clinical studies of IFN-α in conditions such as polycythemia vera. First used experimentally in the 1990s, IFN-α therapy is now used to treat patients with polycythemia vera, melanoma, and essential thrombocythemia [32,33,34].

Hamidi et al. [35] also investigated IFN-α-loaded RBCs as a potential drug-delivery system. Compared with unloaded RBCs and RBCs loaded with water, interferon-loaded cells demonstrated significantly lower resistance to turbulent flow and higher osmotic fragility, indicating that IFN-α-loaded cells are more fragile. However, this is most likely not an interferon-specific effect but rather a consequence of loading proteins into the cells, as the authors noted for bovine serum albumin.

The side effects of IFN-α therapy are well known and should be considered [36]. Since side-effect frequency is linked to IFN-α dose, studies of IFN-α dose-dependent interactions with human cells could support safer, more tailored therapeutic approaches.

2. Materials and Methods

2.1 Endothelial and RBC Cultures

HUVECs were isolated and cultured as described previously [37,38]. Umbilical cords were obtained after uncomplicated deliveries from healthy donors, with written informed consent in accordance with the Declaration of Helsinki. For the experiments, HUVECs were seeded onto coverslips in 24-well plates and incubated at 37 °C and 5% CO_2 until a monolayer formed. The culture medium consisted of medium 199 (Gibco), gentamicin (Gibco), heparin (Merck), 10% fetal calf serum (HyClone), and endothelial growth factor (200 µg/mL) (Gibco). Only first-passage cells were used in the experiments.

Blood was drawn from the antecubital veins of healthy donors (n = 2–3 per experimental set) into tubes containing lithium heparin as the anticoagulant (two 9 mL tubes). All volunteers were informed about the purpose of the study and provided written informed consent in accordance with the Declaration of Helsinki. Experiments were performed within 8 hours of blood collection. Whole blood was cen-

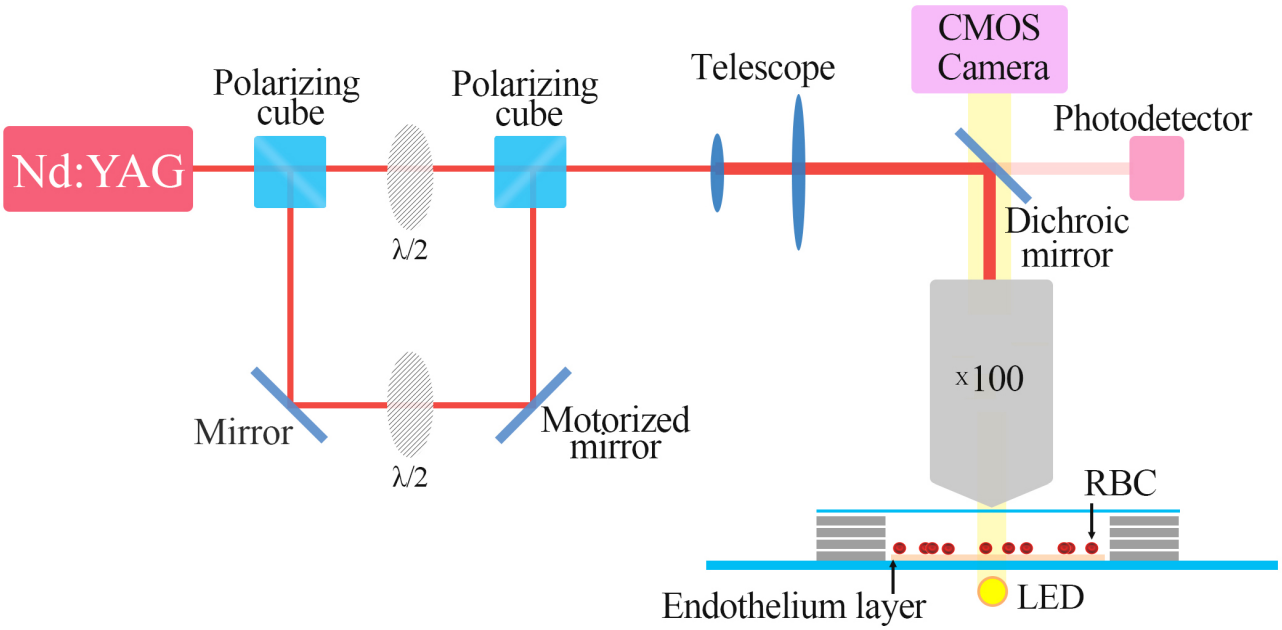


Fig. 1. Schematic representation of dual-channel optical tweezers. A Nd:YAG laser beam is divided into two beams of approximately equal intensity and opposite polarization by a polarizing cube. One beam passes through an additional polarizing cube unchanged and forms the stationary optical trap. The other beam travels through a mirror system that allows independent positional adjustment and creates a movable optical trap. Half-wave plates regulate the laser intensity, controlling the trapping force. A telescope expands the beam to create optimal conditions for trapping red blood cells (RBCs). A dichroic mirror transmits a small fraction of the laser light to a photodetector for real-time intensity measurement. Most of the light is reflected into the objective, which creates the optical traps. A cuvette is positioned beneath the objective and consists of a slide, a monolayer of human umbilical vein endothelial cells (HUVECs), an RBC suspension in autologous plasma at low hematocrit, and a coverslip. LED illumination and a CMOS camera were used to enable sample visualization. LED, light-emitting diode; CMOS, complementary metal-oxide-semiconductor.

trifuged at 170 × g for 10 minutes, and the supernatant was then centrifuged at 3000 × g for 10 minutes to obtain platelet-poor plasma.

To verify the absence of clotting in the stored blood samples, viscosity was measured using the Lamy Rheology RM100 CP1000 rotational viscometer (Lamy Rheology Instruments, France, acquired within the framework of the M.V. Lomonosov Moscow State University Development Program, https://www.lamyrheology.com/en/). All prepared samples met the viscosity acceptance criteria specified by the measurement method.

M. Maksimov and P. Ermolinskiy morphologically confirmed all cell lines by optical microscopy shortly before use. HUVEC viability was assessed by O. Scheglovitova using an MTT assay (Section 2.4). Mycoplasma testing was routinely performed on all HUVEC cultures after each passage. Only mycoplasma-free cells were used in subsequent experiments.

2.2 Interferon Alpha

IFN-α2b (Altevir®, PHARMAPARK, Russia, https://pharmapark.ru/en) was used in this study. The initial solution had a concentration of 300,000 U/mL. The drug was then dissolved in the culture medium and incubated with HUVECs. The resulting sample concentrations were 10, 30, 100, 300, and 1000 U/mL.

2.3 Sample Preparation

Round coverslips bearing HUVEC monolayers were incubated with IFN-α in 24-well culture plates for 24 hours at 37 °C and 5% CO_2. Coverslips with HUVEC monolayers, either treated or untreated with IFN-α, were then placed in glass cuvettes for further measurements. The cuvettes were assembled on a glass slide, with a cover glass bearing an HUVEC monolayer placed on the slide (Fig. 1). A coverslip was then placed on top, and vacuum gel was used to seal the cuvette edges to prevent air exposure and oxidative stress in HUVECs. The cuvettes were filled with blood plasma and RBCs at a low hematocrit (approximately 0.025%), with either IFN-α dissolved in the culture medium or culture medium alone as a control. The resulting IFN-α concentrations in blood plasma containing RBCs were 10, 30, 100, 300, and 1000 U/mL.

2.4 HUVEC Viability Assessment

HUVEC viability was assessed using the MTT reduction assay [39]. In summary, a confluent HUVEC monolayer was prepared in 96-well plates at 15,000 cells per

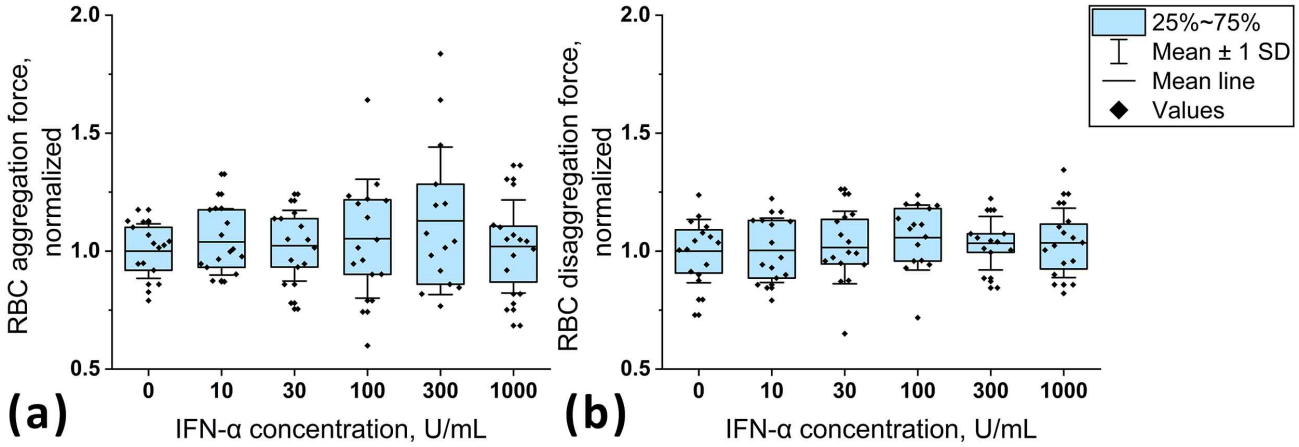


Fig. 2. Normalized aggregation and disaggregation forces of RBCs treated with IFN-α. (a) Normalized RBC aggregation force values at the indicated IFN-α concentrations. The measured forces (in pN) were normalized to the mean value of the corresponding 0 U/mL IFN-α control for each donor, see Section 2.6. for details. The box indicates the Q1–Q3 interval; the horizontal line indicates the mean; whiskers indicate the mean ± 1 standard deviation (SD); points indicate individual values. IFN-α was added to samples before measurements. Each point corresponds to one measurement; n = 3 indicates the number of donors. (b) Normalized RBC disaggregation force values at the indicated IFN-α concentrations. The box indicates the Q1–Q3 interval; the horizontal line indicates the mean; whiskers indicate the mean ± 1 standard deviation (SD); points indicate individual values. IFN-α was added to samples before measurements. Each point corresponds to one measurement; n = 3 indicates the number of donors. IFN-α, interferon alpha.

100 µL. After 24 hours of incubation in a CO_2 incubator, the culture medium was replaced with either the same medium supplemented with IFN-α at different concentrations or medium without IFN-α as a control. After an additional 24 hours of incubation, cell viability was assessed using the MTT assay. The optical density of the solutions was measured using a spectrophotometer at 540 nm. No significant decrease in optical density was observed in the IFN-α-treated samples compared with the control (data not presented), consistent with previous reports [9,15].

2.5 Optical Tweezers

Optical tweezers can trap and manipulate dielectric objects from nanometers to micrometers, including individual living cells. Thus, the method allows measurement of interaction forces between cells without external mechanical contact [40]. The physical principles of optical tweezers are described in Ref. [41]. Our optical tweezers setup includes an Nd:YAG laser (λ = 1064 nm, maximum output power 1 W), polarizing cubes (to create two independent laser beams), a beam-steering device (movable mirror), half-wave plates (to control laser intensity), a telescope (to regulate beam width), an Olympus water-immersion objective with high numerical aperture (100×, NA = 1.00), a CMOS camera, and a photodetector (Fig. 1). The power of each trapping beam interacting with an individual cell is in the range of 5–30 mW at the beam waist. The laser wavelength enables measurements with minimal cytotoxicity because it lies within the low-absorption regions of the hemoglobin spectrum, minimizing cell heating [42]. A similar low-absorption response has been reported for endothelium [43]. Additionally, the blood plasma medium helps dissipate heat due to its high heat capacity.

2.6 Measurements of the Interaction Forces

All measurements were performed at room temperature (22 °C). In these experiments, the interaction forces between individual RBCs and the HUVEC monolayer, as well as those between pairs of individual RBCs, were measured. Only the fixed-beam optical trap was used for force measurements between an RBC and the HUVEC monolayer [44]. The interaction force between an RBC and the HUVEC monolayer (adhesion) is defined as the minimum trapping force required to move an RBC across the HUVEC monolayer. To determine this force, 5–8 individual cells were measured sequentially in each sample for averaging, and the overall measurement process lasted about 30 minutes.

The interaction forces between individual RBCs during aggregation and disaggregation drive the paired aggregation of two adjacent RBCs and the dissociation of two partially aggregated (overlapped) cells, respectively [45]. Specifically, the aggregation force is the minimum force required to halt the overlapping process of two adjacent RBCs sticking together. In contrast, the disaggregation force is the minimum force required to separate partially overlapped (aggregated) cells. These forces were measured at least five times for different pairs of cells in each sample and then averaged. Using optical tweezers, a measurement with one pair of RBCs took approximately 3–4 minutes and did not damage the cells because the optical trapping technique is non-contact [46]. To avoid contact between RBCs and HU-

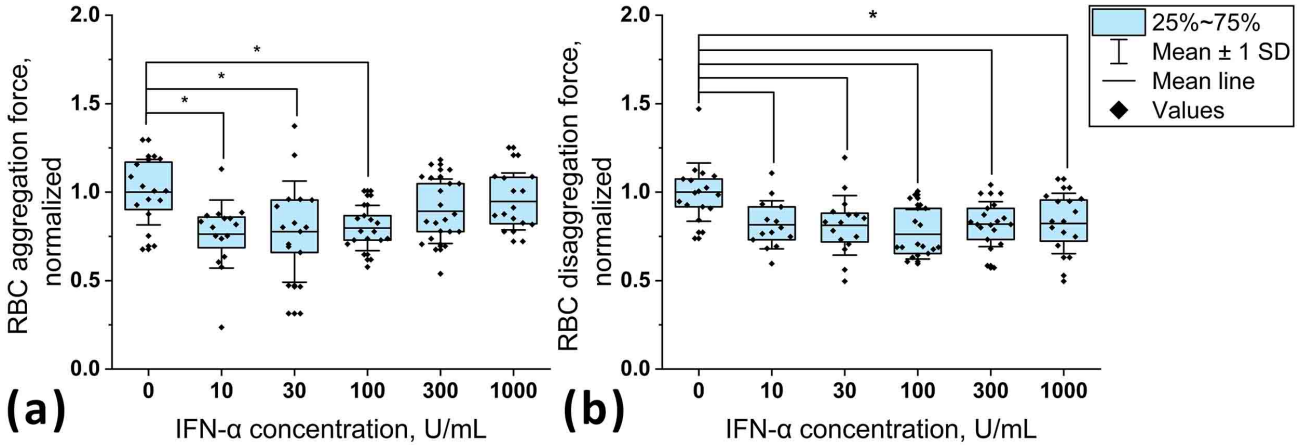


Fig. 3. Normalized aggregation and disaggregation forces of RBCs in the presence of short-term IFN-α-treated HUVECs. (a) Normalized RBC aggregation forces in the presence of HUVECs. The box indicates the Q1–Q3 interval, the horizontal line indicates the mean, whiskers indicate the mean ± 1 standard deviation (SD), and points indicate individual values. IFN-α was added to blood plasma containing RBCs in the cuvettes before measurements. Statistical analyses were performed using the Mann–Whitney U test. The asterisk indicates statistical significance of the difference between samples (* $p < 0.05$). Each point corresponds to one measurement. (b) Normalized RBC disaggregation forces in the presence of HUVECs. The box indicates the Q1–Q3 interval, the horizontal line indicates the mean, whiskers indicate the mean ± 1 standard deviation (SD), and points indicate individual values. IFN-α was added to blood plasma containing RBCs in the cuvettes before measurements. Statistical analyses were performed using the Mann–Whitney U test. The asterisk indicates statistical significance of the difference between samples (* $p < 0.05$). On this graph every sample is statistically significantly lower compared with control values. Each point corresponds to one measurement.

VECs during measurements, RBCs were lifted with optical traps, and the distance between RBCs and HUVECs was maintained at approximately 5–10 µm. Force calibration of the optical tweezers was carried out using the Stokes force calibration method [45]. To reduce variability across individual RBC donors, the data were normalized as follows: For each set of experiments and each RBC donor, the force values were divided by the mean control force (0 U/mL of IFN-α). The data from different donors were pooled only after this procedure. This approach allows estimation of the relative effect across many donors without considering variability in the absolute force values (pN range).

2.7 Statistical Analyses

For each experimental series, blood from 2–3 volunteers was used (two donors were used only for the long-term IFN-α incubation experiments). In total, blood from eight volunteers was used. For each IFN-α concentration, 3–10 aggregation and disaggregation forces between different pairs of RBCs and 5–10 RBC–HUVEC adhesion forces for different RBCs were measured. Statistical analyses were performed using the Mann–Whitney U test (Origin 2021, OriginLab, USA); changes were considered significant if $p < 0.05$.

3. Results

3.1 The Effect of Short-Term IFN-α Treatment on RBC Pair Aggregation/Disaggregation Forces

IFN-α was added at different concentrations to blood plasma containing RBCs in the cuvettes, and RBC aggregation and disaggregation forces were measured; the control sample was not treated with IFN-α (Fig. 2). The total time of RBC exposure to IFN-α ranged from 15 minutes to 1 hour across measurements at each concentration.

Notably, no statistically significant differences were observed between IFN-α-treated and control samples (without IFN-α). The absolute values of RBC aggregation and disaggregation forces ranged from 1 to 4 pN. These results indicate that IFN-α has no direct effect on RBCs.

3.2 Effect of Short-Term IFN-α Treatment of HUVECs on RBC Aggregation/Disaggregation and RBC Adhesion to HUVECs

The coverslips bearing HUVEC monolayers were placed into the cuvettes, and IFN-α was added to blood plasma containing RBCs prior to the measurements. The total time of HUVEC and RBC exposure to IFN-α ranged from 15 minutes to 1 hour across measurements at each concentration. IFN-α was not added to the control samples (Figs. 3,4).

The RBC aggregation forces decreased significantly at IFN-α concentrations of 10, 30, and 100 U/mL, whereas the RBC disaggregation forces were lower at all IFN-α concentrations.

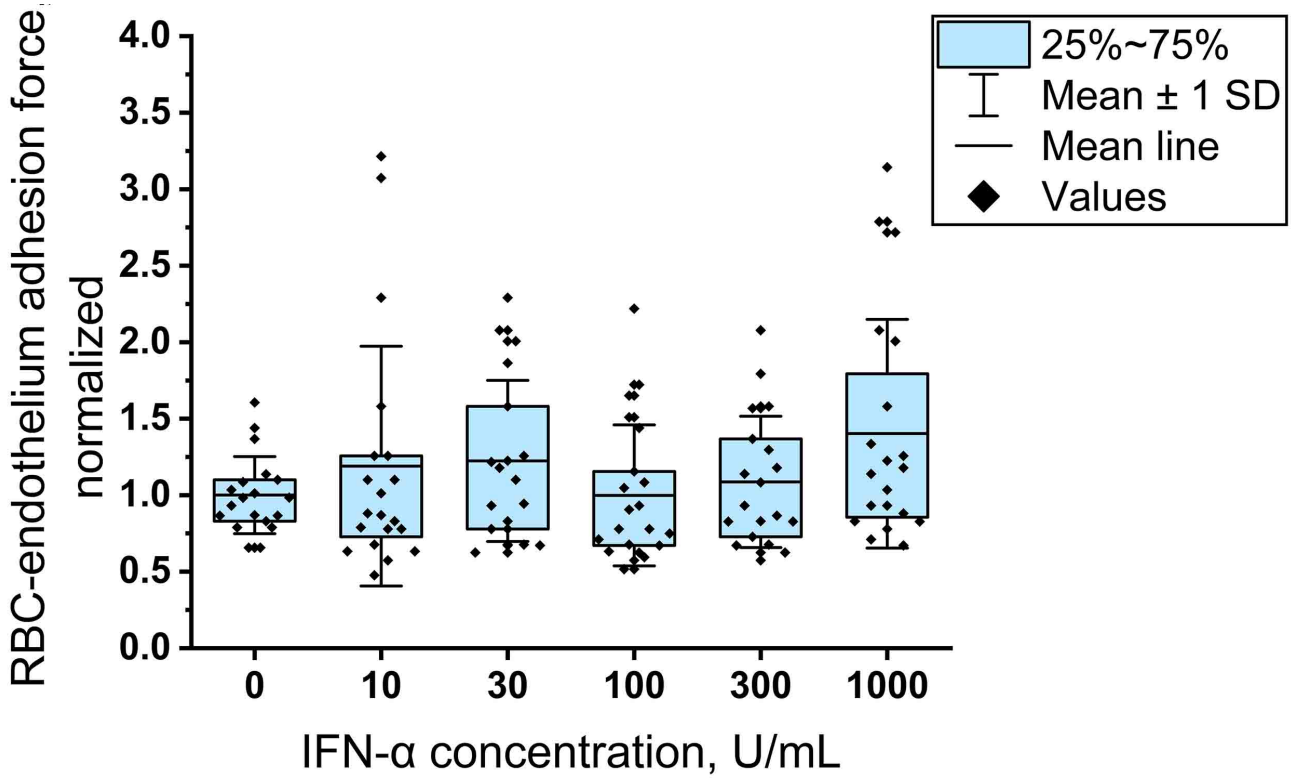


Fig. 4. Normalized forces of RBC–HUVEC adhesion. The box indicates the Q1–Q3 interval, the horizontal line indicates the mean, the whiskers indicate the mean ± 1 standard deviation (SD), and the points indicate individual values. IFN-α was added to blood plasma containing RBCs in the cuvettes before measurements. Each point represents a single measurement.

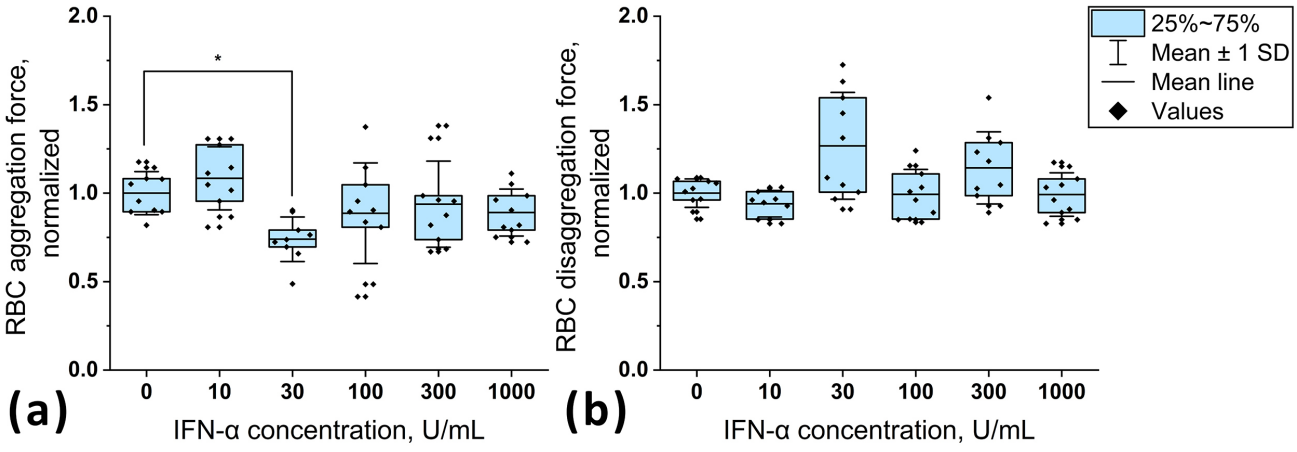


Fig. 5. Normalized aggregation and disaggregation forces of RBCs in the presence of long-term IFN-α-treated HUVECs. (a) Normalized RBC aggregation forces in the presence of HUVECs. The box indicates the Q1–Q3 interval; the horizontal line indicates the mean; whiskers indicate the mean ± 1 standard deviation (SD); points indicate individual values. HUVECs were incubated with the indicated concentration of IFN-α for 24 hours. IFN-α was not added to blood plasma containing RBCs. Statistical analyses were performed using the Mann–Whitney U test. The asterisk indicates statistical significance of the difference between samples (* $p < 0.05$). Each point corresponds to one measurement. (b) Normalized RBC disaggregation forces in the presence of HUVECs. The box indicates the Q1–Q3 interval; the horizontal line indicates the mean; whiskers indicate the mean ± 1 standard deviation (SD); points indicate individual values. HUVECs were incubated with the indicated concentration of IFN-α for 24 hours. IFN-α was not added to blood plasma containing RBCs. Statistical analyses were performed using the Mann–Whitney U test. The asterisk indicates statistical significance of the difference between samples (* $p < 0.05$). Each point corresponds to one measurement.

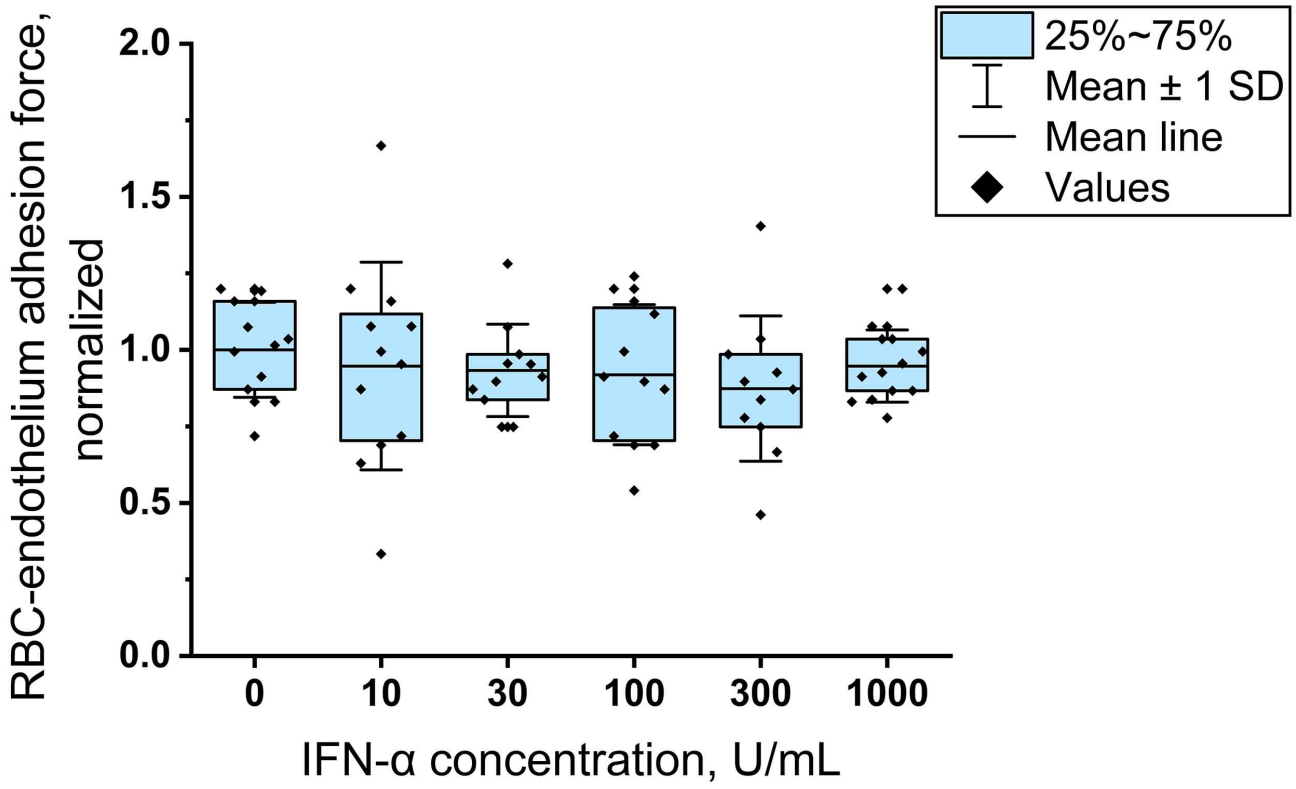


Fig. 6. Normalized forces of RBC–HUVEC adhesion. The box indicates the Q1–Q3 interval, the horizontal line indicates the mean, the whiskers indicate the mean ± 1 standard deviation (SD), and the points indicate individual values. HUVECs were incubated with the corresponding concentration of IFN-α for 24 hours in culture medium. IFN-α was not added to blood plasma containing RBCs in the cuvettes. Each point represents a single measurement.

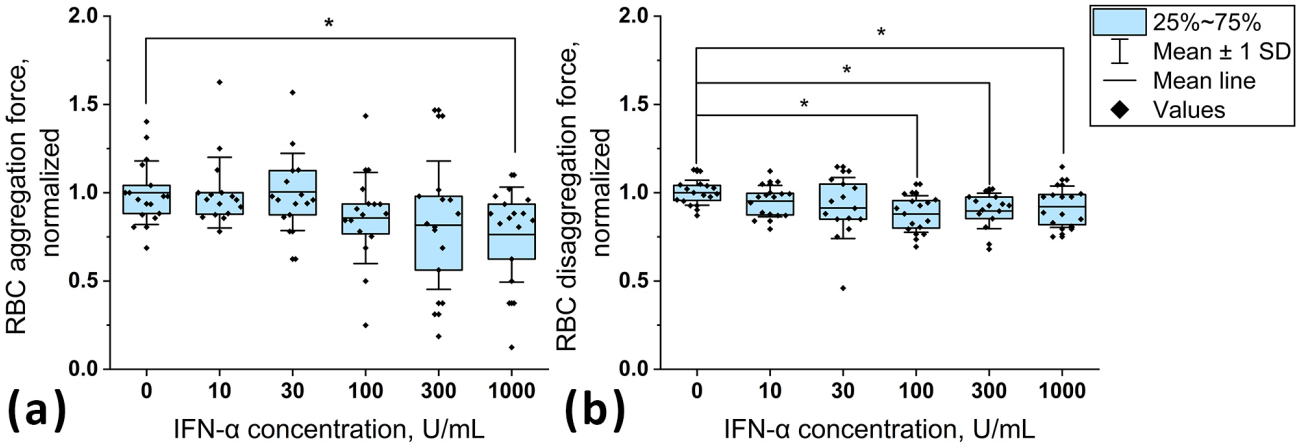


Fig. 7. Normalized aggregation and disaggregation forces of RBCs in the presence of sequentially IFN-α-treated HUVECs. (a) Normalized RBC aggregation forces in the presence of HUVECs. The box indicates the Q1–Q3 interval; the horizontal line indicates the mean; whiskers indicate the mean ± 1 standard deviation (SD); points indicate individual values. HUVECs were preincubated with the corresponding concentration of IFN-α for 24 hours. IFN-α was also added to blood plasma containing RBCs. Statistical analyses were performed using the Mann–Whitney U test. The asterisk indicates statistical significance of the difference between samples (* $p < 0.05$). Each point corresponds to one measurement. (b) Normalized RBC disaggregation forces in the presence of HUVECs. The box indicates the Q1–Q3 interval; the horizontal line indicates the mean; whiskers indicate the mean ± 1 standard deviation (SD); points indicate individual values. HUVECs were preincubated with the corresponding concentration of IFN-α for 24 hours. IFN-α was also added to blood plasma containing RBCs. Statistical analyses were performed using the Mann–Whitney U test. The asterisk indicates statistical significance of the difference between samples (* $p < 0.05$). Each point corresponds to one measurement.

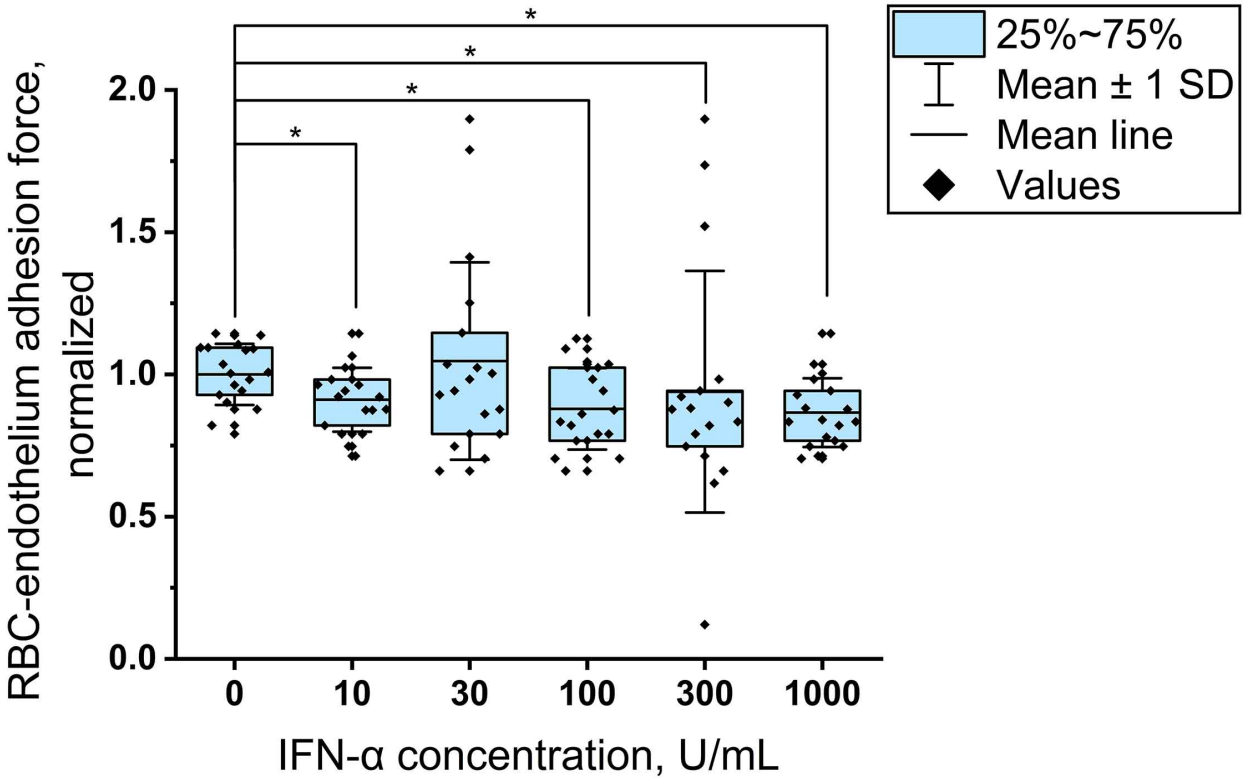


Fig. 8. Normalized forces of RBC–HUVEC adhesion. The box indicates the Q1–Q3 interval; the horizontal line indicates the mean; whiskers indicate the mean ± 1 standard deviation (SD); points indicate individual values. HUVECs were incubated with the corresponding concentration of IFN-α for 24 hours. After placing HUVEC-bearing coverslips in the cuvettes, IFN-α was added to blood plasma containing RBCs before measurements. Statistical analyses were performed using the Mann–Whitney U test. The asterisk indicates statistical significance of the difference between samples (* $p < 0.05$). Each point represents a single measurement.

RBC–HUVEC adhesion did not change significantly in this set of experiments. These results demonstrate that HUVECs release signaling molecules that influence RBC properties in response to IFN-α. The effect of IFN-α occurs rapidly after treatment (<15 minutes).

3.3 Effect of Long-Term Treatment of HUVECs With Interferon on RBC Aggregation/Disaggregation and RBC Adhesion to HUVECs

HUVECs cultured on coverslips in 24-well plates were pretreated with IFN-α for 24 h. Interferon was not added to the control samples. The coverslips with HUVEC monolayers were then placed in cuvettes containing blood plasma and RBCs. IFN-α was not added to the blood plasma. Over 60 minutes, RBC pair aggregation and disaggregation forces, as well as the forces of RBC adhesion to HUVECs, were measured (Figs. 5,6).

The RBC aggregation force at an IFN-α concentration of 30 U/mL was statistically significantly lower than the control value. RBC disaggregation forces did not change significantly compared with the control samples. However, the results were not uniform: in some cases, RBC interaction increased. The statistical analysis reflects only the averaged trends.

Notably, there were no statistically significant differences in RBC and HUVEC adhesion forces compared with the control. Absolute values for RBC aggregation and disaggregation forces ranged from 1 to 3 pN; RBC–HUVEC adhesion forces ranged from 0.4 to 1.3 pN. After 24 hours of HUVEC incubation with IFN-α, RBC aggregation decreased at only one concentration. IFN-α-treated HUVECs showed no statistically significant effects on either RBC disaggregation or RBC–HUVEC adhesion. The effect of long-term IFN-α treatment of HUVECs can be attributed to intracellular processes, such as the inhibition of signaling molecule production, thereby altering RBC properties.

3.4 Impact of Long-Term HUVEC Treatment With IFN-α Followed by Short-Term Treatment on RBC Aggregation and Disaggregation as Well as RBC–HUVEC Adhesion

HUVECs cultured on coverslips in 24-well plates were pretreated with IFN-α for 24 h. Interferon was not added to control samples. The coverslips with HUVEC monolayers were then placed in cuvettes containing blood plasma and RBCs, along with the corresponding concentration of IFN-α. Interferon was not added to the control samples (Figs. 7,8).

The RBC aggregation forces decreased significantly at an IFN-α concentration of 1000 U/mL compared with the control; RBC disaggregation forces decreased significantly at 100, 300, and 1000 U/mL.

RBC–HUVEC adhesion forces decreased across nearly all IFN-α concentrations.

4. Discussion

These results show that IFN-α does not directly affect RBCs, as no changes in RBC aggregation/disaggregation forces were observed in the absence of HUVECs. These findings suggest that IFN-α acts on intercellular interactions through factors produced by IFN-α-stimulated endothelial cells. Moreover, the effect of IFN-α on RBC pair interaction forces and RBC–HUVEC adhesion forces depends on the duration of HUVEC treatment. Short-term treatment (15–60 minutes) reduced RBC interaction forces, whereas long-term treatment (24 hours) exhibited little effect (the decrease in forces was significant only at one IFN-α concentration). Long-term (24 hours) IFN-α HUVEC treatment followed by short-term (15–60 minutes) treatment reduced RBC aggregation forces at a single (the highest) concentration and RBC disaggregation forces at several higher concentrations. RBC–HUVEC adhesion forces decreased only after long-term (24 hours) IFN-α HUVEC treatment followed by short-term treatment (15–60 minutes).

A study combining microfluidics, time-lapse microscopy, and computer simulations revealed details of regulatory network function in single human cells treated with IFN-α for various durations [47]. The IFN-α effect resulted from a gene regulatory network with a fast-responding positive-feedback component (partly via enhanced upregulation of ISGF3 system components) and a delayed negative-feedback component (via enhanced upregulation of USP18). Short-term treatment activated intercellular signaling, whereas long-term treatment activated inhibitors of these signals. Variability in the results was due to different cell cycle phases at the onset of IFN-α exposure. Expression of key signaling molecules stimulated by IFN-α depended on the cell cycle phase. Meanwhile, the cell cycle phase was influenced by compounds present in the culture medium or serum. Regulatory cascades with coupled positive and negative feedback have been identified in many biological processes and, depending on the specific mechanisms and kinetics, can lead to different dynamic changes [48,49].

Overall, this study demonstrates a nonlinear effect of IFN-α on HUVEC function, leading to time-dependent increases or decreases in RBC aggregation/disaggregation and RBC–HUVEC adhesion. The changes in RBC–HUVEC adhesion in our experiments were observed with a two-step sequential treatment. The patterns observed in our work may reflect the impact of IFN-α on endothelial cell production of NO as well as other proaggregant and antiaggregant factors. Thus, these findings generate the hypothesis that interferon-treated HUVECs decrease RBC aggregation through eNOS-mediated NO production in the presence of IFN-α. Our previous publications and those by other authors demonstrate a NO-dependent decrease in RBC aggregation/disaggregation forces [26,50]. Some publications demonstrate the role of IFN-α only in the inhibition of NO production by HUVECs [9]. However, the cells were treated for longer periods in these studies than in our short-term HUVEC treatment experiments.

5. Limitations

The results of *in vitro* studies cannot be directly applied to *in vivo* systems. All measurements were conducted at room temperature (22 °C). This approach has previously been shown to yield adequate results; however, HUVECs may produce fewer signaling molecules at higher temperatures [51,52,53]. Since this study did not measure NO levels directly, it could not determine which signaling molecule is responsible for the decrease in RBC aggregation.

6. Conclusions

IFN-α is used to treat various diseases; thus, understanding the mechanisms underlying its effects on the human body is important for reducing the risk of adverse effects. Our work reveals a complex interplay among interferon, endothelium, and RBCs, suggesting potential strategies to improve the effectiveness of IFN-α in clinical applications. Further studies in animal models are needed to test these hypotheses and assess the clinical relevance of these findings.

Abbreviations

IFN-α, interferon alpha; RBC, red blood cell; HUVEC, human umbilical vein endothelial cell; NO, nitric oxide.

Availability of Data and Materials

All data reported in this paper will be shared by the lead contact upon reasonable request.

Author Contributions

MM, PE, AL, AP, AM and OS designed the research study. OS, MK, IF and NS designed the cultivation protocol, cultivated the endothelium and conducted viability assessment. MM and PE performed the research. AM provided help and advice on experiment design and data interpretation. MM analyzed the data. MM and OS wrote the first draft of the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki. The research protocol was approved by the Ethics Committee of Medical Research and Educational Institute of Lomonosov Moscow State University (Ethics Approval Number: 11/22 05.12.2022), and all participants provided signed informed consent.

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Conflicts of Interest

The authors declare no conflicts of interest.

Declaration of AI and AI-Assisted Technologies in the Writing Process

After the manuscript was written without the use of any AI-assisted technologies, the authors used ChatGPT (GPT-5.6; OpenAI, USA) for English-language editing. All suggestions were reviewed by the authors, who take full responsibility for the final content.

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