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SDF-1α Reduces Human Natural Killer Cell Cytotoxicity against Chronic Myelogenous Leukemia K562 Cells

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ABSTRACT

Stromal cell-derived factor-1 alpha (SDF-1α) has been shown to be up-regulated in a variety of malignancies. So that, its expression is associated with poor prognosis and invasiveness. Natural killer (NK) cells are important effector cells against virus-infected and transformed cells. Especially they play a key role in tumor immune surveillance.

Whereas it was not well understood whether SDF-1α modulates anti-tumor immune response or not, the purpose of the present study was to investigate the effect of SDF-1α on the cytotoxic properties of peripheral blood NK cells.

Human peripheral blood NK cells were freshly isolated using MACSxpess system and cultured in the presence or absence of recombinant human SDF-1α or SDF-1α plus CXCR4 antagonist, AMD3100. CD107a degranulation assay was conducted through the co-culture of NK cells with K562 cells. The percentage of CD107a positive cells was assessed by flowcytometry. Effect of SDF-1α was also examined on the mRNA levels of NKG2A and NKG2D as indicator examples of NK cell inhibitory and activating receptors, respectively. SDF-1α significantly decreased the degranulation activity of NK cells (p=0.04). The mRNA content of NKG2D was down-regulated under the influence of SDF-1α (p=0.03). Moreover, AMD3100 exhibited a trend in recovering the NKG2D mRNA level to its un-treated state (p=0.05).

The present study reveals that SDF-1α has a negative impact on NK cell activity and might is involved in tumor immune-suppression. Thus, it can be concluded that microenvironment manipulations targeting SDF-1α may reinforce current cancer therapies by disturbing one of the immune-suppressive axes in the cancerous milieu.

Keywords: Immunologic cytotoxicity; Killer cells; Natural; NKG2D; SDF-1α

INTRODUCTION

Tumor microenvironment is a complex milieu consisted up of heterogeneous cellular populations including cancerous cells, cancer-associated fibroblasts, endothelial cells, stem cells, and immune cells.

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Existence of various resident cell populations causes various soluble factors to be produced and different crosstalk to take place.[1] Despite some differences in the transcriptomes of distinct malignancies, growth factors and cytokines such as VEGF, FGF, EGF, TGF-β, and IL-10 are shown to be overexpressed in a myriad of solid tumors.[2]

Stromal cell-derived factor-1 alpha (SDF-1α) also known as CXCL12 belonging to CXC family of chemokines, is one of the soluble factors associated with a broad spectrum of neoplasms. The physiologic roles of SDF-1α can be summarized as general lymphocyte trafficking, stem cell homing in bone marrow niche, tissue remodeling, and angiogenesis.[3,4] The up-regulation of SDF-1α in both mRNA and protein levels has been verified in malignancies such as breast, colorectal, gastric, ovarian, and prostate. So that, the expression of SDF-1α has been associated with tumor invasiveness and poor prognosis.[5-7] CXCR4 is the most relevant cognate receptor for SDF-1α.[8]

Reports on the effect of SDF-1α on anti-tumor immune responses are limited and in some cases are controversial. Gil et al reported a decrease in ovarian cancer immune-suppression following the CXCR4/SDF-1α blockage through oncolytic virotherapy.[9] Similarly, Feig et al demonstrated that blocking the SDF-1α synergizes with PD-L1 blockage in controlling the pancreatic cancer.[10] On the other hand, there are implications indicating a positive role for SDF-1α in supporting the survival of T cells and the development of natural killer (NK) cells.[11,12]

The anti-tumor immune response involves various mechanisms, among them, NK cells have crusial roles in tumor immune-surveillance.. Experiences with xenogeneic tumor models in nude mice (nu/nu demonstrated that the tumor induction is only applicable with the cell lines that are unable to stimulate NK cell activity. This is because nude mice contains intact NK cells, but do not have T cells[13]

Although the role of SDF-1α in the homing of immune cells within tumors is somewhat evident, we evaluated cellular functions other than the cell motility induced by SDF-1α in the present study. Regarding the importance of NK cells in tumor immune response and the ambiguity in the literature regarding the effect of SDF-1α on NK cells, the aim of the present study was to investigate the impact of SDF-1α on the cytotoxic properties of NK cells against a human chronic myelogenous leukemia (CML) cell line, K562, *in vitro*.

MATERIALS AND METHODS

Human whole blood MACSxpess NK cell isolation kit was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Human K562 cells were purchased from the Pasteur Institute Cell bank (Tehran, Iran). Recombinant human SDF-1α and CXCR4 antagonist (AMD3100) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Roswell Park Memorial Institute medium (RPMI) 1640 medium, penicillin–streptomycin, fetal bovine serum (FBS), human TNF-α ELISA kit and Revert Aid kit were gained from Thermo Fisher (Auckland, New Zealand). Anti-human CD3-PE, Anti-human CD56-FITC, Anti-human CD107a-PE, Mouse IgG2a-PE, Mouse IgG1-FITC, and Mouse IgG1-PE isotype controls were provided from eBioscience (San Diego, USA). Recombinant human interleukin-2 (IL-2) was obtained from Peprotech (Rocky Hill, USA). RBC lysis solution was gained from Promega (Madison, USA). Accuzol total RNA extraction kit was provided by Bioneer (Daejeon, South Korea) and SYBR Green qPCR master mix was obtained from Takara Bio (Tokyo, Japan).

NK Cell Isolation and Cell Culture

This study was conducted under verifications from the local ethics committee (N. 1395.2967). Blood samples were obtained from five healthy volunteer men. Inclusion criteria for blood donors was an age between 25 and 30 years, lack of any history of recent illness and infections, stress and intense exercise. Exclusion criteria was the use of any types of NSAIDs, steroid medications and nutritional supplements in the last two months, any history of inflammatory diseases and malignancies. All the volunteers were admitted to fill informed consent forms.

Whole blood un-touched NK cell isolation was carried out according to the provider's instruction. Briefly, 4 ml of freshly prepared kit solution was mixed with 8 ml of EDTA-added fresh blood and the tube was placed on a tube roller for 5 minutes. The tube was then located on the magnetic separator for 20 minutes. The supernatant was collected, centrifuged (400g for 5 minutes) and the resulting pellet was used in the experiments after RBC lysis followed by a purity check via flowcytometry (PAS, Partec, Germany). More than 85% of the isolated cell population exhibited $CD56^{+}$, $CD3^{-}$ immune-phenotype (Supplementary Figure 1).

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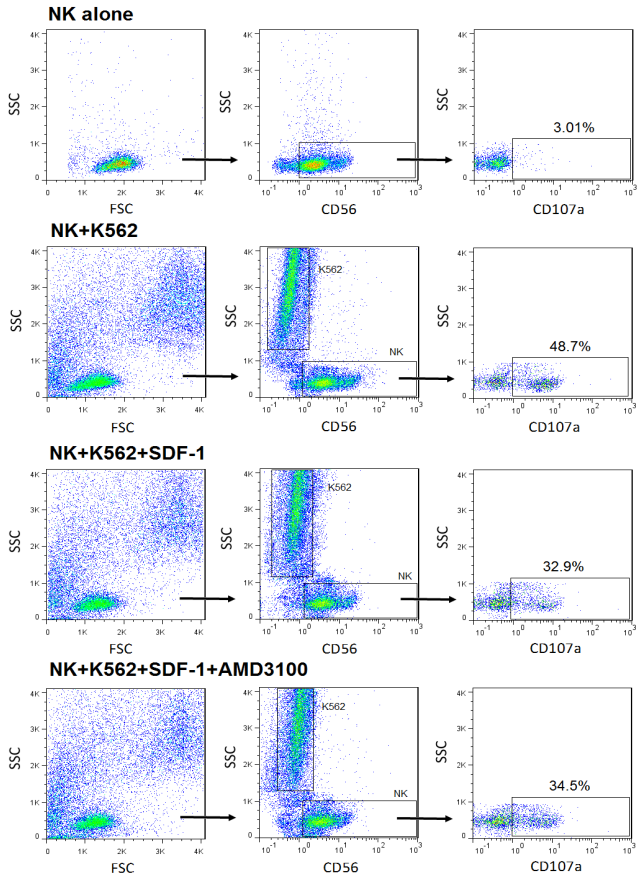

Figure 1. Flowcytometric plots for natural killer (NK) cell CD107a degranulation assay. Freshly isolated NK cells were cultured in the presence or absence of stromal cell-derived factor-1 alpha (SDF1α) (100 nM), SDF-1α and AMD3100 (5μM) for 24 hours. Then, 2×10^5 NK cells were co-cultured with 1×10^5 K562 cells for six hours. The cells were then harvested, stained with anti-CD56-FITC and anti-CD107a-PE antibodies for flowcytometric evaluation. The plots were first arranged with respect to side scattering and CD56 expression, through which, the NK cell and K562 populations are distinguishable. The percentages of $CD107a^+$ cells out of the $CD56^+$ population were then determined for each group.

Table 1. Sequences and amplicon size of the primers used in gene expression analysis

Primer	Length	Amplicon size
NKG2D		
F: 5'- AGGCTTTTATCCACAAGAATC -3'	21	121
R: 5' GAATCCACCCCATCAAATAC-3'	20	
NKG2A		
F: 5'- ATAACCTATGCGGAATTAAAC -3'	21	150
R: 5'-CACAGAGGCCATTAAGATAAG-3'	21	
GAPDH		
F: 5'- AAGCTCATTTCCTGGTATGACG -3'	22	126
R: 5'- TCTTCCTCTTGTGCTCTTGCTGG -3'	23	

The presented primers were used in the quantitative PCR reactions to evaluate the mRNA expression of NKG2D and NKG2A in the study conditions. The expression of GAPDH was used as an internal control to normalize the expression of the mentioned genes.

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NK Cell Degranulation Assay

The isolated NK cells were incubated for 24 hours in the presence of SDF-1α (100 nM) or SDF-1α in combination with CXCR4 antagonist, AMD3100, (5 μM) in RPMI-1640 containing 1% penicillin/ streptomycin, 10% FBS and 2 mM L-glutamine. A total of 2×10^5 NK cells were co-cultured with 1×10^5 K562 cells in each well of round-bottom 96 well plates for six hours (effector to target ratio, 2:1). Then, the supernatants were collected, the cells were harvested, washed with PBS and flowcytometry analyzed immediately after antibody staining. A two-color flowcytometry method was exploited to assess the percentage of $CD107a^+$ cells in each group. In order to interpret the data, the plots were arranged with regard to side scattering and CD56 expression. Afterward, the NK cell population was gated on the basis of the "NK cell alone" clue group. Then, the percentage of $CD107a^+$ cells out of the $CD56^+$ population was determined. Single color compensation controls were used to compensate the spillover of the flowcytometric channels. The flowcytomety results were analyzed by FlowJo software (FlowJo, Ashland, OR, USA).

Gene Expression Analysis

A total number of 2×10^5 freshly isolated NK cells were seeded in each well of six-well plates and were treated with IL-2 (100 IU/ml), SDF-1α alone (100 nM), and SDF-1α in combination with AMD3100 (100 nM and 5 μM, respectively). After 24 hours of treatment, total RNA was extracted according to manufacturer's instruction and the quantity of the yielded RNA was measured by picodrop (PicoDrop, UK). The first-strand cDNA was then synthesized and the quantitative PCR was carried out using Exicycler™ (Bioneer, South Korea) thermal cycler. The sequence and amplicon size of primers used in gene expression experiments are demonstrated in table 1.

Enzyme-Linked Immunosorbent Assay (ELISA)

The supernatants from the co-cultures of NK cell and K562 cells for CD107a assay were collected and stored at -80°C until TNF-α measurements. A calibrator curve was plotted using the standard solution provided by the manufacturer and the test optical densities were converted to concentrations using this calibrator curve.

Statistical Analysis

Regarding that all data sets were passed Kolmogorov-Smirnov normality test, they were analyzed by parametric one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test. Data are shown as mean ± standard deviation. All statistical analyses were performed using GraphPad Prism software (version 6; GraphPad, San Diego, USA).

RESULTS

CD107a Degranulation Assay

K562 cell line was used in this experiment as a standard target of NK cells. Six hours of co-culture with K562 cells caused a significant increase in CD107a expression on NK cells ($p<0.001$). According to our results, treatment with 100 nM of SDF-1α significantly decreased the degranulation activity of NK cells against K562 cells compared to untreated cells ($p=0.044$). Unlike the study hypothesis, AMD3100 at 5 μM concentration did not prevent the SDF-1α-induced alleviation of the NK cell degranulation (Figures 1, 2).

Gene Expression of NKG2D and NKG2A

Obtained from the quantitative RT-PCR experiment, the expression of NK cell activating receptor, NKG2D, was down-regulated in SDF-1α treated cells (p=0.034). Moreover, AMD3100 exhibited a trend in recovering the SDF-1α-induced alleviation of NKG2D to its control state ($p=0.059$). A non-significant decrease in the expression of NK cell inhibitory receptor, NKG2A, in response to SDF-1α was also observed ($p=0.246$; Figures 3a, b). As a positive control, IL-2 at 100 IU/ml concentration increased the NKG2A expression significantly ($p<0.001$), but does not affect the NKG2D expression ($p=0.674$).

TNF-α Secretion from NK Cells

The encounter of NK cells with K562 cells causes a significant TNF-α release from NK cells ($p<0.0001$). Treatment with 100 nM of SDF-1α for 24 hours prior to co-culture, significantly reduced the potential of NK cells to secrete TNF-α upon activation ($p=0.043$). In parallel to the degranulation assay, AMD3100 was not successful in retarding the effect of SDF-1α on TNF-α secretion response (Figure 4).

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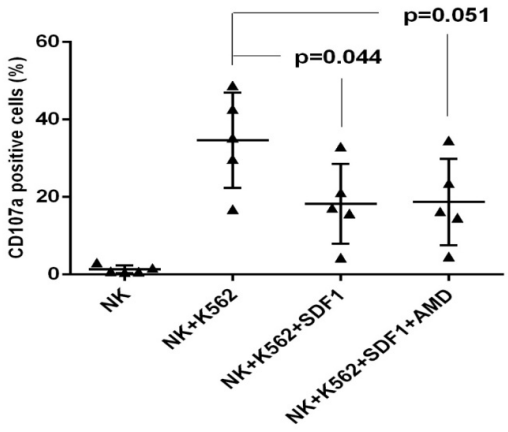

Figure 2. Mean±S.D values for all replications of flowcytometric CD107a degranulation assay. In order to interpret the impact of study conditions on the cytotoxic potential of human NK cells, flowcytometric evaluation of CD107a expression was carried out on the NK cells isolated from five blood donors. Freshly isolated NK cells were cultured in the presence or absence of stromal cell-derived factor-1 alpha (SDF1α) (100 nM), SDF-1α and AMD3100 (5μM) for 24 hours. Then, 2×10^5 NK cells were co-cultured with 1×10^5 K562 cells for six hours. The cells were then harvested, stained with anti-CD56-FITC and anti-CD107a-PE antibodies for flowcytometric evaluation. As an indicator of effector cell degranulation, the expression of CD107a was significantly up-regulated in natural killer (NK) cells co-cultured with K562 cells compared to NK cells alone (p<0.001). NK cells pre-treated with stromal cell-derived factor-1 alpha (SDF1α) (100 nM) for 24 hours showed a decreased potential for degranulation compared to un-treated cells (p=0.044). As a specific antagonist of CXCR4, AMD3100 did not prevent the inhibitory effect of SDF-1α on NK cells. CXCR4: the most specific receptor for SDF-1α; AMD: AMD3100.

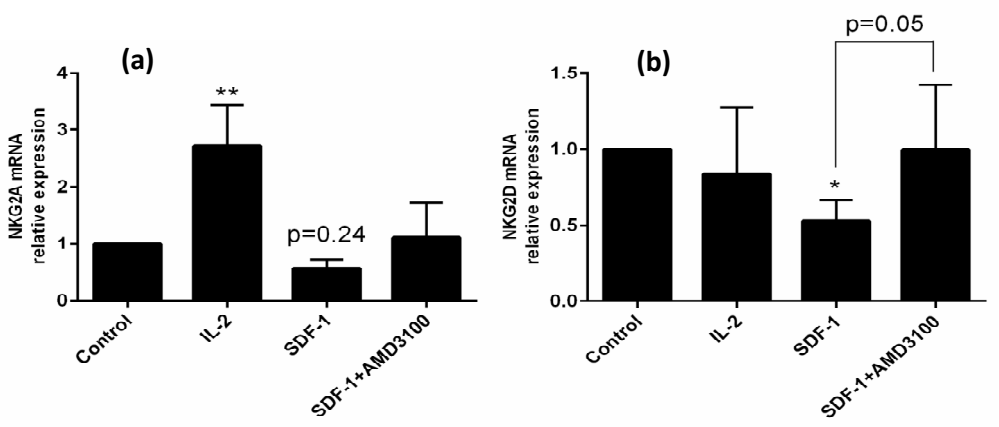

Figure 3. The in vitro effects of SDF-1α on the relative mRNA expression of NKG2A (a) and NKG2D (b) in natural killer (NK) cells. In order to determine the effect of stromal cell-derived factor-1 alpha (SDF1α) on the mRNA levels of NKG2D, as a prominent NK cell activator receptor, and NKG2A, as an NK cell inhibitory receptor, 2×10^5 freshly isolated human NK cells were cultured in the presence of SDF-1α (100 nM) or SDF-1α and AMD3100 (5 μM) for 24 hours. IL-2 (100 IU/mL) was used as a positive control for activating NK cells. The RNA content of NK cells was isolated and was used as a template in the synthesis of the respective cDNA. The quantitative RT-PCR experiment was carried out using the SYBR green method. SDF-1α decreased the expression of NKG2D significantly (p=0.034) but not the expression of NKG2A (p=0.246). * representative of p-values<0.05 and ** representive of p<0.01.

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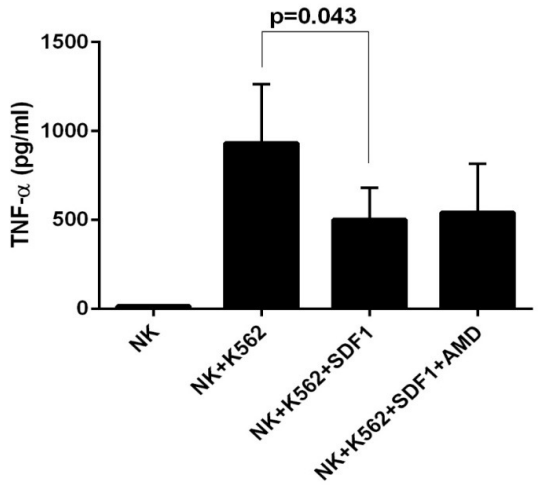

Figure 4. Secretion of TNF-α from natural killer (NK) cells in experimental conditions. Since TNF-α is among the cytokines secreted from activated NK cells, this cytokine was measured in supernatants collected from the co-culture of K562 and NK cells in order to compare the effect of study conditions on the activation state of NK cells. Freshly isolated NK cells were cultured in the presence or absence of stromal cell-derived factor-1 alpha (SDF1α) (100 nM) and SDF-1α plus AMD3100 (5μM) for 24 hours. Then, 2×10^5 NK cells were co-cultured with 1×10^5 K562 cells for six hours and the culture supernatants were collected for enzyme-Linked Immunosorbent Assay (ELISA). The encounter of NK cells to K562 cells caused a notable TNF-α release response ($p<0.001$) but the NK cells pre-treated with SDF-1α (100 nM) exhibited a milder TNF-α release post the encounter with the target cells ($p=0.043$). AMD3100 (5μM) did not prevent the SDF-1α-induced reduction in TNF-α release ($p=0.992$).

DISCUSSION

According to our results, SDF-1α reduced the degranulation activity and the TNF-α resonse of NK cells and decreased the expression of NKG2D, as an activating NK cell receptor. Blocking the CXCR4, as the main receptor of SDF-1α, using AMD3100 did not impede the SDF-1α-induced effects completely and just showed a trend for recovering the expression of NKG2D. The up-regulation of both activating and inhibitory receptors of NK cells upon cell activation has been reported by previous studies.[14,15] In the present study, both NKG2A and NKG2D are exploited as activation markers in order to compare the effects of study conditions on the activation state of NK cells.

Martin-Antonio et al[16] reported that bone marrow stromal cells from multiple myeloma patients which strongly secrete SDF-1α, cause a reduction in the cytotoxicity of cord blood NK cells against multiple myeloma cell lines. This study also indicates that AMD3100 is not able to inhibit the observed changes. Despite the focus of the mentioned study on SDF-1α, it used stromal cells which can trigger various paracrine and juxtacrine inhibitory pathways other than SDF-1 α. The present study aimed at determining the net effect of SDF-1α by taking advantage of recombinant SDF-1α protein.

Feig et al[10] reported that AMD3100 in synergism with anti-PD-L1 antibody benefits pancreatic ductal carcinoma eradication through disturbing the SDF-1α/CXCR4 pathway. While findings of us and the mentioned study about the importance of this pathway are comparable, we did not observe notable outcomes in the case of AMD3100 treatment. However, the aforementioned study did not evaluate the effect of AMD3100 on immune cell functions. In another study, SDF-1α has been reported to modulate the immune-suppression in B cell lymphoma microenvironment through the accumulation of regulatory T cells that was reversible by AMD3100[17]. Gil et al[9] reported that an oncolytic vaccina viruses designed to produce CXCR4-Fc fusion protein in infected ovarian cancer cells increased the IFN-γ/IL-10 ratio in both $CD4^+$ and $CD8^+$ tumor-infiltrating T cells. The CXCR4-Fc fusion protein also increased the cytotoxicity of spleen-derived cytotoxic T cells (CTLs) in various effector to target ratios. Our results about the immune-suppressive properties of SDF-1α are in accordance with the mentioned studies.

Chemokines are known for their key roles in cell trafficking and tissue homeostasis but can also exhibit non-trafficking functions such as roles in angiogenesis and development.[18,19] Chemokine receptor CXCR4 is a G protein-coupled receptor capable of activating a wide range of signaling pathways such as MAP-kinase, PI3 kinase, JAK-STAT, Rho-GTPase, and NF-κb.[20] Regarding that these signaling pathways can be activated through CXCR4, it seems that functions other than the cell trafficking can be expected to occur upon the engagement of CXCR4. In the present study, AMD3100 was expected to block the SDF-1α-induced effects via CXCR4 neutralization. This observation that

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NK cells were not fairly responsive to AMD3100 neutralization may rely on the receptor redundancy of SDF-1α. So that, SDF-1α can transduce its signal through CXCR7 instead of CXCR4.[21] Moreover, the expression of NKG2D and NKG2A were both expected to experience up-regulation in IL-2 treated NK cells, but we only observed an increase in NKG2A expression. This outcome in the case of NKG2D mRNA expression might be regarded as a result of excess NK cell activation.[21]

The limited number of un-expanded NK cells from blood samples was a hurdle to set various concentrations of SDF-1α and AMD3100 for the cells obtained from each individual. In addition, NK cells from other sources such as cord blood do not seem suitable for cancer study models as they exhibit differences with peripheral blood NK cells in their cytotoxic properties.[22] Establishment of SDF-1α knock-out target cells and animal models followed by specific NK cell and CTL evaluations may increase the current knowledge about the role of SDF-1α on anti-tumor immune responses.

The present study is the first in vitro report indicating the influence of recombinant SDF-1α on cytotoxic properties and cytokine response of human peripheral NK cells. Use of fresh, un-touched and un-expanded NK cells provided a possibility to simulate the in vivo condition almost properly and helped to gain accountable results. Along with previous reports, the present study provides the underlying logic for chemokine/chemokine receptor manipulations to overcome the immune-suppressive tumor microenvironment in a way toward enhancing the efficacy of immunotherapies. However, more comprehensive studies are needed to be performed to determine the exact role of chemokines, especially the SDF-1α on the anti-tumor immune responses.

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