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Cervical cancer cells produce TGF-β1 through the CD73-adenosine pathway and maintain CD73 expression through the autocrine activity of TGF-β1

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ABSTRACT

In cancer, the adenosinergic pathway participates in the generation of an immunosuppressive microenvironment and in the promotion of tumor growth through the generation of adenosine (Ado). The present study analyzed the participation of Ado, generated through the functional activity of the cervical cancer (CeCa) pathway in CeCa cells, to induce the expression and secretion of TGF-β1, as well as the participation of this factor to maintain CD73 expression. Ado concentrations greater than 10 μM were necessary to induce an increase of over 50% in the production and expression of TGF-β1 in CeCa tumor cells. Blockade of A2AR and A2BR with the specific antagonists, ZM241385 and MRS1754, respectively, strongly reversed the production of TGF-β1. TGF-β1 produced by CeCa cells was necessary to maintain CD73 expression because the addition of anti-TGF-β neutralizing antibodies or the inhibition of TGF-βRI strongly reversed the expression of CD73 in the CeCa cells. These results suggested a feedback loop in CeCa cells that favors immunosuppressive activity through the production of TGF-β1 and Ado as well as the autocrine activity of TGF–β1 and expression of CD73.

1. Introduction

Cervical cancer (CeCa) represents a major public health problem worldwide, as more than 500,000 new cases and more than 250,000 deaths are detected each year, of which over 80% occur in developing countries [1]. Infection with high-risk human papillomavirus (HR-HPV) is considered the main etiological agent of CeCa [2]. Although the immune response against HPV antigens eliminates most infections and precursor lesions [3], only a small number of women exposed to HR-HPV develop cancer, suggesting that other risk factors may be involved [4]. A growing number of studies have suggested that immunoregulation may play an important role in the carcinogenesis of CeCa. In this context, it has been reported that transforming growth factor-β (TGF-β) plays a crucial role in the development of CeCa. TGF-β generates a local immunosuppressive microenvironment in the cervix infected by HR-HPV to inhibit the proliferation and activation of T cells with antitumor activity [5]. However, TGF-β also favors the persistence of HR-HPV infection and promotes tumor formation, epithelial–mesenchymal transition (EMT), CeCa tumor cell invasion and CeCa tumor cell metastasis [6]. TGF-β has been detected in serum and tissues of patients with HPV-AR infection, in low-grade squamous intraepithelial lesions (SILs), in high-grade SILs and mainly in patients with advanced CeCa. In addition, TGF-β levels correlate directly with the degree of lesion progression [7,8]. The presence of TGF-β in the tumor microenvironment (TME) stabilizes hypoxia-inducible factor 1α (HIF-1α) [9], which in turn induces the expression of CD39 ectonucleotidases (ectonucleoside triphosphate diphosphohydrolase-1, ENTPD1; EC 3.6.1.5) and 5′-ectonucleotidase (CD73, EC 3.1.3.5), which hydrolyze ATP/ADP, AMP

Abbreviations: Ado, adenosine; Anti-TGF-β, anti-human neutralizing antibody against TGF-β1, -β2, and -β3; ARs, adenosine receptors; ATCC, American Type Culture Collection; CeCa, cervical cancer; CTLs, cytotoxicT lymphocytes; EMT, epithelial–mesenchymal transition; HIF-1α, hypoxia-inducible factor 1α; HR-HPV, high-risk human papillomavirus; Ino, inosine; MDSCs, myeloid-derived suppressor cells; MFI, mean fluorescence intensity; NK, natural killer cells; rh-TGF-β1, recombinant human TGF-β1; SILs, low-grade squamous intraepithelial lesions; T regs, regulatory T cells; TGF-β, transforming growth factor-β

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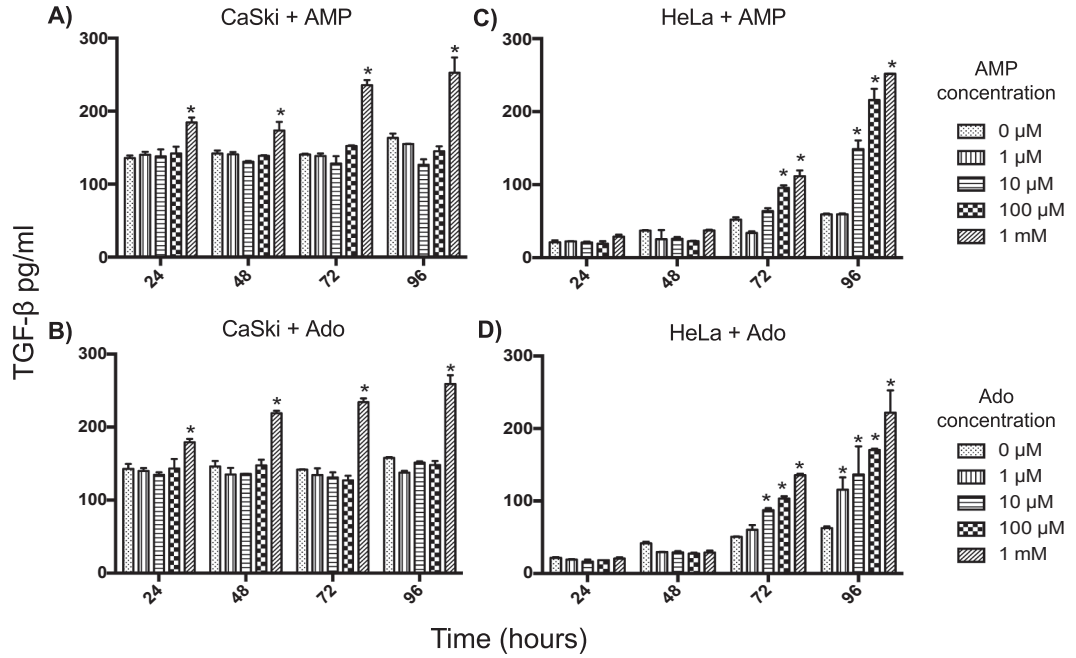

Fig. 1. CeCa cells cultured in the presence of AMP and Ado are induced to secrete TGF-β1. CaSki and HeLa cells (1×10^5) were cultured for 96 h in the presence of 1 μM, 10 μM, 100 μM and 1 mM AMP (A and C) or Ado (B and D). Samples of the supernatants were collected every 24 h to analyze TGF-β1 content by ELISA. Data are representative of three independent experiments, and the averages ± SEM are shown. * Indicates significant difference ($p < 0.05$) in the TGF-β1 content of the supernatants of the treated cells with respect to the supernatant of the untreated cells for each culture time.

and AMP in adenosine (Ado), respectively, and generate high concentrations of Ado in the TME [10–12]. Moreover, TGF-β is a factor that induces the expression of CD39/CD73 in T lymphocytes and dendritic cells [13], and it contributes to the high expression of ectonucleotidases in myeloid-derived suppressor cells (MDSCs) and regulatory T cells (T regs) within the TME [14]. Most of the extracellular signaling activities performed by Ado are mediated by adenosine receptors (ARs; namely, A1, A2A, A2B and A3) coupled to G proteins and found in the membranes of target cells [15]. CD73 expression in tumor cells has been reported in the following different types of cancer: bladder cancer, leukemia, glioma, glioblastoma, melanoma, ovarian cancer, esophagus, thyroid, prostate, breast, colorectal and CeCa [16–29]. It has been estimated that the concentration of Ado in the TME is 10–100 μM. Ado exerts the following effects in the host: suppresses $CD8^+$ cytotoxic T lymphocytes (CTLs), natural killer cells (NK) cells, macrophages, B cells, neutrophils and dendritic cells through interaction with high affinity A2A receptors [15,30]; and promotes tumor growth by inducing the proliferation, invasion and metastasis of tumor cells, mainly through interaction with A1R, A2AR and A2BR [31–33]. When interacting with A2AR and A2BR, Ado induces the production of TGF-β in several cell types to cause immunoregulatory and anti-inflammatory effects [34–36]. Taking into account that we recently reported that CeCa tumor cells are capable of generating large amounts of Ado from the hydrolysis of adenine nucleotides through the hydrolytic activity of CD73 [29], the ability of Ado, generated through the functional activity of the adenosinergic pathway in CeCa cells, to induce the expression and secretion of TGF-β1 as well as maintain CD73 expression in CeCa cells was analyzed in the present study.

2. Materials and methods

2.1. Cell lines

CaSki (HPV16+) and HeLa (HPV18+) cell lines of CeCa were obtained from the American Type Culture Collection (ATCC). The TGF-β-sensitive Mv1Lu cell line was donated by Dr. H. López Muñoz at the UIDCC, FES-Z, UNAM, México.

2.2. Cell cultures

To analyze the participation of the adenosinergic pathway in the secretion and expression of TGF-β in CeCa tumor cells, 1×10^5 tumor cells were cultured in 6-well plates for 96 h in the presence of different concentrations (1 μM, 10 μM, 100 μM and 1 mM) of AMP or Ado (Sigma-Aldrich, St. Louis, MO, USA). Samples of the supernatants were collected every 24 h to analyze TGF-β1 content. To block the interaction of Ado with A2AR and A2BR, 10 μM (final concentration) of the specific antagonists ZM241385 (Sigma-Aldrich) and MRS1754 (Sigma-Aldrich), respectively, were added 30 min before adding AMP or Ado to the cell cultures. Cultures were maintained in Opti-MEM medium (Gibco, CA, USA) supplemented with 1% bovine fetal serum (SFB; Gibco) dialyzed with a 12 kDa cut-off membrane (Sigma-Aldrich), 100 IU/ml of penicillin and 100 μg/ml streptomycin (Gibco) at 37 °C and 5% CO_2.

For induction of CD73, tumor cells were cultured in the presence of either 1 mM Ado or 20 ng/ml recombinant human TGF-β1 (rh-TGF-β1, PeproTech, Rocky Hill, NJ, USA). The effect of TGF-β was blocked by the addition of 2 μg/ml rabbit anti-human neutralizing antibody against TGF-β1, -β2, and -β3 (anti-TGF-β; R&D Systems, Minneapolis, MN, USA) or 25 μM SB505124 (Sigma-Aldrich), selective inhibitor of TGF-β type I receptors.

2.3. Quantification of TGF-β1

To quantify TGF-β1 content in CeCa cell culture supernatants, the human TGF-β1 Quantikine ELISA Kit (R&D Systems) was used according to the manufacturer's protocol.

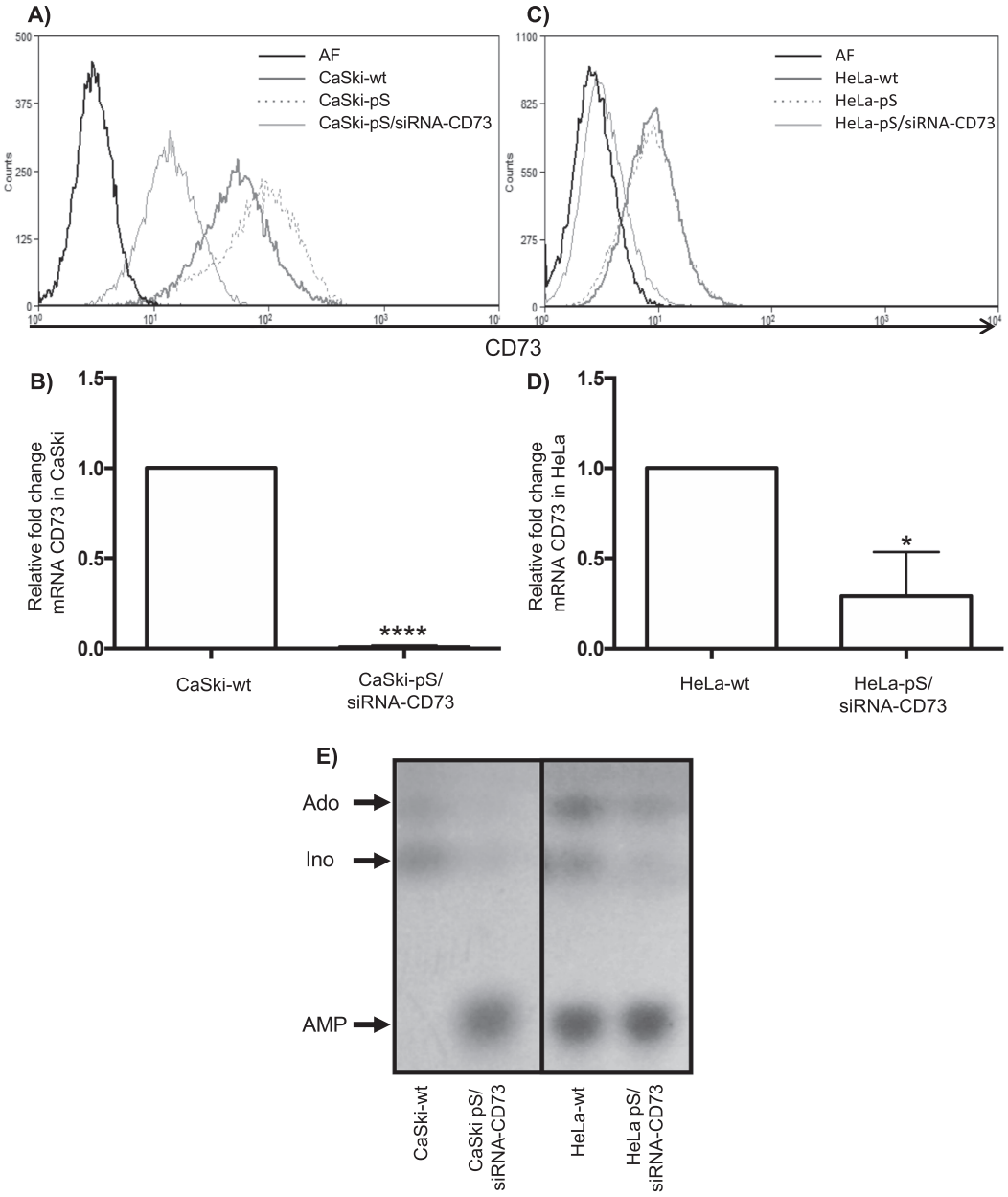

Fig. 2. CD73 inhibition in CeCa cells decreases Ado generation. CaSki and HeLa cells were transfected with the pSiren vector containing a specific siRNA for CD73 (pS/siRNA-CD73) to inhibit CD73 expression. CD73 expression in CaSki-pS/siRNA-CD73 and HeLa-pS/siRNA-CD73 cells was analyzed by flow cytometry (A, C) and quantitative RT-PCR (B, D). (F) The enzymatic activity of CD73 of CaSki-pS/siRNA-CD73 and HeLa-pS/siRNA-CD73 cells was analyzed by TLC and compared with that of their respective wild type (wt) cells. Significant difference are indicated $^{*}(p < 0.05)$ and $^{****}(p < 0.0001)$ with respect to wt.

2.4. mRNA expression

To analyze the mRNA expression of TGF-β1, A2AR and A2BR in CeCa cells, RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was obtained from 500 ng of RNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). TGF-β1 mRNA expression was determined by quantitative RT-PCR, and A2AR and A2BR mRNA expression was determined by RT-PCR at end-point. G6PDH and β-actin genes were used as internal controls. Quantitative RT-PCR was performed using a Light Cycler 480 Real-Time PCR System (Roche Diagnostic, Manheim, Germany) and Universal ProbeLibrary Probes (Roche Diagnostic). Each reaction was performed in a final volume of 10 µl according to the protocol suggested for the LightCycler 480 Probe Master (Roche Diagnostic). The following primers were used: TGF-β1 Forward, 5′ACTACTACGCCAAGGAGGTCAC'3; TGF-β1 Reverse, 5′TGCTTGAACTTGTCATAGATTTCG'3; H6PD Forward, 5′ GCTACGCTCGGATCTTGTTC'3; and H6PD Reverse, 5′CCCAGTGCTTT TCGCTCT'3). Data were analyzed by the ΔCT relative quantification method. The end-point RT-PCR reaction was performed in a volume of 25 µl following the manufacturer's instructions of the Master Mix PCR (Promega, Madison, WI, USA) using a TC1000-G (DLAB NT, Hong Kong). The amplified RT-PCR products were observed by 2% agarose gel electrophoresis (Invitrogen). The gel was stained with GelRed

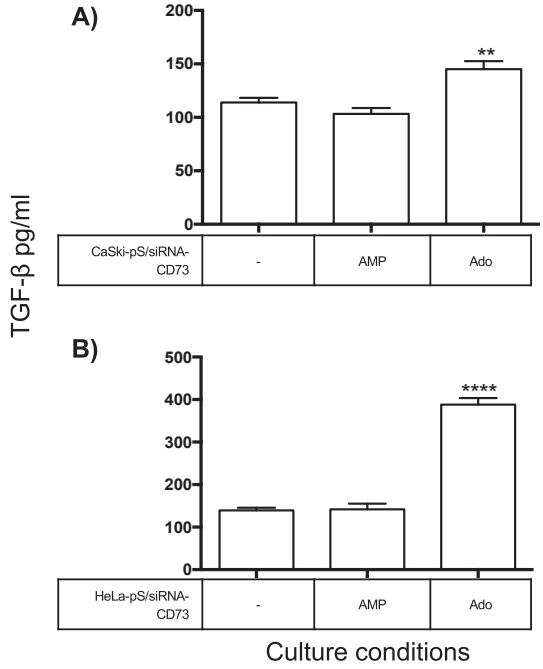

Fig. 3. Ado generated by the hydrolysis of AMP is necessary to induce the production of TGF-β in CeCa cells. CaSki-pS/siRNA-CD73 and HeLapS/siRNA-CD73 cells (1×10^5) were cultured in the presence of 1 mM AMP or Ado. After 96 h, TGF-β1 content was analyzed in the supernatants by ELISA. Data are representative of three independent experiments, and the averages ± SEM are shown. Significant differences in the content of TGF-β1 are indicated. $^{*}(p < 0.001)$ and $^{****}(p < 0.0001)$.

(Biotium, Hayward, CA, USA), and an UV transilluminator (UVP Biodo-H System, Upland, CA, USA) was used to visualize the amplified products. The following primers for A2AR, A2BR and β-actin were used: A2AR Forward, 5′TGACCGCTACATTGCCATC'3; A2AR Reverse, 5′TCC AACCTAGCATGGGAGTC'3; A2BR Forward, 5′TCTGTGTCCCGCTCA GGT'3; A2BR Reverse, 5′GATGCCAAAGGCAAGGAC'3; and β-actin Forward, 5′GGGTCAGAAGGATTCCTATG'3; and β-actin Reverse, 5′ GTCTCAAACATGATCTGGG'3.

2.5. Inhibition of CD73 expression

To inhibit CD73 expression in the CeCa cell lines, the following CD73-specific siRNAs were used (NM_001204813.1 and NM_002526.3): siRNA-1, GAT CCG CCA CTA GCA TCT CAA ATA TTT CAA GAG AAT ATT TGA GAT GCT AGT GGT TTT TTA CGC GTG; and siRNA-2, AAT TCA CGC GTA AAA AAC CAC TAG CAT CTC AAA TAT TCT CTT GAA ATA TTT GAG ATG CTA GTG GCG (RNAi Target Sequence Selector, http://www.clontech.com/MX/Support/Online Tools). The pSIREN-Retro Q vector (Clontech Laboratories, Inc. Mountain View, CA, USA) was used to perform the transfection. Selection of stably transfected cells was performed in the presence of puromycin (Sigma-Aldrich) according to the manufacturer's manual (KnockoutTM siRNA Systems, Clontech Laboratories, Inc.). Because the pSIREN-Retro Q vector contains a puromycin-resistant gene, the empty pSIREN- Retro Q vector was used as a control.

2.6. CD73 expression in CeCa cells

CD73 expression on the membrane of tumor cells was determined using an anti-CD73-PE monoclonal antibody (BD Biosciences, San Diego, CA, USA) and a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). After discarding cell debris, 10,000 events were analyzed and reported as the mean fluorescence intensity (MFI) ± SD. Additionally, CD73 mRNA expression was analyzed by RT-PCR using the methodology described above. The following primers were used: CD73 Forward, 5′GCACTATCTGGTTCACCGTGT'3; and CD73 Reverse, 5′CCTTCCACACCATTATCAAATTC'3).

2.7. Enzymatic activity of CD73

To analyze CD73 hydrolytic activity in CeCa tumor cells and CD73-dysregulated cells, 1×10^6 cells were cultured in the presence of 5 mM AMP in 100 µl of Opti-MEM (Gibco) supplemented with 1% dialyzed FBS. Supernatant was collected after 4 h for CaSki cells and after 8 h for HeLa cells. The presence of Ado was detected by thin layer chromatography (TLC) by placing 1 µl of each supernatant on fluorescent gel-coated plates (Whatman, GE Healthcare, Freiburg, Germany). Samples were eluted for 1 h using a mobile phase composed of isobutanol:isoamyl alcohol:ethoxyethanol:ammonia:water (9:6:18:9:15) [37], and 5 mM AMP, Ado and Inosine (Ino) (Sigma-Aldrich) were used as standard controls. Compounds were visualized using an UV transilluminator.

2.8. Functional activity of TGF-β1

TGF-β1-sensitive Mv1Lu cells [38] were used to evaluate the functional activity of TGF-β1 produced by CeCa cells. Mv1Lu cells (5×10^3 cells) were cultured in triplicate in a 96-well flat bottom plate in the presence of 50% conditioned media from CeCa cells cultured with 1 mM Ado and in the presence or absence of anti-TGF-β neutralizing antibody. Mv1Lu cells cultured in the presence of rh-TGF-β1 (0, 12.5, 25, 50 and 100 ng/ml) or Ado (0 µM, 31.25 µM, 62.5 µM, 125 µM, 250 µM 500 µM, 1 mM and 10 mM) were used as positive and negative controls, respectively. The proliferation of Mv1Lu cells was determined with the CellTiter 96 AQueous One Solution reagent (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions.

2.9. Statistical analysis

The numerical data are presented as the average value ± SEM of three independent experiments. The comparisons were evaluated with multivariate statistical analysis using GraphPad Prism version 6 (GraphPad Prismsoftware, USA). The differences were considered significant when $p < 0.05$.

3. Results

3.1. Ado induces the expression and secretion of TGF-β1 in CeCa cells through interaction with A2AR and A2BR

CeCa tumor cells hydrolyze AMP and generate large amounts of Ado from the enzymatic activity of CD73 [29]. In addition, recent studies have shown that Ado induces the production of TGF-β1 in different cell types through the interaction of A2AR and A2BR [34–36]. To determine if Ado generated through the functional activity of CD73 induces the expression and secretion of TGF-β1 in CeCa cells, 1×10^5 CaSki and HeLa tumor cells were cultured for 96 h in 6-well plates in the presence of different concentrations (1 µM, 10 µM, 100 µM and 1 mM) of AMP. Aliquots of the supernatants were collected every 24 h to determine TGF-β1content by ELISA. At the end of culture, cells were harvested and analyzed for TGF-β1 mRNA expression. CaSki cells significantly increased TGF-β1 production at 24 h in the presence of 1 mM AMP (Fig. 1A). HeLa cells increased TGF-β1 production at 72 h in the

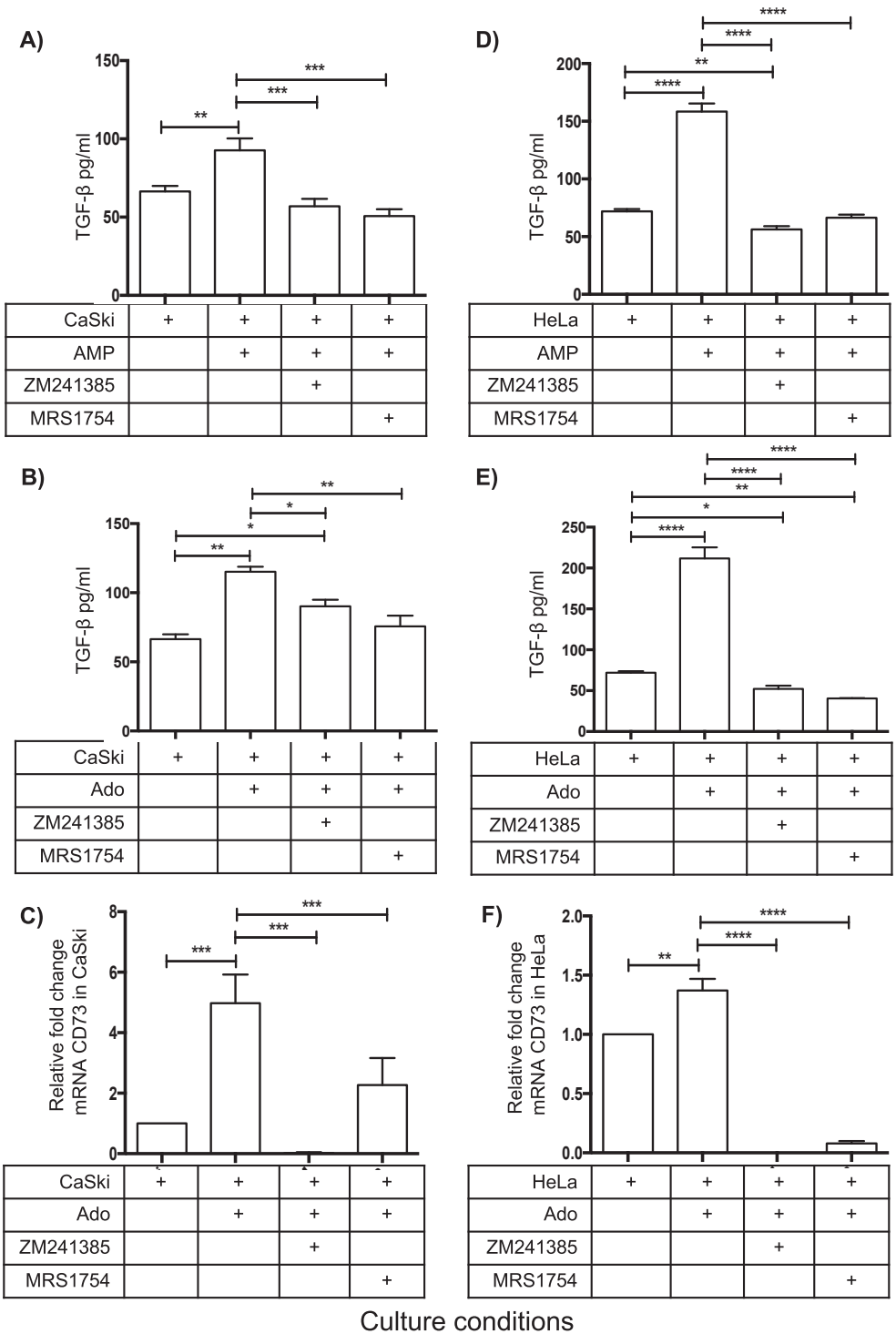

Fig. 4. Ado induces TGF-β production in CeCa cells through interaction with A2AR and A2BR. CaSki and HeLa cells (1×10^5) were cultured for 96 h in the presence of 1 mM AMP (A and D) or Ado (B–C and E–F). Thirty minutes before the addition of AMP or Ado, 10 μM ZM241385 and MRS1754, specific antagonists of A2AR and A2BR, respectively, were added to some cultures. After 96 h, the TGF-β1 content in the supernatants was analyzed by ELISA. TGF-β1 mRNA expression was analyzed by RT-PCR. Data are representative of three independent experiments, and the averages ± SEM are shown. Significant differences are indicated as $^{*}(p < 0.05)$, $^{**}(p < 0.01)$, $^{***}(p < 0.001)$ and $^{****}(p < 0.0001)$.

presence of AMP at concentrations ≥ 10 μM (Fig. 1C). Similar results were obtained when culturing CaSki (Fig. 1B) and HeLa (Fig. 1D) cells in the presence of the same concentrations of synthetic Ado. To corroborate the participation of the CD73-Ado pathway in TGF-β1 production in CeCa cells, the expression of CD73 in CaSki and HeLa cells was inhibited with siRNAs targeting CD73 isoforms (NM_001204813.1 and NM_002526.3) using the pSiren vector (pS/siRNA-CD73). The CaSki-pS/siRNA-CD73 and HeLa-pS/siRNA-CD73 cells showed significantly decreased levels of CD73 protein (Fig. 2A and C) and mRNA (Fig. 2B and D). The ability of these cells to generate Ado by the hydrolysis of AMP (Fig. 2E) was strongly reduced in relation to the wild-type (wt) cells.

When culturing CaSki-pS/siRNA-CD73 and HeLa-pS/siRNA-CD73 cells in the presence of 1 mM AMP or Ado, only Ado induced TGF-β1

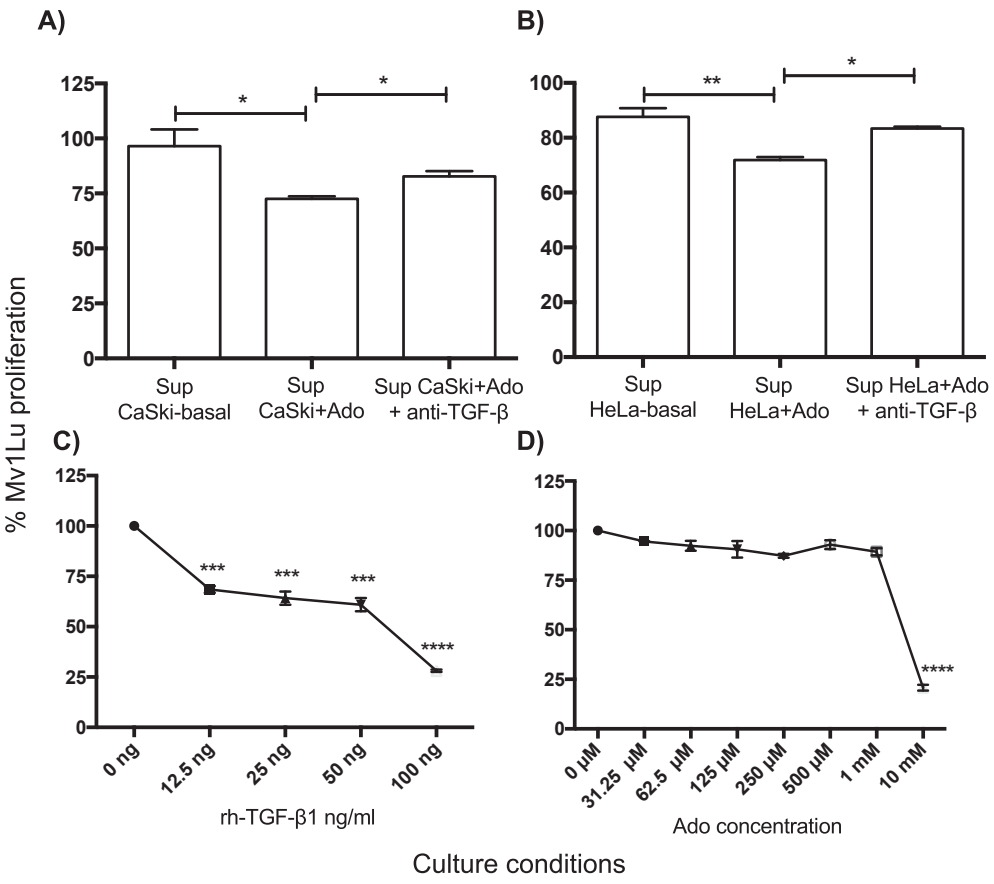

Fig. 5. Functional activity of TGF-β produced by CeCa cells. Mv1Lu cells (5×10^{3}) were cultured for 96 h in the presence of 50% CaSki (A) or HeLa (B) conditioned media that had been cultured with 1 mM Ado and in the presence or absence of neutralizing anti-TGF-β. Mv1Lu cells cultured in the presence of rh-TGF-β1 (0, 12.5, 25, 50 and 100 ng/ml) (C) or Ado (0 μM, 31.25 μM, 62.5 μM, 125 μM, 250 μM 500 μM, 1 mM and 10 mM) (D) were used as positive and negative controls, respectively. The percentage of proliferation was determined. Data are representative of three independent experiments, and the averages ± SEM are shown. Significant differences are indicated as $^{*}(p < 0.05)$, $^{**}(p < 0.01)$, $^{***}(p < 0.001)$ and $^{****}(p < 0.0001)$.

production (Fig. 3), indicating that the induction of TGF-β1 in CeCa cells is dependent on Ado generated through the hydrolysis of AMP.

The expression of A2AR and A2BR receptors was examined by RT-PCR (Supplementary Fig. 1) to analyze their participation in the induction of TGF-β1 production. CaSki and HeLa cells were cultured for 30 min in the presence of 10 μM ZM241385 or MRS1754, selective antagonists of A2AR and A2BR, respectively, and subsequently in the presence of 1 mM AMP or Ado. Interestingly, the ZM241385 and MRS1754 antagonists strongly reversed the production of TGF-β1 induced by the presence of AMP or Ado in the CaSki (Fig. 4A and B) and HeLa (Fig. 4D and E) cells. Similarly, the increase in TGF-β1 mRNA expression induced by Ado was reversed in CaSki (Fig. 4C) and HeLa (Fig. 4F) cells cultured in the presence of ZM241385 or MRS1754. These results suggested that extracellular Ado participates in the induction of TGF-β1 production in CeCa tumor cells through interaction with A2AR and A2BR.

3.2. TGF-β1 produced by CeCa cells is required to maintain CD73 expression

Several studies have shown that TGF-β regulates CD73 expression in different cells of the immune system [13,14]. Because Ado induced the secretion and expression of TGF-β1 in CeCa cells, the ability of this factor produced by CaSki and HeLa cells to regulate CD73 expression was investigated. Initially, the functional activity of TGF-β1 produced by CeCa cells cultured in the presence of Ado was determined using the TGF-β-sensitive Mv1Lu cell line [38]. Supernatants from CaSki (Fig. 5A) and HeLa (Fig. 5B) cells cultured for 96 h in the presence of 1 mM Ado inhibited the proliferation of Mv1Lu cells by more than 30%, which was comparable to the addition of 12.5 ng/ml rh-TGF-β1 (Fig. 5C). In contrast, the addition of anti-TGF-β1-neutralizing antibodies significantly reversed the inhibitory effect of these supernatants. It is important to mention that the presence of synthetic Ado at concentrations < 1 mM inhibited the proliferation of Mv1Lu cells by < 10% (Fig. 5D). To analyze the effect of TGF-β content in the supernatants from CeCa cells cultured in the presence of Ado on CD73 expression in the tumor cells, anti-TGF-β-neutralizing antibodies were added. The increase in CD73 expression (< 30% with respect to basal) observed in CaSki cells cultured in the presence of Ado was reversed by the addition of the anti-TGF-β-neutralizing antibody (Fig. 6A). In contrast, HeLa cells treated with either rh-TGF-β1 or Ado showed no increase in CD73 expression (Fig. 6B). However, the addition of SB505124, selective inhibitor of TGF-β type I receptors, to the cultures of both CaSki (Fig. 7A) and HeLa (Fig. 7B) treated with Ado decreased the expression of CD73 by more than 40% relative to the basal expression of CD73 in both cell lines. In addition, a significant increase in the TGF-β1 content was detected in the supernatants from cells cultured under these conditions (Fig. 7C) and (Fig. 7D). These results suggested that TGF-β autocrinously produced by CeCa cells through the CD73-adenosine pathway may be an indispensable factor in maintaining or inducing CD73 expression in these tumor cells.

4. Discussion

TGF-β is a pleiotropic cytokine that controls several biological functions, such as cell proliferation, apoptosis, stem cell maintenance, EMT and immune response suppression [39,40]. In addition, TGF-β plays an important role in the progression and tumor metastasis of

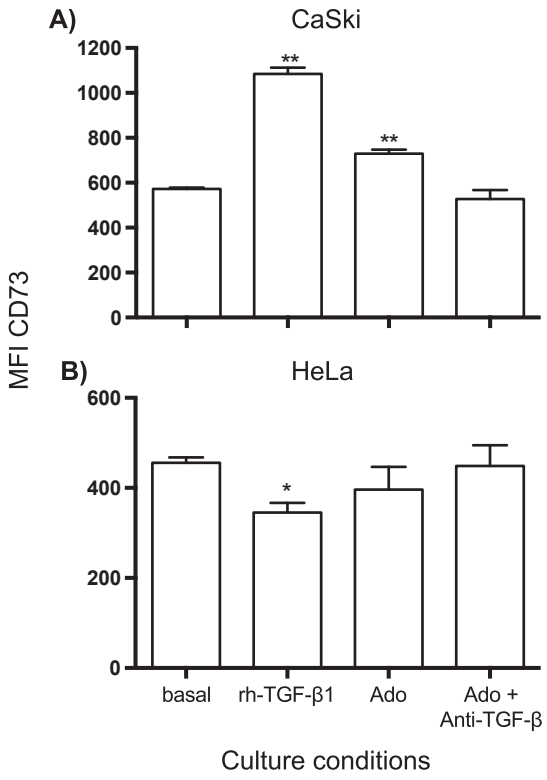

Fig. 6. Anti-TGF-β1 reverses the increase in CD73 expression induced by Ado in CeCa cells. CaSki (A) and HeLa (B) cells (1×10^5) were cultured with 1 mM Ado in the presence or absence of anti-TGF-β. CaSki and HeLa cells cultured in the presence of 20 ng/ml TGF-β-hr were used as the positive control. After 96 h, CD73 expression on the membrane of cells was analyzed by flow cytometry. Data are representative of three independent experiments, and the averages ± SEM are shown. Significant differences in CD73 expression with respect to the basal expression are shown as $^{*}(p < 0.05)$ and $^{**}(p < 0.005)$.

various types of cancer, including prostate, breast, colorectal, liver and CeCa [41,42]. In the particular case of CeCa, several studies have suggested that TGF-β1 expression directly correlates with the degree of disease progression [8,43–45]. In addition, a dual role of TGF-β1 has been suggested during the development of CeCa, either as an anti-oncogenic factor in precancerous cervical lesions or as a tumor promoter in advanced stages [6,46]. In contrast, the expression of TGF-β1 in CeCa has been positively correlated with the expression of HR-HPV oncogenes [47]. In fact, the E6 and E7 oncogenes of HPV-16 induce the activation of the human TGF-β1 promoter through the Sp1 recognition sequence [44]. However, the production of high concentrations of TGF-β in different tumors has been associated with hypoxia conditions that occur in the TME [48], suggesting that other factors can influence the production of TGF-β1 to promote tumor growth. Recent studies have shown that in pathological states, the activation of A2AR or A2BR in different cell types produces TGF-β to exert immunosuppressive and anti-inflammatory effects [34–36]. We previously reported that CeCa cells exposed to AMP generate large amounts of Ado to suppress the effector functions of CTL [29]. The present study provided the first evidence that CeCa cells are induced to produce TGF-β1 through the adenosinergic pathway. CeCa cells cultured in the presence of AMP or Ado were induced to express and secrete TGF-β1, and blockade of A2AR and A2BR with specific antagonists reversed this effect. In addition, the produced TGF-β1 induced or maintained CD73 expression in CeCa tumor cells. It is known that Ado exerts inhibitory effects on the effector cells of the immune system through its interaction with A2AR and A2BR, which are coupled to G proteins and increase the levels of cAMP, thereby decreasing the production of proinflammatory cytokines and increasing the synthesis of immunosuppressive cytokines, such as IL-10 and TGF-β [49–51]. In the present study, concentrations of Ado > 10 μM were necessary to induce an increase greater than 50% in the production of TGF-β1 in CeCa tumor cells with respect to the basal production, and the addition of ZM241385 and MRS1754 strongly reversed the TGF-β1 production induced by Ado. These results suggested that signaling pathways similar to the effector cells of the immune system may occur in CeCa tumor cells to induce the production of TGF-β1 via A2AR and A2BR. Moreover, the present study also demonstrated that the TGF-β1 produced by tumor cells is important for maintaining or inducing CD73 expression in CeCa tumor cells. Addition of neutralizing anti-TGF-β or the inhibition of TGF-βRI strongly reversed CD73 expression in the CeCa cells, suggesting a feedback loop between the adenosinergic pathway and TGF-β1 production to maintain the immunosuppressive status of CeCa cells through generating Ado and TGF-β1, which promotes tumor progression. It is known that TGF-βRI is phosphorylated once TGF-β binds to TGF-βRII to constitute a functional receptor [52]. Recently, a comprehensive molecular and integrative study of CeCa revealed that the *TGFBR2* gene, present exclusively in cervical squamous tumors, is significantly mutated in more than 70% of analyzed tumors [53], thus illustrating the clinical importance of this pathway as a therapeutic target. According to the present study, the alterations reported in the signaling pathway of TGF-β in CeCa [53] may significantly contribute to maintain an active adenosinergic pathway and therefore the immunosuppressive capacity in the TME of CeCa.

5. Conclusions

The present study provided evidence for the first time that the adenosinergic pathway plays an important role in the induction of the secretion and expression of TGF-β1 in CeCa tumor cells through generation of Ado and activation of A2AR and A2BR. Moreover, the present study demonstrated that TGF-β1 produced through this pathway maintains CD73 expression in tumor cells, suggesting a feedback loop to maintain the immunosuppressive status in CeCa through the production of Ado and TGF-β1. These results implied that this route may have clinical importance as a therapeutic target.

Conflict of interests

The authors declare that they have no conflicts of interest.

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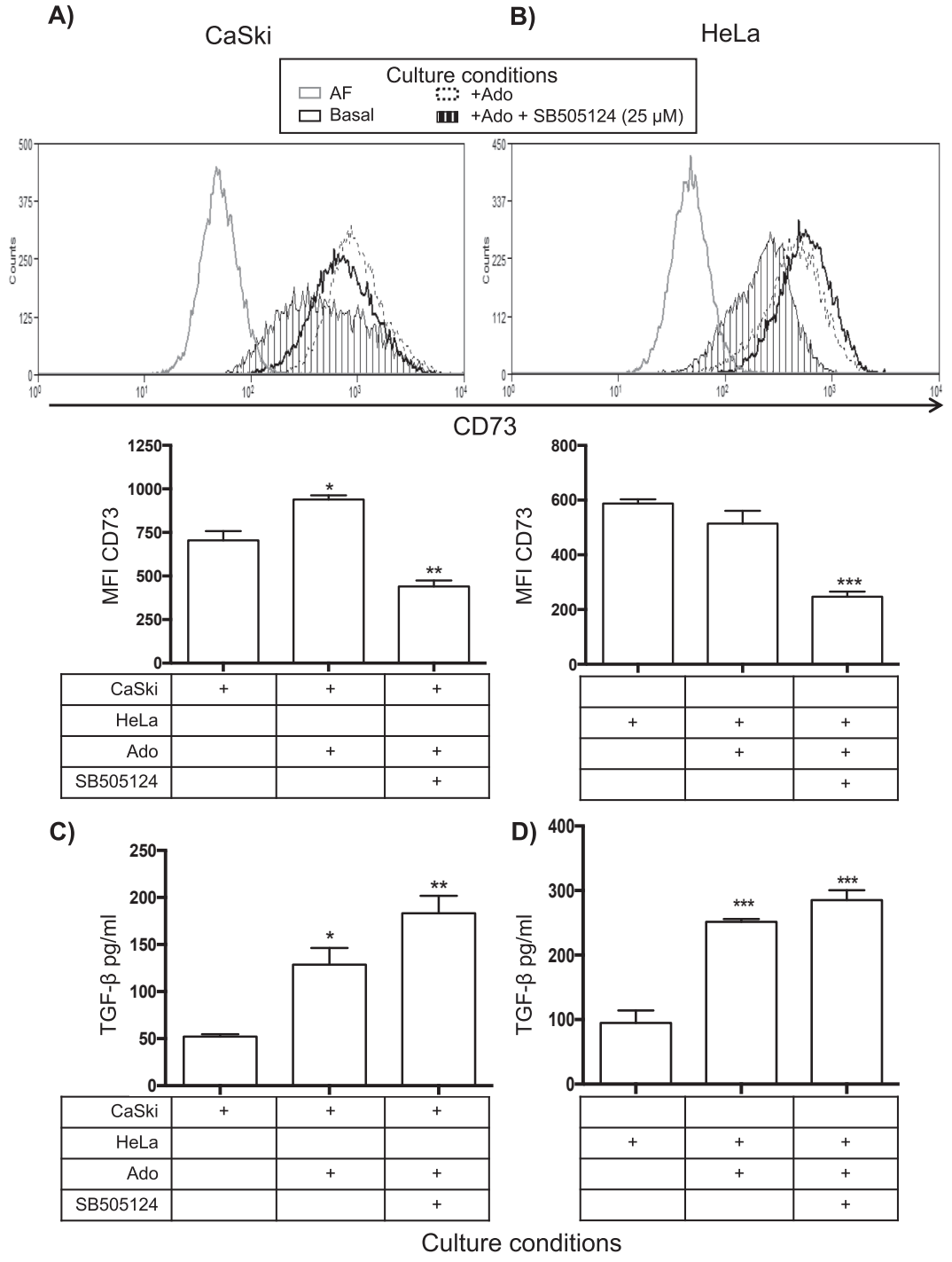

Fig. 7. Blockade of TGF-βRI reduces CD73 expression in CeCa cells. CaSki (A) and HeLa (B) cells (1×10^5) were cultured with 1 mM Ado in the presence or absence of 25 μM SB505124, selective inhibitor of TGF-β type I receptors. After 96 h, CD73 expression on the cell membrane was analyzed by flow cytometry (A and B), and TGF-β1 content was analyzed by ELISA (C and D). Data are representative of three independent experiments, and the averages ± SEM are shown. Significant differences are indicated with respect to cells without treatment (basal expression) as $^{*}(p < 0.05)$, $^{**}(p < 0.005)$, and $^{***}(p < 0.001)$.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2018.09.018.

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