Biochemical and Biophysical Research Communications 435 (2013) 691–695

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Molecular mechanisms of PDGF-AA expression induced by the dsRNA-mimetic poly (I:C) and IL-18

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ARTICLE INFO

*Article history:*
Received 4 May 2013
Available online 21 May 2013

*Keywords:*
dsRNA
IL-18
PDGF-AA
Atherosclerosis

ABSTRACT

Several animal studies suggest a role of platelet-derived growth factors (PDGFs) particularly A and B in atherosclerosis. Previously, it has been shown that viral infections have the ability to initiate and accelerate atherosclerosis in animal models. Recently, it has been reported that IL-18 has a pro-atherogenic character. Moreover, viral infections have been shown to be associated with induction of IL-18 bioactivity. By using human predendritic KG1 cells, we sought to assess PDGF-AA production under the influence of IL-18 and the byproduct of viral replication, dsRNA-mimetic poly (I:C). Here we demonstrate that poly (I:C) and IL-18 have the ability to induce PDGF-AA expression. In addition, costimulation of KG-1 cells with both IL-18 plus poly (I:C) shows an additive effect on PDGF-AA production. Furthermore, we demonstrate that neither p38 nor SAPK/JNK is required for PDGF-AA production by both PIC and IL-18. However, the expression of PDGF-AA has been found to be associated with increased activation of NF-κB and enhancement of DNA-binding capacity of NF-κB as shown by electrophoretic mobility shift assay (EMSA) and supershift analysis. Collectively, this study demonstrates that the byproduct of viral replication, dsRNA [poly (I:C)], and IL-18 have the ability to induce PDGF-AA in NF-κB-dependent manner. Furthermore, dsRNA act in an additive way with IL-18 to induce PDGF-AA which plays a major role in atherosclerosis. These data might help to understand the pro-atherogenic character of IL-18 and molecular mechanisms of viral infection-induced atherosclerosis.



## 1. Introduction

The platelet-derived growth factor (PDGF) family is composed of four isoforms (PDGF-A, -B, -C, and -D). These isoforms dimerize via disulfide linkages to form the homodimers PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD and the heterodimer PDGF-AB [1–3]. They act via two receptor chains (PDGFR-alpha and -beta). The expression of PDGF has been shown in most cell types of the atherosclerotic arterial wall, as well as in infiltrating inflammatory cells [4]. PDGFs particularly A and B have been found in atherosclerotic lesions at high levels compared with normal vessel wall [4]. The mechanisms involved in PDGF and PDGFR expression in conjunction with atherosclerosis are not known. It has been reported that inflammation mediated by both innate and adaptive immunity plays a major role in the development of atherosclerotic lesions [5]. Recently, it has been shown that IL-18 which is a proinflammatory cytokine with important regulatory functions in the innate immune response system has a pro-atherogenic character [6]. Furthermore, animal studies support the concept that IL-18 participates in the pathogenesis of atherosclerosis as demonstrated by a reduction in atherosclerosis in IL-18-deficient ApoE$^{-/-}$ mice [7]. In addition, IL-18 has been shown to mediate release of matrix metalloproteinase-9 which plays a crucial role in atherosclerosis [8,9]. The classic risk factors for atherosclerosis include, cigarette smoking, diabetes, hypertension, and hypercholesterolemia. Recently, viral infections have been reported as additional risk factor. Furthermore, it has been shown that herpes viruses are able to initiate and accelerate atherosclerosis in animal models [10]. Moreover, viral infections have been shown to be associated with induction of IL-18 bioactivity [11]. By using human predendritic KG1 cells, we sought to investigate PDGF-AA production under the influence of IL-18 and the dsRNA-mimetic polyinosinic:polycytidylic acid [poly(I:C)] which is commonly used to investigate the cellular effets of dsRNA [12–16].

## 2. Materials and methods

### 2.1. Materials

IL-18. and poly (I:C) were obtained from R&D Systems/MBL (Wiesbaden, Germany) and Amersham Biosciences (Freiburg,

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http://dx.doi.org/10.1016/j.bbrc.2013.05.044

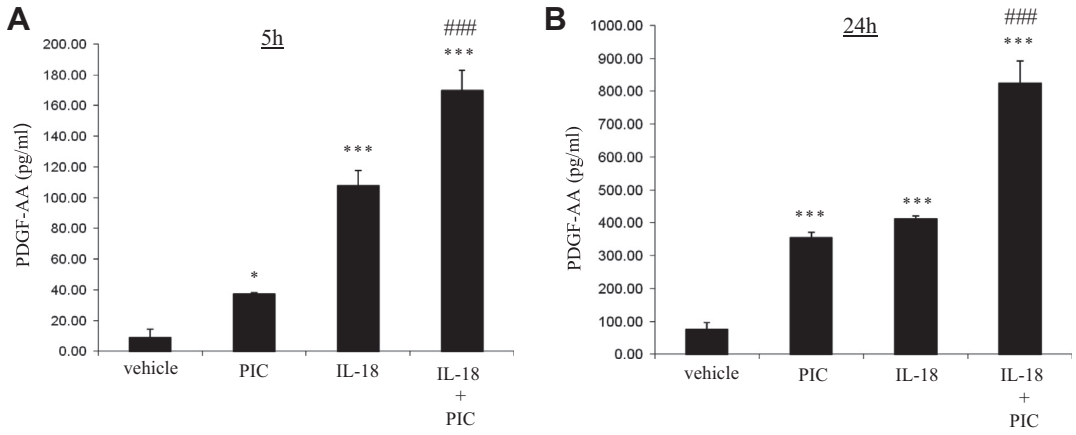


**Fig. 1.** PIC and IL-18 have the ability to release PDGF-AA in cellular supernatant. KG-1 cells were stimulated with vehicle (control) or either PIC (30 μg/ml), IL-18 (10 ng/ml) alone or in combination with PIC. PDGF-AA release was monitored by ELISA after 5 h (A) and 24 h (B). Data represent means ± SD ($n$ = 3), $^{*}p < 0.05$, $^{***}p < 0.001$ versus control, $^{\#\#\#}p < 0.001$ versus IL-18 alone.

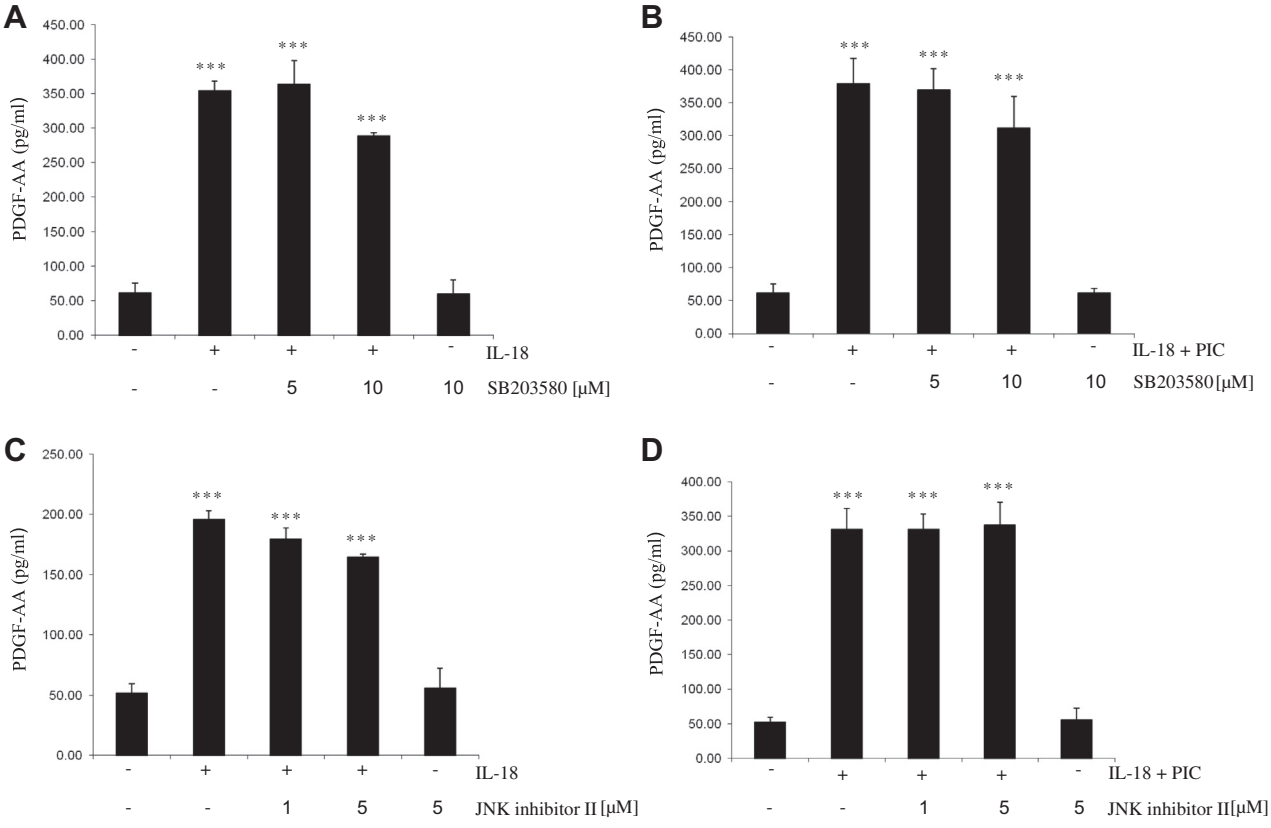


**Fig. 2.** Neither P38 MAPK nor SAPK/JNK is required for PDGF-AA production by IL-18 and PIC in KG-1 cells. (A) KG-1 cells were stimulated with vehicle (control) or either SB203580 (10 μM), IL-18 (10 ng/ml) alone or in combination with the indicated concentrations of SB203580. (B) KG-1 cells were stimulated with vehicle (control) or either SB203580 (10 μM), IL-18 (10 ng/ml) + PIC (30 μg/ml) alone or in combination with the indicated concentrations of SB203580. (C) KG-1 cells were stimulated with vehicle (control) or either JNK inhibitor II (10 μM), IL-18 (10 ng/ml) alone or in combination with the indicated concentration of JNK inhibitor II. (D) KG-1 cells were stimulated with vehicle (control) or either JNK inhibitor II (10 μM), IL-18 (10 ng/ml) + PIC (30 μg/ml) alone or in combination with the indicated concentrations of JNK inhibitor II. After 5 h the release of PDGF-AA was determined by ELISA. Data represent means ± SD ($n$ = 3), $^{***}p < 0.001$ versus control.

Germany), respectively. SB203580, c-jun-amino-terminal kinase (JNK) inhibitor II, and inhibitor of NF-κB (IκB) kinase (IKK) inhibitor VII (IKKVII) were from Calbiochem-Novabiochem (Bad Soden, Germany). Antibodies: IκB-α (rabbit polyclonal, Santa Cruz Biotechnology, Heidelberg, Germany); β-actin (mouse monoclonal, Sigma–Aldrich).

### 2.2. Cell culture

The human AML-derived predendritic cell line KG1 was from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Cells were cultivated in RPMI 1640 supplemented with 100 U/mL of penicillin, 100 g/mL of streptomycin,

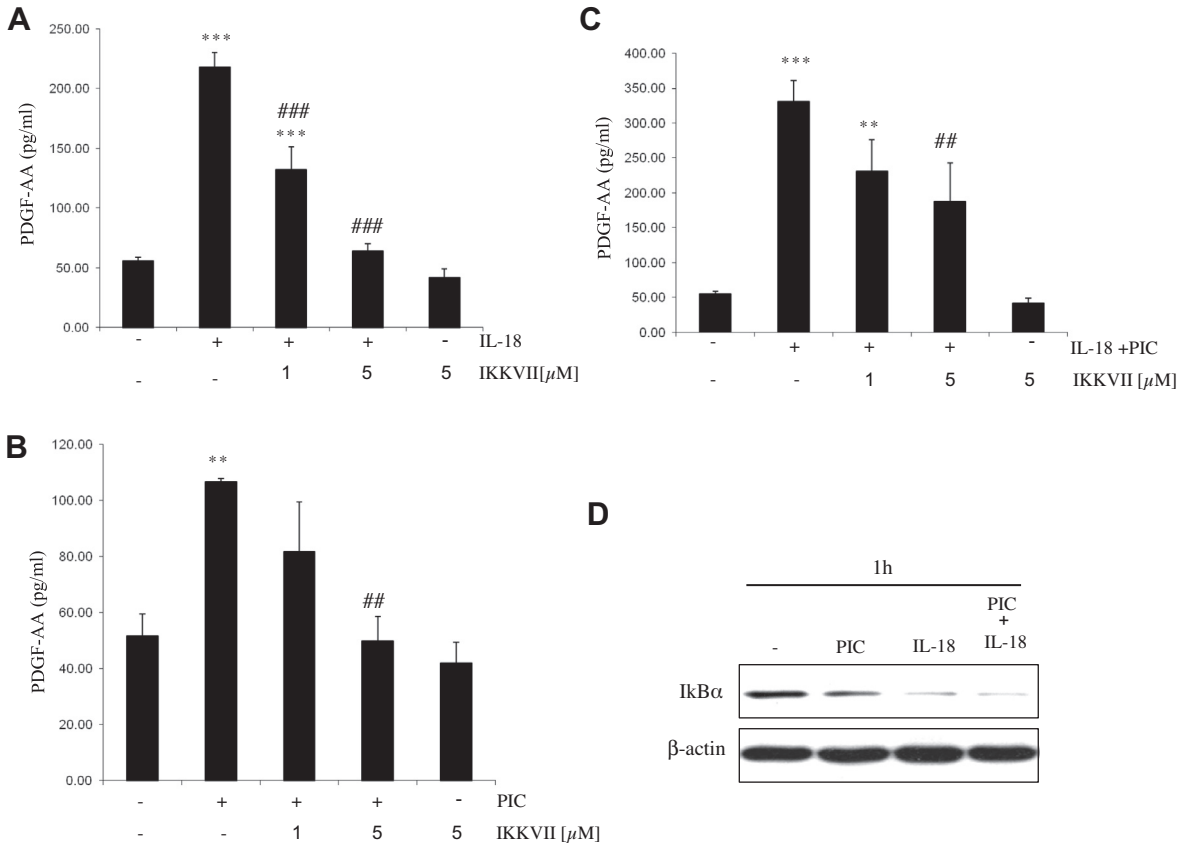


**Fig. 3.** NF-κB is essential for PDGF-AA production by IL-18 and PIC in KG-1 cells. KG-1 cells were stimulated with vehicle (control) or either IKKVII (5 μM), IL-18 (10 ng/ml) alone or in combination with the indicated concentrations of IKKVII (A) or either IKKVII (5 μM), PIC (30 μg/ml) alone or in combination with the indicated concentrations of IKKVII (B) or either IKKVII (5 μM), IL-18 (10 ng/ml) + PIC (30 μg/ml) alone or in combination with the indicated concentrations of IKKVII (C). After 5 h the release of PDGF-AA was determined by ELISA. Data represent means ± SD ($n = 3$), $^{**}p < 0.01$, $^{***}p < 0.001$ versus control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus either PIC, IL-18 or IL-18 + PIC. (D) KG-1 cells were stimulated with vehicle (control) or either PIC (30 μg/ml), IL-18 (10 ng/ml) alone or in combination with PIC for the indicated time period. Total protein (50 μg) was subjected to Western blot analysis and probed with anti–IκBα-specific polyclonal antibody. For ascertainment equal loading the blots were stripped and reprobed with the antibody raised against β-actin. One representative of three independently performed experiments is shown.

and 10% heat-inactivated FCS (Invitrogen, Karlsruhe, Germany). For experiments, cells were seeded at a density of $6 \times 10^6$ cells/2 mL in 6-well polystyrene plates (Greiner, Frickenhausen, Germany) using the aforementioned culture medium.

### 2.3. Elisa

Levels of PDGF-AA in cell-free culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (BD Bioscience/Pharmingen, Heidelberg, Germany).

### 2.4. Western blotting analysis

For detection of IκB-α, and β-actin, cells were harvested using lysis buffer [(150 mmol/L NaCl, 1 mmo

l/L CaCl2, 25 mmol/L Tris–Cl (pH 7.4), 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), DTT, Na3VO4, PMSF (each 1 mmol/L), and NaF (20 mmol/L)]. After transfer onto PVDF membranes and blocking, blots were probed with primary antibodies overnight (4 °C) followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany). Detection was achieved by a chemoluminescence-based kit (Amersham Biosciences) according to the manufacturer's instructions.

### 2.5. EMSA

Preparation of crude nuclear extracts and subsequent electrophoretic mobility shift assay (EMSA) was performed using consensus oligonucleotides for NF-κB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) as described previously [17]. For supershift analysis, 2 μl of supershift antibody was preincubated for 1 h at room temperature with the nuclear extracts before the binding reaction with the labeled oligonucleotides.

### 2.6. Statistical analysis

Results are expressed as means ± SD. Statistical analysis was performed using Student's $t$ test and for multiple comparisons the ANOVA test for significance. $p$-values below 0.05 were considered as indication for statistically significant differences between conditions compared.

## 3. Results and discussion

The involvement of PDGF in atherosclerosis has been supported by several animal studies. Specifically, in different animal models of acute arterial injury by balloon catheterization neointimal SMC accumulation was reduced by the administration of different PDGF pathway inhibitors including neutralizing PDGF antibodies [18], PDGFR kinase inhibitors [19] and PDGFR neutralizing antibodies [20,21]. The expression of PDGF has been shown in most

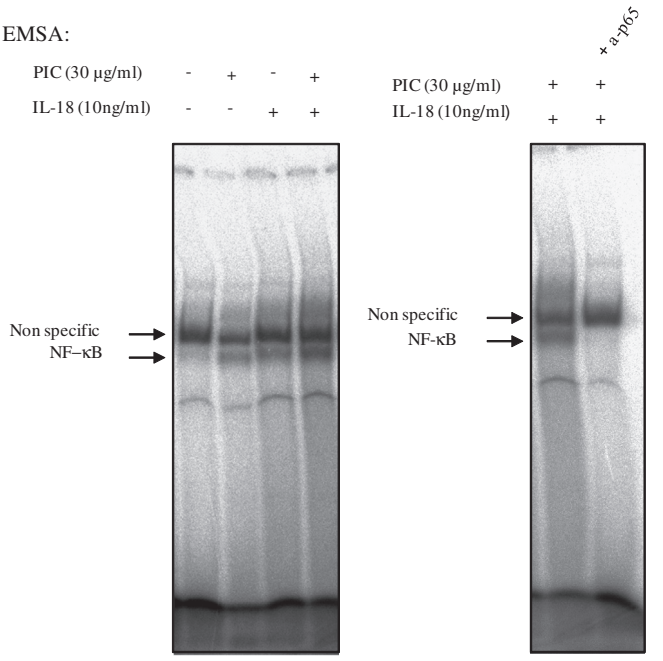


**Fig. 4.** PIC increases the DNA-binding activity of NF-κB induced by IL-18 in KG-1 cells. The DNA binding of NF-κB was analyzed by electrophoretic mobility shift assay (EMSA) using consensus NF-κB oligonucleotides. KG-1 cells were stimulated with vehicle (control) or either PIC, IL-18 alone or in combination with PIC at the indicated concentrations for 2 h. KG-1 cells were harvested for nuclear extract preparations. DNA–protein complexes were resolved from unbound DNA by non-denaturating gel electrophoresis as described in "Materials and Methods". For supershift analysis the nuclear lysates were preincubated with p65 antibody overnight at 4 °C prior to the addition of the labeled oligonucleotides.

cell types of the atherosclerotic arterial wall, as well as in infiltrating inflammatory cells [4]. PDGFs particularly A and B have been found in atherosclerotic lesions at high levels compared with normal vessel wall [4]. The mechanisms involved in PDGF and PDGFR expression in conjunction with atherosclerosis are not known. Inflammation mediated by both innate and adaptive immunity has been reported to play a major role in the development of atherosclerotic lesions [5]. IL-18 which is a proinflammatory cytokine with important regulatory functions in the innate immune response system has been shown to have a pro-atherogenic character [9]. Furthermore, animal studies support the concept that IL-18 participates in the pathogenesis of atherosclerosis as demonstrated by a reduction in atherosclerosis in IL-18-deficient ApoE$^{-/-}$ mice [10]. In addition, IL-18 binding protein (the endogenous inhibitor of IL-18) has been shown to inhibit or interfere with the progression of atherosclerotic plaques [22]. Moreover, IL-18 has been shown to mediate release of matrix metalloproteinase-9 which plays a crucial role in atherosclerosis [8,9]. In addition to the classic risk factors for atherosclerosis like cigarette smoking, diabetes, hypertension, and hypercholesterolemia, viral infections have been reported as additional risk factor. Furthermore, herpes viruses have been reported to intiate and accelerate atherosclerosis in animal models [10]. Moreover, viral infections have been shown to be associated with induction of IL-18 bioactivity [11]. By using human predendritic KG1 cells, we sought to investigate PDGF-AA production under the influence of IL-18 and poly(I:C) a synthetic analogue of dsRNA. In order to investigate the potential cross communication between dsRNA and IL-18, KG1 cells were treated with poly(I:C) [PIC], IL-18 or IL-18 in combination with PIC for 5 and 24 h. Time course experiments revealed that PIC and IL-18 have the ability to release PDGF-AA in cellular supernatants. Furthermore, costimulation of KG-1 cells with both IL-18 plus PIC shows an additive effect on PDGF-AA levels at 5 h (Fig. 1A) and 24 h (Fig. 1B). The level of PDGF-AA in cellular supernatants is increased by time. Previously, it has been shown that not only IL-18 [23–29] but also PIC [29] have the ability to activate p38 MAPK as well as JNK. Therefore, the involvement of p38 and JNK in PDGF-AA production was investigated using the pharmacological inhibitors SB203580 and JNK inhibitor II, respectively. Preincubation of KG-1cells with SB203580 as well as JNK inhibitor II before stimulation with IL-18 or Il-18 in combination with PIC did not show any changes in the levels of PDGF-AA released into cellular supernatants (Fig. 2), indicating that neither p38 nor SAPK/JNK is required for PDGF-AA production by both PIC and IL-18. NF-κB has been shown to cooperatively activate PDGF-A chain through protein–protein interaction with the transcription factor Krüppel-like factor 5 (KLF5) [30]. Therefore, the involvement of NF-κB in the production of PDGF-AA by both PIC and IL-18 was investigated. Pretreatment of KG-1 cells with the NF-κB inhibitor IKK inhibitor VII (IKKVII) before stimulation with either IL-18, PIC, or IL-18 plus PIC significantly reduced the expression of PDGF-AA in dose-dependent manner (Fig. 3A–C) indicating that NF-κB is essential for the expression of PDGF-AA. Furthermore, Western blot analysis displayed a marked decrease in IκBα protein (the inhibitory protein of NF-κB) level by PIC and IL-18 after 1 h stimulation (Fig. 3D). In addition, to evaluate whether the increasing in IκB-α degradation induced by PIC and IL-18 (Fig. 3D) is functionally linked to a rise in DNA-binding capacity of NF-κB we performed EMSA analysis using consensus oligonucleotides for NF-κB. As shown in Fig. 4, PIC and IL-18 have the ability to induce the DNA-binding activity. Moreover, PIC increased the DNA-binding activity induced by IL-18. The identity of the DNA-bound complex was confirmed by Supershift analysis. Supershift analysis indicated that the lower complex contained p65 because a strong reduction of this complex was evident when nuclear lysates were preincubated with the p65 antibody. In the present work, we could show that the byproduct of viral replication, dsRNA [poly (I:C)], and IL-18 have the ability to induce PDGF-AA expression in NF-κB-dependent manner. Furthermore, dsRNA act in an additive way with IL-18 to induce PDGF-AA which plays a major role in atherosclerosis [4,18–21]. These data might help to understand the pro-atherogenic character of IL-18 and molecular mechanisms of viral infection-induced atherosclerosis. To the best of our knowledge this is the first time that IL-18 or PIC is shown to induce the expression of PDGF-AA.

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