

Original Paper

# CX3CL1(+) Microparticles Mediate the Chemoattraction of Alveolar Macrophages toward Apoptotic Acute Promyelocytic Leukemic Cells

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## Key Words

Apoptosis • Acute lung injury • Acute promyelocytic leukemia • Chemotaxis • CX3CL1 • CX3CR1 • Differentiation syndrome • Fractalkine • Phagocytosis • Microparticles • Resolution

## Abstract

**Background/Aims:** During the resolution phase of inflammation, release of “find-me” signals by apoptotic cells is crucial in the chemoattraction of macrophages toward apoptotic cells for subsequent phagocytosis, in which microparticles derived from apoptotic cells (apo-MPs) are involved. A recent study reports that CX3CL1 is released from apoptotic cells to stimulate macrophages chemotaxis. In this study, we investigated the role of CX3CL1 in the apo-MPs in the cell-cell interaction between alveolar macrophage NR8383 cells and apoptotic all-trans retinoic acid-treated NB4 (ATRA-NB4) cells. **Methods/Results:** Apoptotic ATRA-NB4 cells and their conditioning medium (CM) enhanced the chemoattraction of NR8383 cells as well as their phagocytosis activity in engulfing apoptotic ATRA-NB4 cells. The levels of CX3CL1(+) apo-MPs and CX3CL1 were rapidly elevated in the CM of ATRA-NB4 cell culture after induction of apoptosis. Both exogenous CX3CL1 and apo-MPs enhanced the transmigration of NR8383 cells toward apoptotic ATRA-NB4 cells. This pro-transmigratory activity was able to be partially inhibited either by blocking the CX3CR1 (CX3CL1 receptor) of NR8383 cells with its specific antibody or by blocking the surface CX3CL1 of apo-MPs with its specific antibody before incubating these apo-MPs with NR8383 cells. **Conclusion:** CX3CL1(+) apo-MPs released by apoptotic cells mediate the chemotactic transmigration of alveolar macrophages.

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## Introduction

All-trans retinoic acid (ATRA) has been used successfully in the treatment of acute promyelocytic leukemia (APL) by inducing APL cell differentiation into mature granulocytes. However, this treatment can be complicated by the occurrence of differentiation syndrome (DS) in 25% of APL patients [1]. DS has the full clinical manifestations associated with acute lung injury including fever, dyspnea, hypoxemia, a diffuse pulmonary infiltrate detectable on chest x-ray and a massive infiltration of APL cells into the alveolar spaces [1]. Previous works have shown that the granulocytic differentiation and functional activation in ATRA-treated APL (ATRA-APL) cells contribute to the transmigration of circulating ATRA-APL cells into alveolar spaces, resulting in the development of acute lung injury in DS patients. Several chemokines are involved in the pathogenesis of DS, such as interleukin-8 and monocyte chemoattractant protein -1 [1-3]. Recently, we reported that the alveolar macrophage (AM $\phi$ ) is able to engulf apoptotic ATRA-APL cells; this may contribute to the clearance of apoptotic APL cells in the alveolar space during the resolution phase of DS [4]. However, the mechanism underlying the chemoattraction of AM $\phi$  toward apoptotic APL cells has not been investigated in detail.

AM $\phi$  is strategically located in the alveolar space and functions primarily against invading pathogens, microorganisms, or environmental toxins [5]. During the resolution phase of acute lung injury, AM $\phi$  are able to undertake phagocytosis of apoptotic leukocytes, contributing to the clearance of leukocytes from the lung and the restoration of epithelial barrier function [5]. Before the tethering/engulfment phases of AM $\phi$ -mediated clearance of apoptotic cells, AM $\phi$  are required to navigate effectively to sites of apoptotic cells. Regarding this, the apoptotic cells are able to release many "find-me" signals such as ribosomal protein S19, endothelial monocyte-activating polypeptide II (EMAPII), and lysophosphatidylcholine, at an early apoptotic stage to chemoattract AM $\phi$  for subsequent phagocytosis [6, 7]. Recent studies have reported that microparticles (MPs) released from apoptotic cells play a role in navigating mononuclear phagocytes toward apoptotic cells for subsequent phagocytosis [8, 9].

CX3CL1, also known as fractalkine, is a chemokine and intercellular adhesion molecule. CX3CL1 is a type I transmembrane protein, the extracellular portion of which comprises a chemokine domain attached to a mucin stalk [10]. The membrane-bound full-length surface CX3CL1 functions as an adhesion molecule and enables the integrin-independent capture and firm adhesion of leukocytes via the CX3CL1 receptor, which is called CX3CR1 [10]. Under inflammatory status, the extracellular chemokine domain of CX3CL1 can be cleaved/shed by a disintegrin and metalloproteinase domain (ADAM) to generate a soluble chemokine from stimulus-exposed cells [10]. Recently, Truman et al. reported that CX3CL1 is released from apoptotic lymphocytes to stimulate the macrophage's chemotactic transmigration, and a part of CX3CL1 is in association with MPs released by those apoptotic lymphocytes [9]. However, whether CX3CL1 mediates the chemoattractant property of MPs derived from apoptotic cells (apo-MPs) has been rarely studied.

Based on this evidence, it is logical to test the hypothesis that apoptotic cells release CX3CL1(+) apo-MPs to stimulate the chemoattraction of macrophages toward apoptotic cells. In the present study, we used apoptotic ATRA-treated NB4 (ATRA-NB4) cells to represent apoptotic ATRA-APL cells in the alveolar spaces among patients with DS. Using an in vitro cell co-culture model, we investigated the role of CX3CL1(+) apo-MPs in the cell-cell interactions between AM $\phi$  (NR8383) cells and apoptotic ATRA-NB4 cells.

## Materials and Methods

### *Cell culture and the preparation of conditioned medium (CM)*

NB4 cells are a human APL cell line (a gift from M. Lanotte, INSERM U-301, SDI No 15954.1 CNRS, Centre Hayem, Paris, France). NR8383 cells (CRL-2192; ATTC, Manassas, VA) are a well-characterized, semi-

adherent AM $\phi$  cell line that is derived from normal Sprague-Dawley (SD) rat lung and can be extrapolated to freshly derived AM $\phi$  of various species [11]. Both NB4 cells and NR8383 cells were cultured in RPMI-1640 medium (GIBCO, Grand Island, NY) and in Ham's F12K medium, respectively, as described previously [4]. ATRA treatment can induce NB4 cells into the process of granulocytic differentiation [1]. NB4 cells ( $1 \times 10^5$  cells/ml) were treated with ATRA (1  $\mu$ M; Sigma, St. Louis, MO, USA) for 3 days before receiving UV irradiation. ATRA-NB4 cells and NR8383 cells were adjusted to the cell densities of  $1 \times 10^6$  cells/ml and  $2 \times 10^6$  cells/ml, respectively, before further experiments.

To prepare CM, supernatants from ATRA-NB4 cell cultures, both before and after UV irradiation, were harvested and then centrifuged at 250g for 5 minutes to remove all cellular components. Finally, these CMs either underwent MP purification or were stored as aliquots at  $-20^\circ\text{C}$ .

#### *Generation of apoptotic cells*

To induce apoptosis, ATRA-NB4 cells were irradiated with 254 nm wavelength UV light (2000 mJ/cm $^2$ ) using a UV lamp (Spectroline; Westbury, NY) [12]. Subsequent to the irradiation, the cells were incubated for up to 24 hours at  $37^\circ\text{C}$ . Apoptosis was detected by double-staining the washed UV-treated ATRA-NB4 (UV-ATRA-NB4) cells with annexin V (NXPE; R&D Systems, Minneapolis, USA) and 7-amino-Actinomycin-D (7-AAD; BD Bioscience Pharmingen, San Diego, USA), which allows early apoptotic cells to be distinguished from late apoptotic cells by flow cytometry analysis [13].

#### *MPs preparation and flow cytometry analysis*

MPs from UV-ATRA-NB4 cell cultures were prepared as reported by Gasser et al. [14]. The CM of ATRA-NB4 cell culture (ATRA 1  $\mu$ M for 3 days) was collected and centrifuged at 1,500g for 5 minutes prior to further ultracentrifugation at 100,000g for 1 hour (Optima<sup>TM</sup> L-100XP Ultracentrifuge; Beckman Coulter, Fullerton, California, USA). The MP pellet was washed once and re-suspended in PBS. For flow cytometry analysis, the MPs were stained with annexin V (NXPE; R&D Systems, Minneapolis, USA) or anti-CX3CL1 (MAB365; R&D System, Minneapolis, USA) antibodies. The flow-cytometric analysis of the MP preparations showed the expected heterogeneous populations, with sizes varying approximately between 0.1 and 2  $\mu$ m as verified by the use of control beads (2- $\mu$ m each; ACBP-20-10; Becton Dickson). The numbers of MPs within the same gated area of MPs in the flow cytometry dot plots, as shown in Figure 3A, were then calculated [15, 16].

#### *Flow cytometry analysis of ATRA-NB4 cells and NR8383 cells*

The non-permeabilized ATRA-NB4 cells and NR8383 cells were stained with either rabbit CX3CL1 polyclonal antibody (MAB365; R&D System, Minneapolis, USA) and rabbit CX3CR1 polyclonal antibody (AF5825; R&D System, Minneapolis, USA), respectively, before analysis by FAC Scan as reported previously [4, 15].

#### *Transmigration assay*

The transmigration assay was performed in a co-culture system (BD Falcon<sup>TM</sup>, San Jose, CA) using an upper insert with membrane (8  $\mu$ m pore size,  $1 \times 10^5$  pores/cm $^2$ ) [2]. NR8383 cells were transferred to the upper insert of the transmigration assay system, while either apoptotic ATRA-NB4 cells or their CMs were added into the lower plate. The upper insert and lower plate were assembled and incubated for 2 hours. The diagram of transmigration assay has been demonstrated in our previous reports [2, 3]. Transmigration cell numbers were calculated as described previously [2]. The results were expressed as a migration index indicating a fold increase relative to the number of NR8383 cells transmigrating toward either apoptotic cells or their CM.

#### *Phagocytosis of apoptotic NB4 cells*

NR8383 cells were cultured in plates at  $37^\circ\text{C}$  for 3 days before being co-cultured with either UV-untreated or UV-treated ATRA-NB4 cells ( $2 \times 10^6$  cells/well) at  $37^\circ\text{C}$  for 2 hours before assaying for phagocytosis. Non-ingested cells were removed after washing with PBS. After myeloperoxidase staining, the number of NR8383 cells containing ingested NB4 cells was counted by light microscopy [4]. The results were expressed as phagocytosis index indicating a fold increase relative to the percentage of NR8383 cells engulfing UV-untreated ATRA-NB4 cells as control.

*Phagocytosis of fluorescence-labeled apoptotic cells*

The fluorescent membrane stains Alexa Fluor 488 phalloidin (green; Invitrogen, Oregon, OR, USA) and PKH-26 (red; Sigma-Aldrich, St. Louis, MO, USA) were used to label NR8383 cells and apoptotic UV-ATRA-NB4 cells, respectively. Thereafter, the labeled NR8383 cells were co-cultured with labeled apoptotic UV-ATRA-NB4 cells ( $2 \times 10^6$  cells) at 37°C for 2 hours before being photographed under a fluorescent microscope [12].

*Western blotting*

ATRA-NB4 cells were irradiated and then incubated for up to 24 hours at 37°C before performing protein extraction and Western blots as described previously [15]. Mouse polyclonal antibodies against human CX3CL1 (MAB3561; R&D Systems, Minneapolis, USA) and monoclonal antibody against Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma, St. Louis, Missouri, USA) were used for the Western blotting. The results were first normalized against the GAPDH present in the same cells and then expressed as a fold increase relative to the UV un-irradiated ATRA-NB4 cells as control [4, 15].

*Measurement of CX3CL1*

The level of CX3CL1 in the CM was determined using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems; Minneapolis, MN, USA) following the manufacturer protocol [17, 18].

*Statistical analysis*

The results were evaluated by one way ANOVA followed by the Fisher's least significant difference (LSD) procedure where appropriate. A value of  $p < 0.05$  was considered significant. All results are presented as mean  $\pm$  SD.

**Results***NR8383 cells chemoattractants are produced by apoptotic ATRA-NB4 cells*

We first determined the effect of UV irradiation on the induction of apoptosis in ATRA-NB4 cells at 4-hours, 16-hours and 24-hours after UV irradiation. Table 1 demonstrates that, after UV irradiation, the percentage of early apoptotic ATRA-NB4 cells significantly increased at 4-hour after UV irradiation ( $p < 0.001$ ), and the percentage of both early and late apoptotic cells also increased significantly in a time-dependent manner ( $p < 0.001$  &  $p < 0.001$ : respectively).

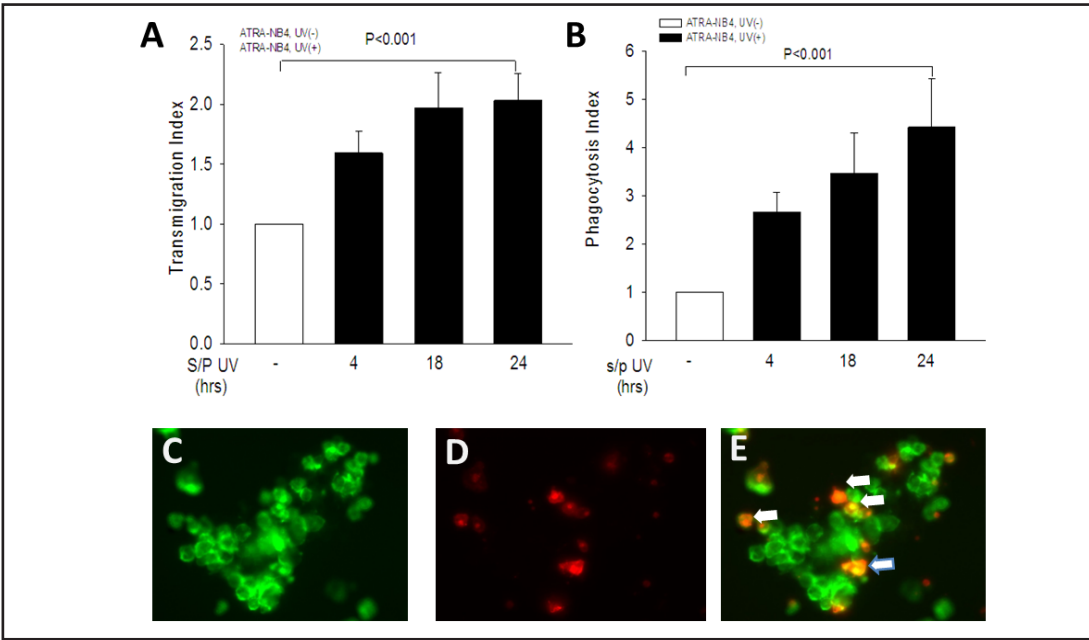
We then determined the trans migratory activity of NR8383 cells toward apoptotic ATRA-NB4 cells which had been induced into the process of apoptosis by UV-irradiation at 4 to 24 hours previously. Figure 1A demonstrates that the trans migratory activity of NR8383 cells toward apoptotic ATRA-NB4 cells was significantly higher than those cells toward UV-untreated ATRA-NB4 cells; and this trans migratory activity increased in a time-dependent manner when the duration after irradiation of the apoptotic ATRA-NB4 cells was increased ( $p < 0.001$ ).

Thereafter, we determined the phagocytosis activity of NR8383 cells on engulfing apoptotic ATRA-NB4 cells. Consistent with our previous study [4], Figure 1(B-E) demonstrates that NR8383 cells had a higher phagocytic activity in engulfing apoptotic ATRA-NB4 cells than in engulfing living ATRA-NB4 cells; and that the phagocytic activity increased in a time-dependent manner as the time after UV irradiation of the co-cultured apoptotic ATRA-NB4 cells increased ( $p < 0.001$ ).

It now became important to determine whether chemo-attractants were released by the apoptotic ATRA-NB4 cells after triggering of the apoptotic program. CMs, harvested from apoptotic ATRA-NB4 cell cultures, were added into the lower plates of the trans migratory system to determine their chemotactic activity. Figure 2A demonstrates that CM of apoptotic ATRA-NB4 cell culture harvested at 4 hours after UV irradiation had an enhanced chemoattractant activity on NR8383 cells than CM harvested from UV-untreated cells ( $p < 0.001$ ), and this activity continued to increase gradually up to 24 hours after UV irradiation.

Hours after UV Tx	Early apoptosis (%) <sup>+</sup>	Late apoptosis (%) <sup>+</sup>
0	0.5±0.2	1.4±0.4
4	11.2±2.5 <sup>#</sup>	10.0±2.4
18	13.3±1.7 <sup>#</sup>	33.2±6.5 <sup>##,*</sup>
24	12.1±1.0 <sup>#</sup>	42.0±14.0 <sup>##,**</sup>
<i>p</i> value <sup>++</sup>	<i>p</i> <0.001	<i>p</i> <0.001

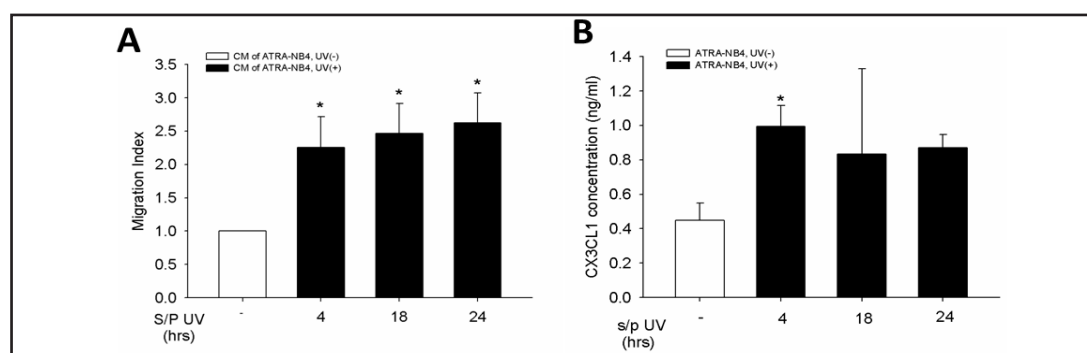
**Table 1.** Ultra-violet irradiation induced apoptosis in the ATRA-NB4 cells. <sup>+</sup>Early apoptosis is defined as cells expressing annexin V, but not 7-AAD; and late apoptosis: defined as cells expressing both annexin V and 7-AAD, <sup>++</sup>: ANOVA; <sup>#</sup>: *p* <0.001 vs. 0-hour (early apoptosis); <sup>##</sup>: *p* <0.001 vs. 0-hour (late apoptosis); <sup>\*</sup>: *p* <0.05 vs. 4-hour (late apoptosis); <sup>\*\*</sup>: *p* <0.001 vs. 4-hour (late apoptosis)



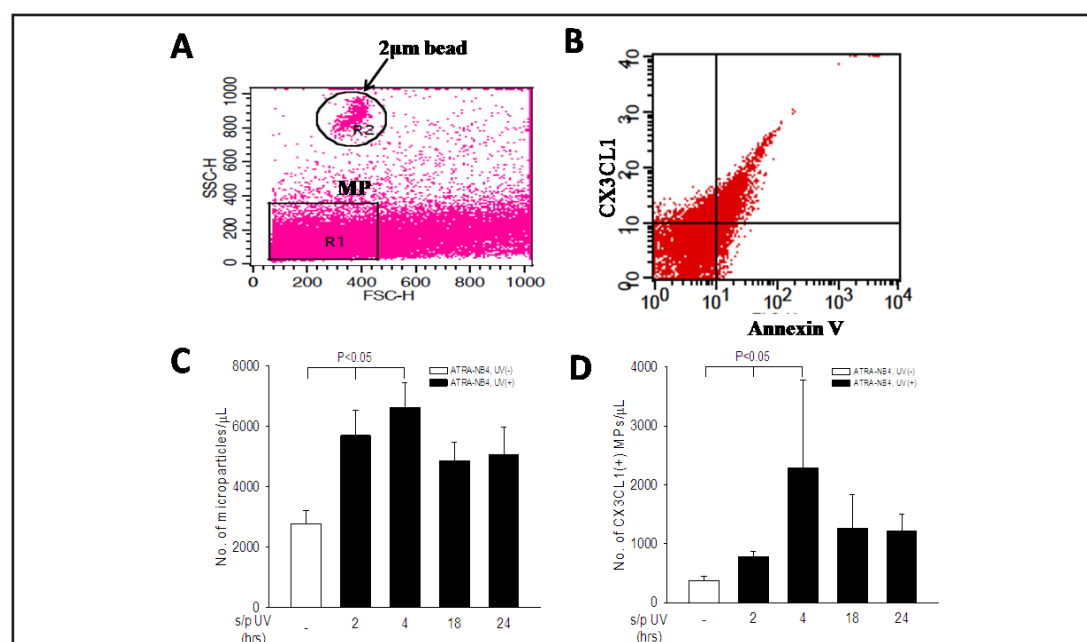
**Fig. 1.** Transmigration of NR8383 cells toward apoptotic ATRA-NB4 cells for subsequent phagocytosis. (A) NR8383 cells were added into the upper insert of trans migratory assay system, and UV-unirradiated ATRA-NB4 cells (blank bar) or UV-irradiated ATRA-NB4 cells (4–24 hours after UV; black bar) were added into the lower plates. The upper insert and lower plate were assembled to determine the chemotactic activity (Materials and Methods section). The results were expressed as migration index indicating a fold increase relative to the number of NR8383 cells trans migrating toward UV-untreated ATRA-NB4 cells as control. (B) NR8383 cells were cultured in plates for 3 days before UV-ATRA-NB4 cells were added into such plates for phagocytosis (Materials and Methods). The results were expressed as phagocytosis index indicating a fold increase relative to the percentage of NR8383 cells engulfing UV-untreated ATRA-NB4 cells as control. The results are presented as the means±SD from six (A) and four (B) independent experiments, respectively. The results were analyzed by ANOVA. (C) The Alexa Fluor 488 phalloidin-labeled NR8383 cells (green) and (D) PKH26-labeled apoptotic UV-ATRA-NB4 cells (red) were co-cultured for 2 hours before being photographed by fluorescent microscope. (E) Overlay of photograph C and D. White arrows indicate apoptotic ATRA-NB4 cells were engulfed by NR8383 cells (yellow). Magnification x 300.

We then examined whether CX3CL1 was released into the CMs by apoptotic ATRA-NB4 cells. Figure 2B demonstrates that CX3CL1 was constitutively released by ATRA-NB4 cells; the level of CX3CL1 was significantly increased 4 hours after induction of apoptosis by UV irradiation (*p* <0.05), and this level remained persistently elevated thereafter.





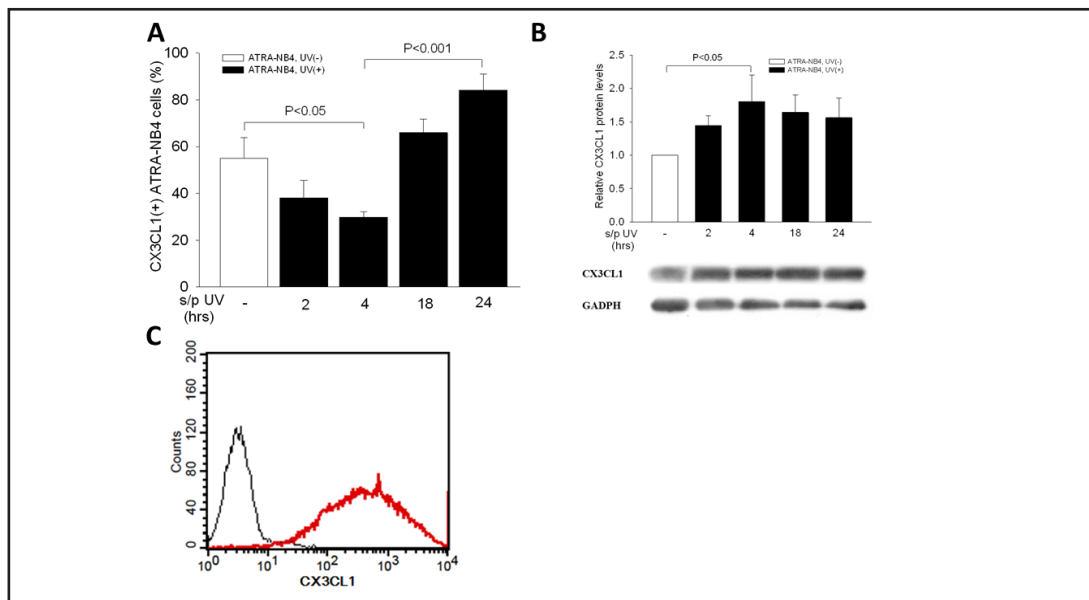
**Fig. 2.** Chemotactic activity and the level of CX3CL1 in the conditioning medium harvested from apoptotic ATRA-NB4 cell cultures. (A) The CMs were harvested from UV-ATRANB4 cell cultures, either before (blank bar) or 4–24 hours after (black bar) UV irradiation, which were added into the lower plate of transmigration assay system, while NR8383 cells were added separately on the upper insert. The upper insert and lower plate were assembled to determine the chemotactic activity (Materials and Methods section). The results were expressed as migration index indicating a fold increase relative to the number of NR8383 cells transmigrating toward CM harvested from UV-untreated ATRA-NB4 cell culture as control. \*:  $p < 0.001$  vs. CM harvested from UV-untreated ATRA-NB4 cell culture; (B) The level of CX3CL1 in the CMs was determined by ELISA. \*:  $p < 0.05$  vs. UV-untreated ATRA-NB4 cells. The results are presented as the means  $\pm$  SD from six (A) and five (B) independent experiments, respectively.



**Fig. 3.** Flow cytometric analysis of MPs released from apoptotic ATRA-NB4 cells. MPs were purified from CM of ATRA-NB4 cell cultures at either before or 2–24 hours after UV irradiation. (A) Dot plot showing the heterogeneity in MPs size; circled dots refer to 2μm beads run in parallel as internal control. (B) Expression of CX3CL1 and annexin V on MPs in the flow cytometric analysis was determined by using antibody specific for CX3CL1 and annexin V. The total numbers of apo-MPs (C) and CX3CL1(+) apo-MPs (D), in the 20 ml CM harvested from UV-treated ATRA-NB4 cell cultures, were calculated using a known concentration of flow count beads. These represent the means  $\pm$  SD from five independent experiments (C, D).

#### *CX3CL1(+) apo-MPs are released by apoptotic ATRA-NB4 cells*

We subsequently purified apo-MPs from the CMs of apoptotic ATRA-NB4 cell cultures. Figure 3 demonstrates that, as compared with MPs released by living cells, the level of apo-MPs released by apoptotic ATRA-NB4 cells increased progressively over the first 4 hours



**Fig. 4.** Characterization of CX3CL1 expression on ATRA-NB4 cells by flow cytometry and western blotting, and CX3CR1 expression on NR8383 cells by flow cytometry. (A) ATRA-NB4 cells were first treated with UV-irradiation 2-24 hours before determining their surface expression of CX3CL1 by flow cytometric analysis. The results are presented as the means $\pm$ SD from six independent experiments, and were analyzed by ANOVA. (B) The levels of CX3CL1 protein in the whole cells of ATRA-NB4 cells were determined by western blotting. The results were first normalized against GAPDH from the same cells and subsequently expressed as a fold increase relative to the UV-untreated ATRA-NB4 cells. The results are presented as the means $\pm$ SD from five independent experiments, and were analyzed by ANOVA. (C) Surface expression of CX3CR1 on the NR8383 cells as determined by flow cytometric analysis. This is a representative picture from four independent experiments.

after induction of apoptosis by UV-treatment ( $p < 0.05$ ), and thereafter this level decreased gradually until the 24-hour point after UV irradiation. Both CX3CL1 and annexin V were expressed on the surface of these apo-MPs (Fig. 3B); and the level of CX3CL1(+) apo-MPs in the CMs of apoptotic ATRA-NB4 cell cultures was also significantly elevated at 4-hours after UV irradiation than those MPs released by the living cells ( $p < 0.05$ ; Fig. 3D).

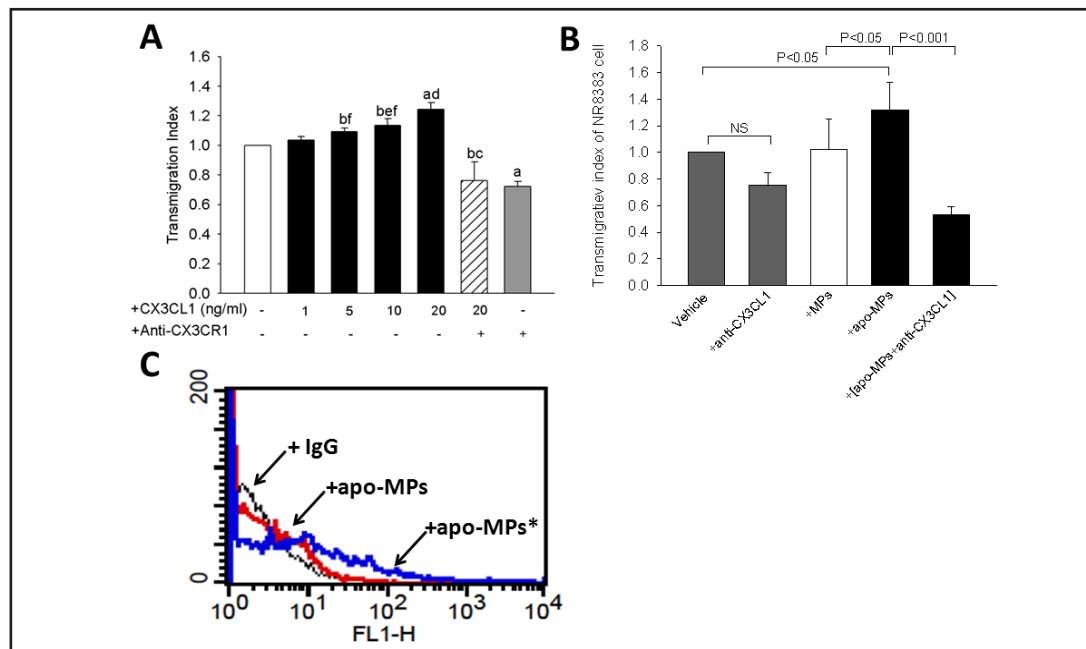
#### *Expression of CX3CL1 on UV-ATRA-NB4 cells and the expression of CX3CR1 on NR8383 cells*

Further flow cytometry analysis of the surface expression of apoptotic ATRA-NB4 cells indicated that CX3CL1 was expressed on the UV-untreated ATRA-NB4 cells, and that its level initially decreased at 4-hours after induction of apoptosis by UV irradiation. However, this level then increased when measured at 18- and 24-hours after UV irradiation (Fig. 4A). We also determined the protein level of CX3CL1 in the apoptotic ATRA-NB4 cells by the Western blotting assay. Figure 4B shows that, as compared with UV-untreated cells, the level of CX3CL1 protein was found to increase progressively in the apoptotic ATRA-NB4 cells over the first 4 hours after UV irradiation ( $p < 0.05$ ), but then underwent no significant change thereafter up to the end of the UV irradiation study.

Flow cytometry analysis also demonstrated that CX3CR1 was expressed on the surface of NR8383 cells (Fig. 4C).

#### *CX3CL1 mediates the pro-transmigratory property of CX3CL1(+) apo-MPs released by apoptotic-ATRA-NB4 cells*

Figure 5A demonstrates that transmigratory activity of NR8383 cells toward apoptotic ATRA-NB4 cells was inhibited when the CX3CR1 of the NR8383 cells were previously blocked



**Fig. 5.** CX3CL1 mediates the pro-transmigratory property of CX3CL1(+) apo-MPs released by apoptotic ATRA-NB4 cells. (A) NR8383 cells were treated with exogenous CX3CL1 (1–20 ng/ml; black bar) before being added to the upper insert of the transmigration assay system. Part of NR8383 cells were either treated with anti-CX3CR1 antibody (grey bar) or treated with anti-CX3CR1 antibody followed by exogenous CX3CL1 treatment (striped bar). The results were expressed as migration index indicating a fold increase relative to the number of NR8383 cells transmigrating toward UV-untreated ATRA-NB4 cells in the absence of anti-CX3CR1 antibody and exogenous CX3CL1 treatment (blank bar). a:  $P < 0.001$  vs. blank bar; b:  $P < 0.05$  vs. blank bar; c:  $P < 0.001$  vs. CX3CL1 20 ng/ml only; d:  $P < 0.001$  vs. CX3CL1 1 ng/ml only; e:  $P < 0.05$  vs. CX3CL1 1 ng/ml only; f:  $P < 0.05$  vs. CX3CL1 20 ng/ml only (B) NR8383 cells were firstly incubated with vehicle (grey bar), anti-CX3CL1 (grey bar), MPs derived from UV un-irradiated ATRA-NB4 cell cultures (+MPs; blank bar), apo-MPs derived from apoptotic ATRA-NB4 cell cultures (+apo-MPs; black bar) or apo-MPs that had been pretreated with antibody specific against CX3CL1 (+apo-MPs + anti-CX3CL1; black bar), respectively, before being added to the upper insert of the transmigration assay system. The apoptotic ATRA-NB4 cells (4 hours after UV irradiation) were added into the lower plate (A & B). Upper insert and lower plate were assembled for migratory assay (Materials and Methods section). The results were expressed as migration index indicating a fold increase relative to the number of vehicle-treated NR8383 cells transmigrating toward UV-untreated ATRA-NB4 cells. The results are the means  $\pm$  SD from five independent experiments (A & B). NS: not significant. (C) Apo-MPs, harvested from apoptotic ATRA-NB4 cell cultures (4 hours after UV irradiation), were either labeled (apo-MP\*) or not labeled (apo-MP) with fluorescent FITC-conjugated CX3CL1 monoclonal antibody. Subsequently, NR8383 cells were incubated with either apo-MPs, apo-MP\* or IgG (as control) before flow cytometry analysis. This is a representative picture from four independent experiments.

by its specific antibody ( $p < 0.05$ ). We then demonstrate that exogenous CX3CL1 protein could enhance the transmigration activity of NR8383 cells in a dose-dependent manner ( $p < 0.05$ ), and this enhancement was also inhibited when CX3CR1 on the NR8383 cells were previously blocked by its specific antibody before treatment with exogenous CX3CL1 (Fig. 5A).

Next, the question whether apo-MPs, released by apoptotic ATRA-NB4 cells, have pro-transmigratory activity relative to NR8383 cells was explored. To do this, apo-MPs were incubated with the NR8383 cells before transmigration assays. Figure 5B shows that NR8383 cells incubated with apo-MPs, derived from apoptotic ATRA-NB4 cells, had a significantly higher transmigration activity than those cells incubated with either vehicle



alone or MPs derived from UV-untreated ATRA-NB4 cells ( $p < 0.05$  and  $p < 0.05$ ; respectively); and this pro-transmigratory activity could be significantly inhibited by blocking the surface CX3CL1 of apo-MPs with its specific antibody before incubating these apo-MPs with NR8383 cells ( $p < 0.001$ ). Finally, apo-MPs were labeled with a fluorescent FITC-conjugated CX3CL1 monoclonal antibody before being incubated with NR8383 cells for flow cytometry analysis. Figure 5C demonstrates that the fluorescent FITC-labeled conjugates were able to be transported from the labeled apo-MPs to the surface of recipient NR8383 cells, indicating that apo-MPs are able to adhere and/or fuse to the cell membrane of the recipient NR8383 cells.

## Discussion

Release of “find-me” signals by apoptotic cells is crucial to the docking of macrophages with apoptotic cells for subsequent phagocytosis during the final stage of the apoptosis program [19]. The present study demonstrates CX3CL1(+) apo-MPs, released by apoptotic APL cells, contribute to the chemoattraction of AM $\phi$  toward apoptotic cells.

We demonstrate that the release of CX3CL1(+) apo-MPs and CX3CL1 by apoptotic ATRA-NB4 cells was enhanced during the early apoptotic process, and this was accompanied with a simultaneous decrease in the surface expression of CX3CL1 on the apoptotic ATRA-NB4 cells. To confirm the crucial role of CX3CL1-CX3CR1 axis, our data indicate that CX3CL1 enhances the transmigration of NR8383 cells toward apoptotic cells, and this is dependent on the presence of CX3CR1 on the NR8383 cells (Fig. 1,2,5).

Release of MPs is a well-known characteristic of cells undergoing apoptosis, and these MPs are involved in the chemoattraction of mononuclear phagocytes to apoptotic cells [7]. To extend this finding, we demonstrated that an increased amount of CX3CL1(+) apo-MPs were released by apoptotic ATRA-NB4 cells to enhance the transmigration of NR8383 cells toward apoptotic cells. We confirmed that apo-MPs released during the apoptotic process differ phenotypically and quantitatively from those MPs released during cellular activation [20, 21]. Recent studies indicate that MPs released by activated neutrophils or differentiated ATRA-APL cells are associated with anti-inflammatory property in which the anti-inflammatory mediator annexin A1 is involved [15, 16, 22]. In contrast to these studies, we demonstrate that apo-MPs released by apoptotic ATRA-NB4 cells induced a higher pro-transmigratory activity on NR8383 cells than those MPs released by living ATRA-NB4 cells (Fig. 5), and this was in parallel with a higher amount of CX3CL1(+) MPs released by apoptotic ATRA-NB4 cells than released by the living cells. CX3CL1 on the surface of apo-MPs is likely to act as an adhesion molecule to promote the adhesion of apo-MPs to recipient NR8383 cells via the CX3CL1-CX3CR1 axis (Fig. 5C) [23], this may result in the release of a larger amount of CX3CL1 from apo-MPs to the surface of NR8383 cells to build up a chemotactic gradient in navigating NR8383 cells toward apoptotic ATRA-NB4 cells. To further support the crucial role of CX3CL1 in the CX3CL1(+)MPs, our results demonstrate that pro-transmigratory property of apo-MPs released by apoptotic ATRA-NB4 cells was inhibited by blocking their surface CX3CL1 with its specific antibody (Fig. 5). Taken together, our results indicate that CX3CL1 mediates the pro-transmigratory property of the CX3CL1(+) apo-MPs released by apoptotic ATRA-NB4 cells in the transmigration of NR8383 cells toward apoptotic ATRA-NB4 cells.

CX3CL1 released by apoptotic cells is also able to promote the macrophage's capacity in engulfing apoptotic cells. In this context, CX3CL1 enhances the binding of macrophages to apoptotic cells through up-regulating MFG-E8 (milk fat globule epidermal growth factor 8), prevents macrophages from apoptosis and enhances monocyte recruitment into alveolar space to expand the AM $\phi$  pool [24-28]. On the other hand, we also demonstrate that, when ATRA-NB4 cells progressed from early to late apoptotic stage, the surface expression of CX3CL1 was increased (Fig. 4), which may act as an adhesion molecule to promote the binding of those apoptotic cells to NR8383 cells, via CX3CL1-CX3CR1 axis, for subsequent

phagocytosis, and this will prevent late apoptotic cells from releasing toxic molecules to cause further tissue damage. In agreement with a previous study [9], we also demonstrated that total cellular CX3CL1 protein level was persistently elevated in the apoptotic ATRA-NB4 cells (Fig. 4B), indicating that CX3CL1 protein production is enhanced to expand the cytoplasmic pool after induction of the apoptotic process. The cytoplasmic CX3CL1 is likely to be mobilized to plasma membrane to expand the surface CX3CL1 in apoptotic ATRA-NB4 cells after induction of apoptotic process because neither the level of total cellular CX3CL1 protein nor the level of CX3CL1 or CX3CL1(+) apo-MPs in the CM had a significant change during the later stage of apoptotic process (Fig. 2,3). However, this warrants further study.

Previous studies in DS have focused on investigating the role of cytokines in the pathogenesis of acute lung injury during acute inflammatory phase. In this context, IL-8, MCP-1, IL-1 $\beta$  and GRO- $\alpha$  play an important role in inducing transmigration of differentiated APL cells from blood stream into alveolar space [1-3, 29, 30]. In contrast to these studies, we studied the mechanism underlying the clearance of apoptotic APL cells during resolution phase in DS patients. Based on our results, we postulate that apoptotic ATRA-APL cells are likely to release CX3CL1(+) MPs and CX3CL1 to enhance transmigration of AM $\phi$  toward apoptotic cells allowing subsequent phagocytosis in the alveolar spaces in DS patients with acute lung injury. However, this warrants further study to determine the level of CX3CL1(+) MPs and CX3CL1 in the bronchioalveolar lavage in patients with DS or in the animal DS model. In addition, we have reported previously that, after interacting with apoptotic cells, AM $\phi$  release lipoxin A4 to enhance the phagocytic activity of AM $\phi$ , promote monocyte recruitment and inhibit neutrophils trafficking into alveolar space [26, 28], this may further contribute to the clearance of apoptotic cells from alveolar spaces during the resolution phase in DS patients. Therefore, we conclude that CX3CL1, released by apoptotic APL cells, plays a crucial role in the clearance of apoptotic APL cells from alveolar space during resolution phase in DS patients. Our work also provides a rationale for further clinical studies aimed at defining the pathophysiological role of CX3CL1 in the resolution of acute lung injury caused by other etiologies.

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