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Original Article

Role of CX3CL1 in the chemotactic migration of all-trans retinoic acid-treated acute promyelocytic leukemic cells toward apoptotic cells

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Abstract

Background: Phagocytic clearance of apoptotic neutrophils by tissue macrophages is a crucial component in the resolution phase of acute inflammation. However, the number of tissue macrophages is low and not likely to cope satisfactorily with the excess number of dying neutrophils. Although recent studies have reported that neutrophils are able to engulf apoptotic neutrophils, the mechanisms by which living neutrophils are attracted to apoptotic neutrophils are poorly defined. Increased amounts of CX3CL1 and microparticles (MPs) are rapidly released by apoptotic cells, and are involved in the chemoattraction of mononuclear phagocytes toward apoptotic cells. The current study investigated the role of CX3CL1 in the chemoattraction of all-trans retinoic acid (ATRA)-treated NB4 (ATRA-NB4) cells toward apoptotic cells.
Methods: Conditioning medium and MPs were harvested from apoptotic ATRA-NB4 cell cultures to determine their effects on living ATRA-NB4 cells by transmigration assay and adhesion assay. The cytokine levels in the conditioning medium were determined by enzyme-linked immunosorbent assay. Expression of CX3CR1 (a receptor of CX3CL1) on ATRA-NB4 cells was determined by flow cytometric analysis.
Results: ATRA-NB4 cells transmigrated toward the apoptotic ATRA-NB4 cells, and this chemoattraction was partially inhibited when the CX3CR1 on ATRA-NB4 cells was blocked by its specific antibody. Both exogenous CX3CL1 and MPs released by apoptotic ATRA-NB4 cells were able to enhance the chemoattraction of ATRA-NB4 cells toward apoptotic cells or the adhesion of ATRA-NB4 cells to endothelial cells. CX3CL1 was expressed on the surface of MPs, and blocking this CX3CL1 with its specific antibody was able to partially inhibit the chemoattractive property of MPs.
Conclusion: CX3CL1, in either the free or MP form, is released rapidly by apoptotic ATRA-NB4 cells after induction of apoptosis to mediate the chemoattraction of living ATRA-NB4 cells toward apoptotic cells.
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Keywords: acute promyelocytic leukemia; apoptosis; chemotaxis; CX3CL1; microparticles

1. Introduction

Inflammation is a beneficial host response to infections or toxic injury. In the phase of an acute inflammation, large numbers of polymorphonuclear neutrophils are rapidly recruited from the bloodstream to the site of infection or injury via transmigration through the vascular endothelium. Neutrophils are considered primary effector cells in infection-induced

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acute inflammatory reactions where they destroy invading pathogens.[1] Although the life span of neutrophils is extended in inflamed tissues,[2] apoptosis of infiltrating neutrophils has been documented *in vitro* as well as *in vivo*. Therefore, numerous tissue neutrophils die in the inflammatory sites. Because uncontrolled release of toxic substances from dead neutrophils can propagate the inflammatory response leading to tissue destruction, early recognition, and phagocytosis of dying inflammatory neutrophils by macrophages is crucial in the resolution of the inflammatory response.[3] Although it is known that apoptotic inflammatory neutrophils can be phagocytosed by macrophages,[3] macrophages are rare at sites of acute inflammation where the number of neutrophils within the tissue can be extremely high. In recent studies, neutrophils were able to act in a form of cellular cannibalism and participate in the clearance of aged or apoptotic neutrophils by phagocytosis in inflamed tissue.[4,5] This indicates that activated neutrophils also play a crucial role in the clearance of apoptotic cells during the resolution phase of acute inflammation. However, the mechanism underlying the chemotactic transmigration of living neutrophils toward apoptotic neutrophils has been rarely studied to any great extent.

CX3CL1, also known as fractalkine, is a chemokine and intercellular adhesion molecule. Previous studies have reported that CX3CL1 is implicated in the pathogenesis of acute inflammation.[6,7] In this context, CX3CL1 is able to chemoattract and activate leukocytes such as lymphocytes, natural killer cells, dendritic cells, and monocytes/macrophages, resulting in the enhancement of leukocyte attachment to and migration through the vascular endothelium and causing mononuclear cell accumulation in the inflammatory sites.[6] However, whether CX3CL1 is involved in the chemoattraction of living granulocytes to apoptotic ones is still not clear.

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.[8] 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.[8] Previous studies have shown that the granulocytic differentiation and functional activation in ATRA-treated APL (ATRA-APL) cells contributes to the transmigration of circulating ATRA-APL cells into alveolar spaces and the development of acute lung injury in DS patients.[8–10] Previous studies have also demonstrated that the ATRA-APL cell model is an excellent *in vitro* study model of the cell–cell interaction between granulocytes and other inflammatory cells.[9–13]

Based on this evidence, it is logical to test the hypothesis that CX3CL1 contributes to the chemoattraction of living ATRA-APL cells toward apoptotic cells. In the present study, using an *in vitro* cell co-culture model, we were able to examine the cell–cell interactions between ATRA-treated APL NB4 (ATRA-NB4) cells and apoptotic ATRA-NB4 cells. We have specifically addressed the pivotal role of CX3CL1-CX3CR1 axis in the chemoattraction of ATRA-NB4 cells toward apoptotic cells.

2. Methods

2.1. Cell culture and the preparation of conditioning medium

NB4 cells (a human APL cell line; a gift from M. Lanotte, INSERM U-301, SDI No 15954.1 CNRS; Centre Hayem, Paris, France) were cultured in RPMI-1640 medium (GIBCO, Grand Island, NY, USA) as described previously.[12] NB4 cells (1×10^5 cells/mL) were treated with ATRA (1μM; Sigma, St. Louis, MO, USA) for 3 days (ATRA-NB4 cells) to induce the process of granulocytic differentiation prior to receiving UV irradiation.[8]

To prepare conditioning medium (CM), supernatants from ATRA-NB4 cell cultures were harvested and then centrifuged at 250*g* for 5 minutes to remove all cellular components. This CM was stored as aliquots at −20°C.

2.2. 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, USA).[5] Subsequent to the irradiation, the cells were incubated for up to 24 hours at 37°C. Apoptosis was detected by staining the washed UV-irradiated ATRA-NB4 (UV-ATRA-NB4) cells with annexin V (NXPE; R&D Systems, Minneapolis, MN, USA).[14]

2.3. Microparticle preparation and flow cytometry analysis

Microparticles (MPs) from ATRA-NB4 cell cultures were prepared as reported by Gasser et al.[15] The CM of ATRA-NB4 cell culture (ATRA 1 μM for 3 days) was collected and centrifuged at 1500*g* for 5 minutes prior to further ultracentrifugation at 100,000*g* for 1 hour (Optima L-100XP Ultracentrifuge; Beckman Coulter, Fullerton, CA, USA). The MP pellet was washed once and resuspended in phosphate-buffered saline (PBS). For flow cytometry analysis, the MPs were stained with annexin V (NXPE; R&D Systems, Minneapolis, MN, USA) or anti-CX3CL1 (MAB365; R&D Systems) antibodies. The flow-cytometric analysis of the MP preparations showed the expected heterogeneous populations, with sizes varying approximately between 0.1 μm and 2 μm as verified by the use of control beads (2-μm each; ACBP-20-10; Becton Dickson); the representative figure has been published previously.[11] The numbers of MPs within the same gated area of MPs in the flow cytometry dot plots were then calculated.[11,16]

2.4. Transmigration assay

The transmigration assay was performed in a co-culture system (BD Falcon, San Jose, CA, USA) using an upper cell culture insert with membrane (8 μm pore size, 1×10^5 pores/

cm^2) and a companion 24-well plate at the bottom. Briefly, ATRA-NB4 cells (ATRA 1μM for 3 days) were pretreated with CM harvested from apoptotic ATRA-NB4 cells, exogenous CX3CL1 (R&D System, Minneapolis, MN, USA), MPs harvested from apoptotic ATRA-NB4-cell cultures or an antibody against CX3CR1 (AF5825; R&D System) prior to being added into the upper insert. The lower plate was then filled with apoptotic ATRA-NB4 cells. The upper insert and lower plate were assembled and incubated for 2 hours. After incubation, the membrane was then removed and stained with Wright stain. The transmigrated ATRA-NB4 cells present were then counted using light microscopy as described previously.[9] The results were expressed as a migration index indicating a fold increase relative to UV-untreated ATRA-NB4 cells as control.

2.5. Adhesion test by colorimetric assay

Human umbilical vein endothelial cells were seeded into 24-well culture dishes at a density of 5×10^4 cells/well and were incubated at 37°C with 5% CO_2 and 100% humidity until complete confluence was observed. ATRA-NB4 cells were first incubated with either CM or MPs. Thereafter, the washed cells were dyed with 0.25% Rose Bengal (Alfa Aesar, Ward Hill, MA, USA) for 15 minutes and then were washed twice with PBS to remove unincorporated dye. Finally, the dyed ATRA-NB4 cells were adjusted to 1×10^6 cells/mL prior to being added to each well to adhere to the human umbilical vein endothelial cells for 1 hour. Each well was washed three times to remove unbound cells and then cells were treated with a solution of ethanol/PBS (1:1) for 30 minutes to release the stain. Absorbance at 570 nm for each well was determined using an Infinite M1000 microplate reader (TECAN, AG, Männedorf, Switzerland). The results were expressed as an adhesion index indicating a fold increase relative to UV-untreated ATRA-NB4 cells as control.

2.6. Measurement of interleukin-8, transforming growth factor-β1, and tumor necrosis factor-α

The levels of interleukin (IL)-8, transforming growth factor (TGF)-β1 and tumor necrosis factor (TNF)-α in the CM was determined using enzyme-linked immunosorbent assay kits (R&D Systems) following the manufacturer's protocol.[17,18]

2.7. Flow cytometry analysis

The nonpermeabilized ATRA-NB4 cells were stained with rabbit CX3CR1 polyclonal antibody (AF5825; R&D System, Minneapolis, MN, USA) prior to analysis by FAC Scan (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) as previously reported.[11,12]

2.8. Statistical analysis

The results were evaluated by one-way analysis of variance followed by the Fisher's least significant difference procedure where appropriate. A p value < 0.05 was considered significant. All results are presented as mean ± standard deviation.

3. Results

3.1. CX3CL1 released by apoptotic ATRA-NB4 cells stimulates living ATRA-NB4 cells chemotaxis

In a previous study, we reported that ATRA-NB4 cells are induced into apoptosis in a time-dependent manner during the 24-hour period after UV irradiation; the percentage of early and late apoptotic ATRA-NB4 cells increased from 0.5% ± 0.2% and 1.4 % ± 0.4% prior to UV-irradiation, to 11.2% ± 2.5% and 10.0% ± 2.4% at 4 hours after UV irradiation, and to 12.1% ± 1.0% and 42.0% ± 14.0% at the 24-hour interval after UV irradiation, respectively. In this study, we determined whether apoptotic ATRA-NB4 cells were able to stimulate living ATRA-NB4 cells in either chemotactic transmigration toward them or adhesion to endothelial cells. We first harvested CM from apoptotic cell cultures. ATRA-NB4 cells were then incubated with CM prior to testing migration assay and adhesion assay. Fig. 1 demonstrates that, as compared with CM harvested from living ATRA-NB4 cell culture, CM harvested from apoptotic cell cultures was able to enhance ATRA-NB4 cells in both the transmigration and

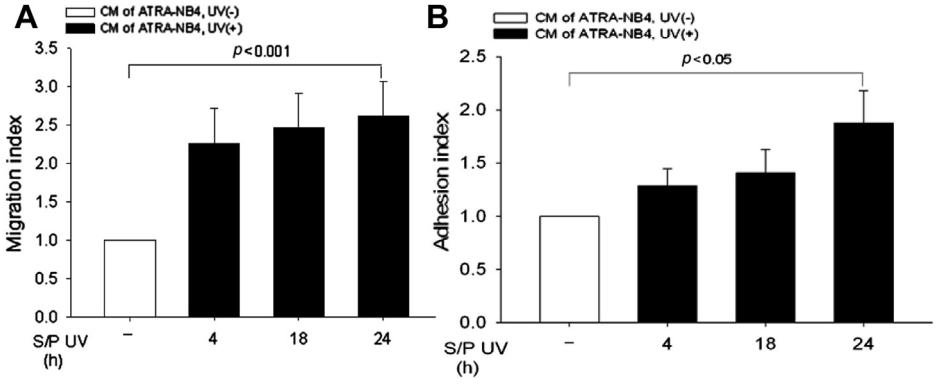

Fig. 1. Promigratory and proadhesive activity in the conditioning medium (CM) harvested from apoptotic all-trans retinoic acid (ATRA)-treated NB4 cell cultures. The CM was harvested from ATRA-NB4 cell cultures, either prior to (blank bar) or 4–24 hours after UV irradiation (black bar). ATRA-NB4 cells were incubated first with CM prior to testing with (A) transmigratory assay and (B) adhesive assay (see Methods in main text). The results are presented as the mean ± standard deviation from (A) six and (B) five independent experiments, respectively.

adhesion to endothelial cells, and the promigratory activity and proadhesive activity in the CM increased rapidly immediately after induction of apoptosis by UV-irradiation and increased in a time-dependent manner relative to how long after UV irradiation the CM were harvested ($p < 0.001$ and $p < 0.05$, respectively).

We further determined the level of cytokines in the CM of apoptotic cell cultures by enzyme-linked immunosorbent assay. In a recent study, we reported that the level of CX3CL1 in the CM of apoptotic ATRA-NB4 cell cultures increased from 447 ± 100 pg/mL prior to UV-irradiation to 994 ± 121 pg/mL at 4 hours after UV irradiation, and this level remained persistently elevated thereafter. Fig. 2 demonstrates that the level of IL-8 decreased in a time-dependent manner over 24 hours after UV irradiation ($p < 0.05$); the level of TGF-β1 decreased in a time-dependent manner during the initial 4 hours after induction of apoptosis by UV irradiation ($p < 0.001$) but increased thereafter, and the levels of TNF-α had no significant change during the 24 hours after induction of apoptosis.

We also demonstrated that CX3CR1 was expressed on the surface of living ATRA-NB4 cells by flow cytometry analysis (Fig. 3), and the transmigration of ATRA-NB4 cells toward apoptotic ATRA-NB4 cells was inhibited when CX3CR1 on the surface of living ATRA-NB4 cells was previously blocked by its specific antibody prior to transmigratory assay ($p < 0.05$; Fig. 3). We then demonstrated that exogenous CX3CL1 protein could enhance the transmigration of ATRA-NB4 cells toward apoptotic cells in a dose-dependent manner ($p < 0.05$), and this enhancement was also inhibited when CX3CR1 on the ATRA-NB4 cells was previously blocked by its specific antibody prior to treatment with exogenous CX3CL1 ($p < 0.001$; Fig. 3).

3.2. MPs released by apoptotic ATRA-NB4 cells stimulate living ATRA-NB4 cells chemotaxis

Recently, we also reported that the level of MPs released by ATRA-NB4 cells increased from 2770 ± 450 MPs/μL prior to UV irradiation to 6630 ± 816 MPs/μL at the 4-hour mark after UV-irradiation ($p < 0.05$). Both CX3CL1 and annexin V were expressed on the surface of these apo-MPs; and the level of CX3CL1(+)MPs released by ATRA-NB4 cells increased from 447 ± 136 MPs/μL prior to UV irradiation to 2287 ± 1498 MPs/μL at 4-hours after UV irradiation.

Next, we explored whether MPs released by apoptotic cells have proinflammatory activity relative to ATRA-NB4 cells. To do this, we incubated the ATRA-NB4 cells with MPs concentrates first prior to testing with transmigratory assay and adhesion assay. As compared with cells incubated with either vehicle alone or MPs derived from UV-untreated ATRA-NB4 cells, ATRA-NB4 cells incubated with MPs harvested from apoptotic cells had a significantly higher transmigratory activity ($p < 0.05$ and $p < 0.05$, respectively, Fig. 4A) and adhesion activity ($p < 0.05$ and $p < 0.05$, respectively Fig. 4B); and the promigratory and proadhesive property of MPs harvested from apoptotic cells, could be significantly inhibited by blocking their surface CX3CL1 with its specific

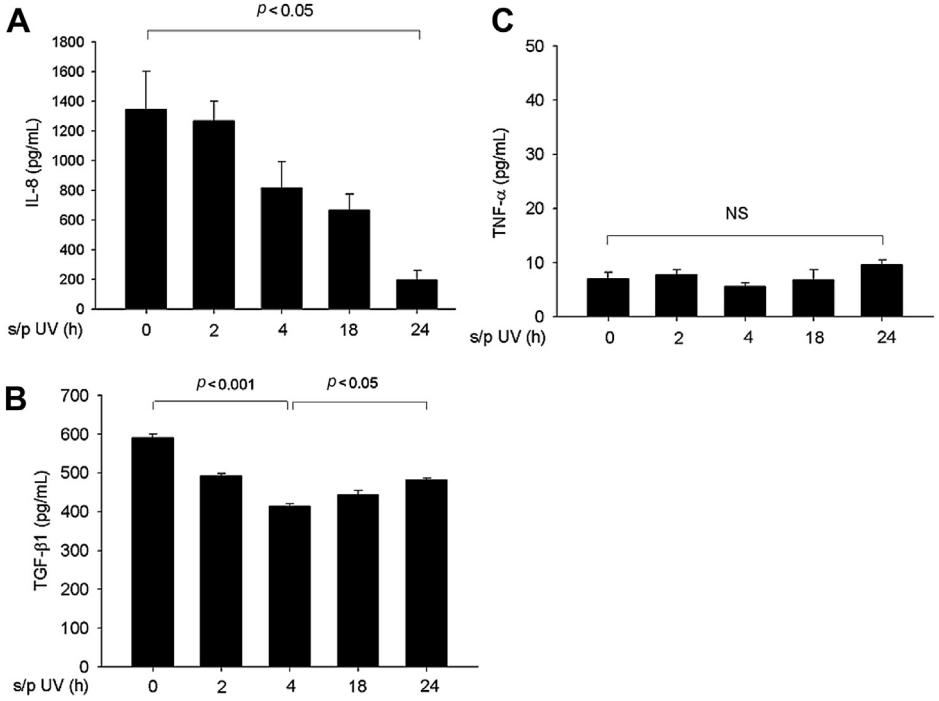

Fig. 2. Level of interleukin (IL)-8, transforming growth factor (TGF)-β1 and tumor necrosis factor (TNF)-α in the conditioning medium harvested from apoptotic all-trans retinoic acid (ATRA)-treated NB4 cell cultures. The level of (A) IL-8, (B) TGF-β1 and (C) TNF-α in the CM was determined by enzyme-linked immunosorbent assay. The results are presented as the mean ± standard deviation from six independent experiments.

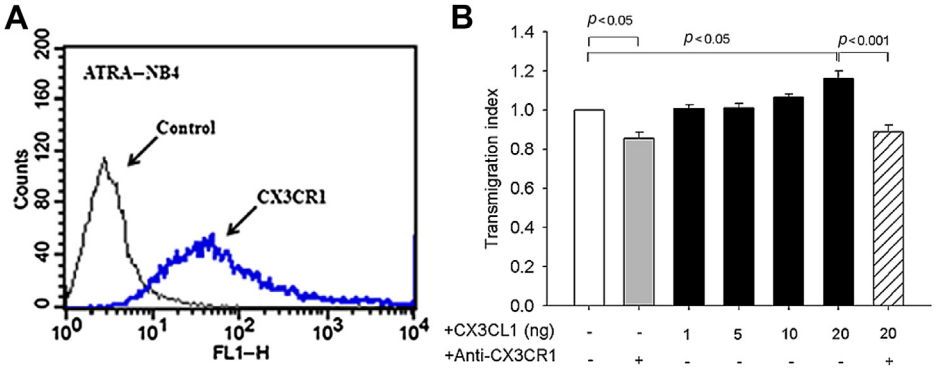

Fig. 3. CX3CL1–CX3CR1 axis in the transmigration of living all-trans retinoic acid (ATRA)-treated NB4 cells toward apoptotic cells. (A) Surface expression of CX3CR1 on the ATRA-NB4 cells was determined by flow cytometric analysis. This is a representative picture from four independent experiments. (B) ATRA-NB4 cells were treated with exogenous CX3CL1 (1–20 ng; black bar), in which part of ATRA-NB4 cells were pretreated with an antibody specific to CX3CR1 (gray bar and striped bar) prior to treatment with exogenous CX3CL1 (striped bar). Thereafter, the treated ATRA-NB4 cells were tested with transmigratory assay (see Methods in main text). The results are presented as the mean ± standard deviation from five independent experiments.

antibody prior to incubating those MPs with ATRA-NB4 cells ($p < 0.001$ and $p < 0.05$; respectively).

4. Discussion

In the current study, *in vitro* co-culture studies were carried out to investigate the mechanism underlying the chemoattraction of living ATRA-NB4 cells toward apoptotic ones. In this study, we present evidence that CX3CL1 released by apoptotic ATRA-NB4 cells is able to mediate the chemoattraction of living ATRA-NB4 cells toward apoptotic cells and the adhesion of ATRA-NB4 cells to the endothelial cells. To support further the crucial role of CX3CL1-CX3CR1 axis in the chemoattraction of living ATRA-NB4 cells toward apoptotic cells, we demonstrated that: (1) CM harvested from apoptotic ATRA-NB4 cell cultures was able to enhance the living ATRA-NB4 cells was both in transmigration and in adhesion to endothelial cells; (2) the chemoattraction of living ATRA-NB4 cells toward apoptotic cell was dependent on the presence of CX3CR1 on the ATRA-NB4 cells; and (3) exogenous CX3CL1 was able to enhance the transmigration of ATRA-NB4 cells in a dose-dependent manner. Consistent with our results, previous studies also reported that CX3CL1 released by apoptotic cells mediates the chemoattraction of mononuclear phagocytes or alveolar macrophages toward apoptotic cells.[19,20] In a recent study, CX3CL1 was expressed on the surface of apoptotic ATRA-APL cells, which may contribute to the binding of apoptotic cells to living ATRA-APL cells via CX3CL1-CX3CR1 axis. Along this line, Esmann et al[5] reported that the activated neutrophils are able

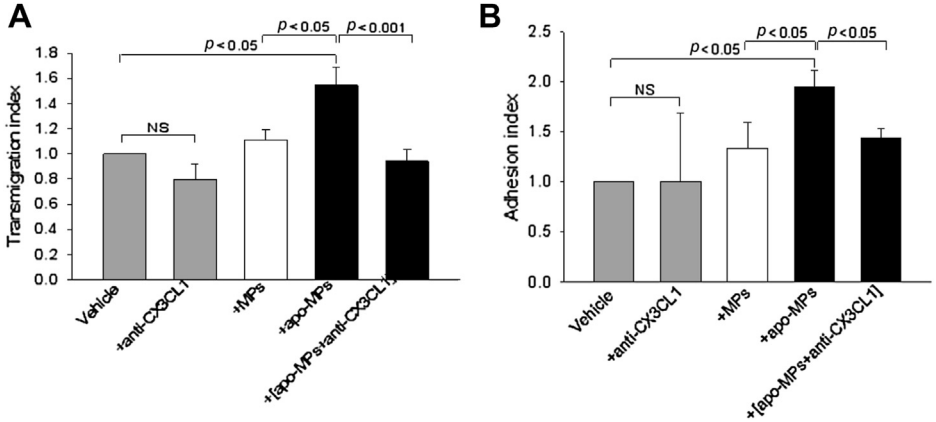

Fig. 4. Promigratory and proadhesive activity of microparticles released from apoptotic all-trans retinoic acid (ATRA)-treated NB4 cells. ATRA-NB4 cells were first incubated with one of the followings: vehicle (gray bar), anti-CX3CL1 (gray bar), microparticles (MPs) harvested from UV unirradiated ATRA-NB4 cell cultures (blank bar), MPs harvested from apoptotic ATRA-NB4 cell cultures (+apo-MPs; black bar) or MPs that had been pretreated with anti-CX3CL1 antibody (+apo-MPs + anti-CX3CL1; black bar), respectively, prior to being added to (A) the upper insert of the transmigration system for transmigration assay or (B) the human umbilical vein endothelial cells-coated plates for adhesion assay. (A) In the transmigration system, the apoptotic ATRA-NB4 cells were added into the lower plates which were subsequently assembled with the upper plates as mentioned above (see Methods in main text). The results are expressed as (A) migration index and (B) adhesion index indicating a fold increase relative to the number of vehicle-treated ATRA-NB4 cells either transmigrating toward UV-untreated ATRA-NB4 cells or adhesive to human umbilical vein endothelial cells, respectively. The results are the means ± standard deviation from five independent experiments. NS = no significance.

to engulf apoptotic cells for phagocytosis during the resolution phase of inflammation, suggesting that the chemoattraction of living ATRA-NB4 cells toward apoptotic cells may result in the phagocytic clearance of apoptotic cells and prevent them from releasing toxic molecules to cause further tissue damage. However, this warrants further study. Taken together, we hypothesized that the CX3CL1 released by apoptotic ATRA-APL cells plays a crucial role in the chemoattraction of living ATRA-APL cells toward apoptotic cells for subsequent phagocytosis during the resolution phase of DS.

Release of MPs is a well-known characteristic of cells undergoing apoptosis; these MPs are involved in the chemoattraction of mononuclear phagocytes to apoptotic cells, in which CX3CL1 mediates the chemoattractive property of these MPs.[21] Consistent with this, we have demonstrated that the MPs released by apoptotic ATRA-NB4 cells were also able to enhance the living ATRA-NB4 cells in their transmigration toward apoptotic cells and in their adhesion to endothelial cells (Fig. 4). As an intercellular adhesion molecule, CX3CL1 on the surface of MPs is likely to promote the binding of MPs to recipient ATRA-NB4 cells via the CX3CL1-CX3CR1 axis, resulting in a more efficient release of a larger amount of CX3CL1 from such MPs to the surface of living ATRA-NB4 cells and building up a chemotactic gradient in navigating living ATRA-NB4 cells toward apoptotic ATRA-NB4 cells. To support this notion, Mack et al[22] have indicated that the presence of adhesion molecules on MPs is able to enhance the targeted cell binding and cell−cell interaction.

Recent studies have reported that IL-8, TNF-α, and TGF-β1 are able to enhance the transmigration of neutrophils.[23,24] We have also reported that treating APL cells with ATRA can induce APL cells into the granulocytic differentiation process as well as releasing many chemokines, such as IL-8, TNF-α, and monocyte chemotactic protein-1, which thereafter contribute to the functional activation of differentiated APL cells in both migration and adhesion activity.[8−10] In this study, we also demonstrated that the level of IL-8, TNF-α, and TGF-β1 in the CM of apoptotic ATRA-NB4 cell cultures was not increased after induction of apoptosis by UV irradiation, suggesting that these mediators are not likely to be involved in the cell−cell interaction between living and apoptotic ATRA-NB4 cells.

We conclude that CX3CL1, in either free or MP form, is released by apoptotic ATRA-APL cells to mediate the chemoattraction of living ATRA-APL cells toward apoptotic cells for subsequent phagocytic clearance. Our work provides a rationale for further clinical studies aimed at defining the pathophysiological role of the CX3CL1-CX3CR1 axis in both DS and acute inflammation caused by other etiologies. In addition, CX3CL1 seems to be a promising target for a therapeutic regimen in treating acute inflammation, which also supports the need for further clinical studies.

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