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Functional expression of P2X family receptors in macrophages is affected by microenvironment in mouse T cell acute lymphoblastic leukemia

Shayan Chen [a], Wenli Feng [a], Xiao Yang [a], Wanzhu Yang [a], Yongxin Ru [a], Jinfeng Liao [a], Lina Wang [a], Yongmin Lin [a], Qian Ren [a], Guoguang Zheng [a,b,*]

[a] State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin 300020, China
[b] Center for Stem Cell Medicine, Chinese Academy of Medical Sciences, Beijing 100730, China

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ABSTRACT

Nucleotides are important players in intercellular signaling communication network. P2X family receptors (P2XRs) are ATP-gated plasma membrane ion channels with diverse biological functions. Macrophages are important components in the microenvironment of hematopoiesis participating in both physiological and pathological processes. However, the role of P2XRs in macrophages in leukemia has not been established. Here we investigated expression pattern and functions of P2XRs in macrophages from bone marrow (BM) and spleen of Notch1-induced T-ALL mice. Real-time PCR showed that P2XRs except P2X5R were expressed in BM and spleen macrophages. Furthermore, with the development of leukemia, the expression of P2X7R increased in both BM and spleen macrophages whereas expression of P2X1R increased in spleen macrophages. Live cell imaging recoding the Ca^{2+} response demonstrated that P2X7R expressed in macrophages was functional. TUNEL and electron microscopy analysis found that apoptotic macrophages were frequently observed in BM and spleen at late stage of leukemia, which was partly contributed by the activation of overexpressed P2X7R. Our results suggested that the intercellular communication mediated by nucleotides might orchestrate in the pathological process of leukemia and could be a potential target for the treatment of leukemia.

1. Introduction

Nucleotides, as a ubiquitous family of extra cellular signaling molecules, exert different effects through the interaction with P2 receptors, which are classified into P2X and P2Y families [1]. P2X receptors (P2XRs) are a family of ionotropic ATP-gated plasma membrane ion channels, which form non-selective cationic channels opening imparting significant permeability to Ca^{2+} upon stimulation [2]. P2XRs including seven distinct subtypes (P2X1R–P2X7R) are widely distributed with various functions including flux of Ca^{2+} and Na^{+} [3], plasma membrane blebbing [4], rapid release of interleukin-1 [5], mediating micro vesicle shedding and cell fusion [6], cell proliferation [7], cell death [8], and bone formation/resorption [9].

Macrophages are not only an essential cellular component of the host defense system [10] but also important components in the hematopoietic microenvironment and are regarded as a kind of niche for hematopoietic stem cells [11]. Meanwhile macrophages have remarkable plasticity and are educated by specific microenvironment. The expression of P2XRs by macrophages seems to be organ-dependent. P2X4R and P2X7R were found to be expressed in most macrophages [12] while P2X1R, P2X3R and P2X5R were also detected in mouse alveolar macrophages [13] and P2X1R was reported in resident peritoneal macrophages [14]. Moreover, it was reported that P2X7R was highly expressed in macrophages and microglia [15].

The roles of P2XRs in macrophages are mainly focused on P2X7R since it participates in macrophages-mediated physiological and pathological processes. Activation of P2X7R facilitates the secretion of lysosomes in monocytes and macrophages, which was known as a secretion pathway for IL-1β [5]. In mouse bone marrow-derived macrophages (BMDMs) ATP increased cellular tissue factor activity and the release of microparticles in a P2X7-dependent manner [16]. Study with P2X7R knockout mice demonstrated that P2X7R mediated killing of intracellular parasite and *Toxoplasma gondii* by

* Corresponding author at: State Key Laboratory of Experimental Hematology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin 300020, China. Fax: +86 22 23909402.
E-mail address: zhengggtjchn@aliyun.com (G. Zheng).

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human and murine macrophages [17]. In tuberculosis (TB) infection in humans, P2X7R gene with the 1513C allele was a possible risk factor for human susceptibility to TB [18]. In inflammation, the activation of P2X7R was a powerful event in the regulation of the caspase-1 inflammasome [19]. In immune defense, P2X7R function had been linked to the ability of macrophages to handle pathogens. For example, activation of P2X7R was shown to induce the killing of intracellular mycobacteria in macrophages, which might be correlated with the promotion of phagosome–lysosome fusion [20]. P2X7R was required for manifestations of liver injury in acetaminophen-induced hepatotoxicity [21].

The activation of P2X7R induced shedding of microvesicles from plasma membrane and the formation of membrane pores permeable to large molecules, which were possibly responsible for ATP-dependent cell death. However, the molecular mechanisms leading to P2X7R-induced cell death were not yet fully understood. It was reported that P2X7R activation induced a rapid receptor dependent cytolysis [22] or a delayed apoptosis [8] whereas evidence also implicated P2X7 as a survival/growth-promoting receptor [7].

Though increased expression of P2X7R was found in macrophages in response to infection of parasites [23], the expression and function of P2XRs in macrophages under pathological conditions has not been fully elucidated. Yet, little is known about the expression and function of P2XRs in macrophages in leukemia. In this paper, we analyzed the expression of P2XRs, P2X7R-mediated calcium response and apoptosis of bone marrow (BM) and spleen macrophages with the development of leukemia using a Notch1-induced murine T-cell acute lymphoblastic leukemia (T-ALL) model. We found that macrophages expressed P2XRs except P2X5R. Furthermore, increased expression and elevated calcium response of P2X7R were detected in macrophages at late stage of leukemia. Moreover, more apoptotic macrophages were observed at late stage of leukemia. Thus, we provided the first systemic study of P2XRs in macrophages in hematopoietic malignancies, which will help the better understanding of leukemia and finding of potential therapeutic targets.

2. Materials and methods

2.1. Mice

C57BL/6J mice were provided by the SPF-certified Animal Centre of the Institute of Hematology & Blood Diseases Hospital, CAMS & PUMC. Six to eight wks old male mice were used and maintained in sterile squirrel cages in a positive pressure room in the same center. The procedures for the animal experiments involved in this study were approved by the Animal Care and Use Committee at the institutions.

2.2. Notch1 induced T-ALL mouse model

Notch1-induced mouse leukemia model was established and described previously [24,25] and a non-irradiated Notch1-induced mouse leukemia model was used in this study. In brief, GFP^+ leukemia cells were isolated from BM or spleen of leukemia mice and transplanted into mice without irradiation. Based on the preliminary experiment, tail vein injection of 1×10^6 leukemia cells were performed to make sure all mice suffered leukemia with a survival time of 35–40 days. The mice were sacrificed by cervical dislocation at the indicated time points. The BM and spleen were collected for further analysis.

2.3. Isolation of macrophages

BM cells were obtained by flushing femurs and tibias, and suspended in PBE. Spleen cells were harvested by milling spleen and suspended in PBE. Red cells were removed using ammonium chloride lysing buffer. For gene expression analysis, macrophages were sorted by flow cytometry. For P2X7 receptor-mediated calcium response analysis, cells were directly cultured in RPMI 1640 supplemented with 20% FBS at 37 °C in a CO_2 incubator for 2 h. Then, non adherent cells were removed by washing three times.

2.4. Flow cytometric analysis and cell sorting

Cells were stained with a combination of antibodies including antibodies against mouse F4/80 (APC conjugated, BM8), Gr-1(PerCP-Cy5.5 conjugated, MR5D3) and CD115 (PE conjugated, AFS98), which were purchased from BioLegend. For cell apoptosis analysis, cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained following the instruction of in situ cell death detection kit (TUNEL technology, FITC labeled, Roche). Finally, cells were washed and resuspended in PBE containing 0.5% FBS and subjected to FACS analysis or cell sorting. An LSR II flow cytometer (BD Biosciences) was used for FACS analysis and a FACS Aria III (BD Biosciences) was used for cell sorting. Standard protocols were followed for all experiments and Flow Jo software (version 7.6.1) was also used for data analysis. Macrophages were gated or sorted as the $SSC^{int/lo}$ sub-population in the $Gr\text{-}1^{lo}F4/80^+CD115^{int}$ population [26] and apoptotic macrophages were gated as the $FITC^+$ macrophages sub-population.

2.5. Real-time polymerase chain reaction (PCR) analysis

Total RNA was extracted from macrophage using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and reverse transcribed using the

Table 1
Primers used in real-time PCR.

Genes	Primer sequences	Product (bp)	GenBank accession
GAPDH	Forward 5′-CACTTGAAGGGTGGAGC-3′ Reverse 5′-GGGCTAAGCAGTTGGTG-3′	145	NM_008084
P2X1	Forward 5′-GACAAACCGTCGTCACCTCT-3′ Reverse 5′-ATCCCAGAGCCGATGGTAGT-3′	114	NM_008771
P2X2	Forward 5′-ATCATCAATCTGGCCACTGCT-3′ Reverse 5′-CAAGGGTCACAGGCCATCTA-3′	163	NM_153400
P2X3	Forward 5′-CGTGAACTACAGCTCTGTCCG-3′ Reverse 5′-TGAAGAGAGGGAAACGGATGC-3′	143	NM_145526
P2X4	Forward 5′-GTGACGTCATAGTCCTCTACTGT-3′ Reverse 5′-TGCTCGTAGTCTTCCACATACTT-3′	82	NM_011026
P2X5	Forward 5′-GCAGCTCACCATCCTGTTGTA-3′ Reverse 5′-AGAGTCGTTCCCCAAGCATC-3′	149	NM_033321.3
P2X6	Forward 5′-CTCATCCCTACGGCCATCAC-3′ Reverse 5′-AGTCGTCTTTGGGGCTCTTG-3′	150	NM_011028
P2X7	Forward 5′-GGTGGGGTGACGAAGTTAGG-3′ Reverse 5′-ATACTCAGGACACAGCGTCT-3′	135	NM_011027

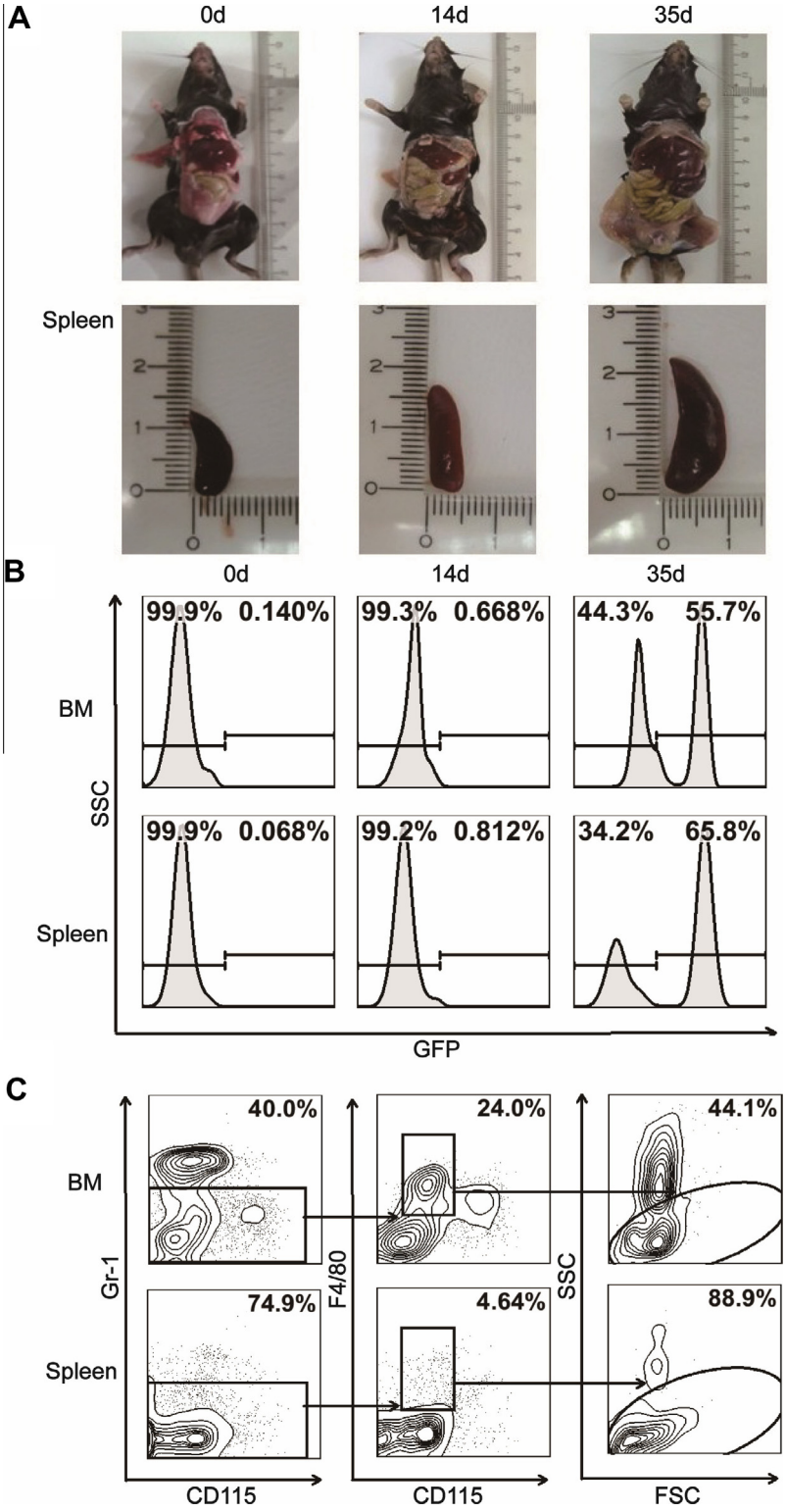

Fig. 1. Isolation of macrophages from BM and spleen of Notch1-induced T-ALL mice. (A) Mice were sacrificed on day 0, 14 and 35 and the size of mice is shown. (B) GFP^+ leukemia cells in BM and spleen were monitored at the indicated time points by flow cytometry analysis. (C) Macrophages from BM and spleen of mice were sorted as the $SSC^{int/lo}$ sub-population in the $Gr\text{-}1^{lo}F4/80^+CD115^{int}$ population by three-color flow cytometry analysis.

Super Script First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) following the manufacturers' protocols. The expression level of target genes was analyzed by the RQ value calculated through ΔΔCt method [$\Delta\Delta Ct = (Ct_{TARGET} - Ct_{GAPDH})_{sample} - (Ct_{TARGET} - Ct_{GAPDH})_{calibrator}$]. The primer sequences of all genes were listed in Table 1.

2.6. Analysis of P2X7 receptor-mediated calcium response

All procedures in this part were kept out of the light. The adherent macrophages in dishes were washed with Lock's solution and loaded with Flura 3/AM (Sigma) at a concentration of 1 mM for 30 min. Then, cells were washed and kept in 200 μl Lock's solution

each dish. For blocking experiments, cells were pretreated with 1 mM 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62, Sigma) for 1 h. The fluorescence of cells were recorded by UltraVIEW VoX 3D Live Cell Imaging System (PerkinElmer) every 2 s. 2′-3′-O-(4-benzoyl) benzoyl-ATP (BzATP, Sigma) was added to the final concentration of 100 μM. At the end of each measurement, Triton X-100 was added to obtain maximal fluorescence and then excess EGTA was added to obtain minimal fluorescence. The integrated optical density (IOD) of macrophages was further analyzed by the software Volocity (PerkinElmer).

2.7. Transmission electron microscopy analysis

Femurs and spleens were fixed in 6% glutaraldehyde in PBS (pH 7.4) for 1 h. BM tissues were taken out from femurs, washed in PBS, post-fixed in 1% osmium tetroxide. Then, tissues were dehydrated in graded alcohol, and embedded in Epon 812. The ultrathin sections were stained with uranyl acetate and lead citrate and finally examined with a Hitachi electron microscope (H-600).

2.8. Statistical analysis

The results were expressed with means ± SD. Analysis was done using the SPSS 16.0 software package (SPSS, Chicago, IL, USA) or Prism (GraphPad Software Inc, La Jolla, CA, USA). Statistical significance was accepted when the *P* values were less than 0.05.

3. Results

3.1. Analysis of macrophages in BM and spleen from the Notch1-induced T-ALL mice

Over-expression of intracellular domain of Notch1 (ICN1) in hematopoietic stem cells causes acute T lymphoblastic leukemia (T-ALL) in murine [25] and transplantation of 1×10^6 leukemia cells to non-irradiated mice causes mice to suffer the same type of leukemia and died within 40 days. Mice were sacrificed and splenomegaly were observed (Fig. 1A). The steady increase of GFP^+ leukemia cell population in BM and spleen (Fig. 1B) were also observed. Flow cytometry analysis was used to detect the distribution of macrophages in BM and spleen, and macrophages were defined as $SSC^{int/lo}$ sub-population in the Gr-1^{lo}F4/80^+CD115^{int} population. The typical flow cytometry analysis is shown in Fig. 1C.

3.2. Dynamic gene expression of P2XRs in macrophages

Macrophages were isolated from BM and spleen of control mice or mice at middle (14 days) and late (35 days) stages of leukemia and the expression of P2XRs were detected by real time RT-PCR. Macrophages expressed P2XRs except P2X5R. Furthermore, with the development of leukemia, up-regulation of P2X1R and P2X7R expression was observed in spleen macrophages, while up-regulation of P2X7R expression was observed in BM macrophages. Moreover, spleen macrophages from leukemic mice expressed higher level of P2X1R and P2X7R than BM macrophages (Fig. 2).

3.3. P2X7R-mediated Ca^{2+} response in macrophages

P2X7R-mediated calcium responses in macrophages on day 0, 14 and 35 were recorded by live cell imaging upon stimulation with 100 μM P2X7R complete and specific agonist BzATP. The fluorescence of macrophages increased to peak within several seconds after the administration of BzATP and then decreased gradually. The typical states of macrophages at quiescence, peak fluorescence and reduced fluorescence are shown in upper panel of Fig. 3A. Moreover, when macrophages were pretreated with P2X7R antagonist KN-62, no obvious fluorescence change could be observed upon stimulation with BzATP (lower panel of Fig. 3A). These results suggested that the calcium response was P2X7R mediated. To show the whole process, the fluorescence of macrophages was analyzed by the software Volocity and plotted (Fig. 3B and C). The results show that much higher increase in fluorescence is observed in macrophages from day 35 leukemic mice than that from control mice or day 14 leukemic mice, which is in accordance with our observation that macrophages express higher level of P2X7R at late stage of leukemia.

3.4. Apoptosis of macrophages

Apoptosis is one of responses triggered by P2X7R. To detect apoptotic macrophages, macrophages were detected by in situ cell

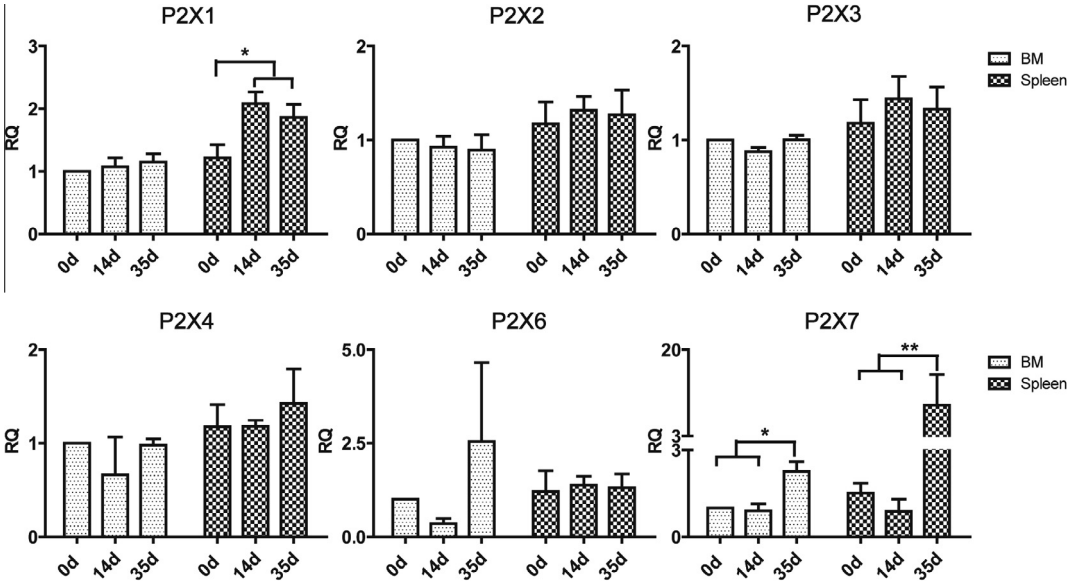

Fig. 2. Dynamic gene expression of P2X family receptors in macrophages from BM and spleen during the development of leukemia. Mice were sacrificed at the indicated time points and macrophages from BM and spleen were sorted by flow cytometry. The gene expression of P2X family receptors was detected by real-time RT-PCR from 3 independent experiments. All data represent the means ± SD. ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

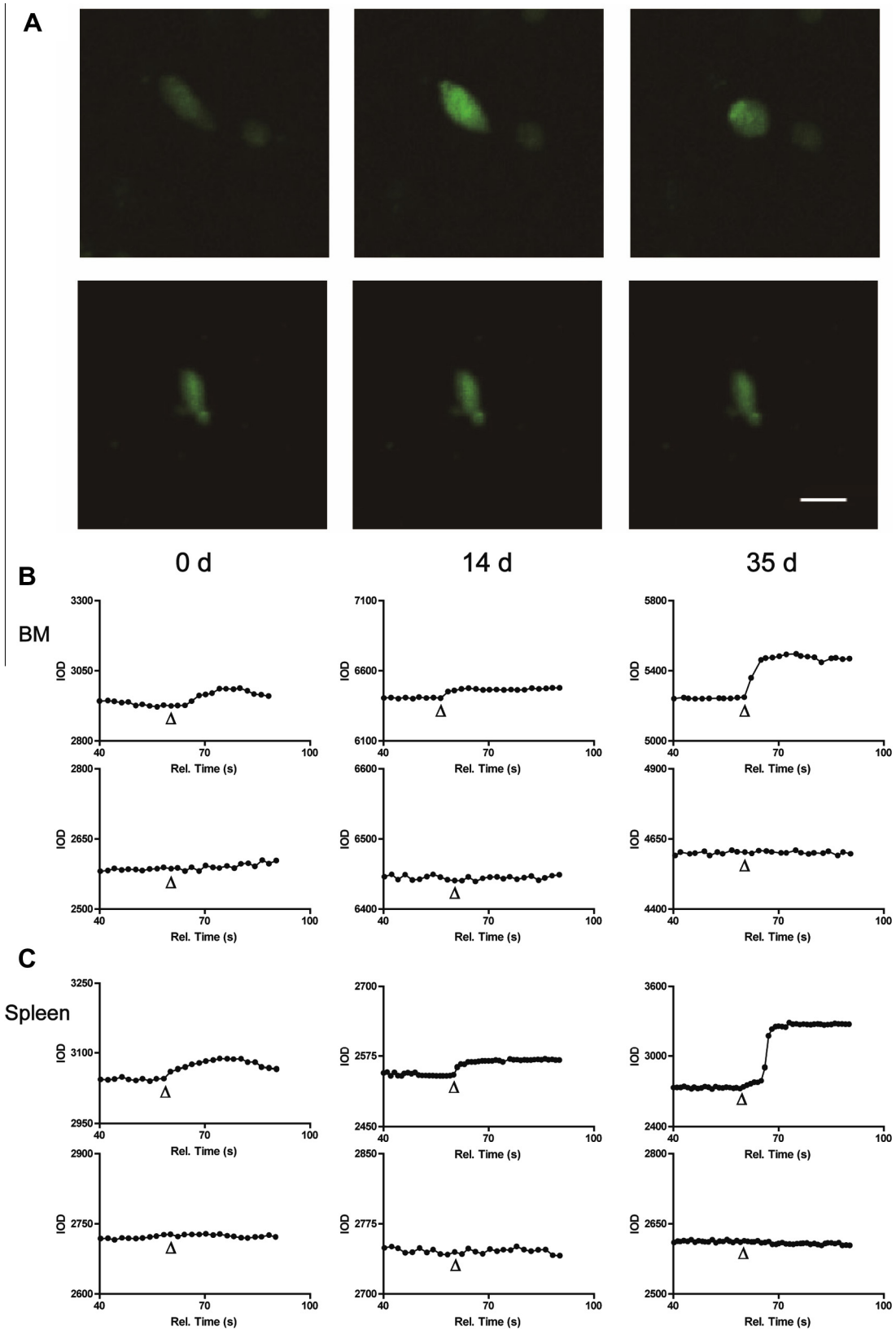

Fig. 3. P2X7 receptor-mediated intracellular Ca^{2+} change in macrophages from BM and spleen. Macrophages were isolated from BM (B) or spleen (C) of mice on day 0, day 14, and day 35 before loaded with Flura 3/AM. The intracellular free Ca^{2+} upon stimulation with BzATP without or with the pretreatment of KN-62 was recorded by confocal microscopy. (A) The typical images of macrophages upon stimulation. Upper: without KN-62; lower: with KN-62. Left: before stimulation; middle: 5 s after stimulation; right: 100 s after stimulation. A scale bar of 15 μm was indicated. (B–D) The real time intracellular free Ca^{2+} upon stimulation with 100 μM BzATP at the indicated time point (△) was analyzed by the software Volocity Demo and ploted. Upper: without KN-62; lower: pretreated with KN-62.

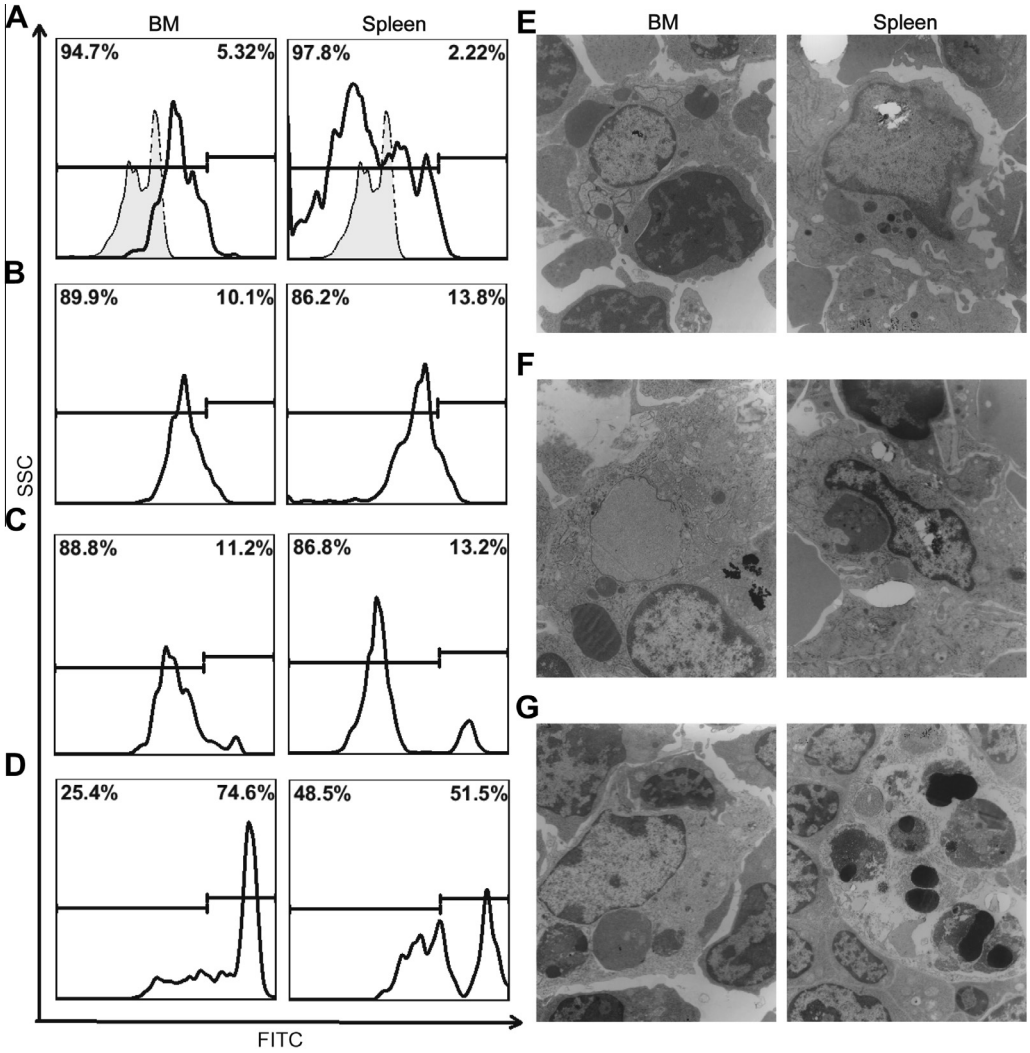

Fig. 4. Apoptosis of macrophages in BM and spleen. Single cell suspension was obtained from the BM (left) or spleen (right) of control mice on day 0 (A and B) or leukemia mice on day 14 (C) and day 35 (D), respectively and the apoptotic macrophages were detected by flow cytometry after TUNEL staining. Apoptotic macrophages were gated as $FITC^+$ macrophages sub-population. (A) Negative control samples stained without TUNEL reagents (shadow) or with TUNEL reagents but not enzyme solution (full line) (B–D) Apoptotic macrophages on day 0, day14 and day 35, respectively. Sections of BM (left) or spleen (right) from mice on day 0 (E, BM and spleen ×5000), day 14 (F, BM ×8000, spleen ×6000) and day 35 (G, BM ×4000, spleen ×3500) were prepared, respectively, before detection by electron microscopy.

death detection kit, and apoptotic macrophages were defined as $FITC^+$ macrophages sub-population. Significant increase in apoptotic rate was observed in both BM and spleen macrophages on day 35 (Fig. 4C) whereas no significant increase was observed on day 14 (Fig. 4D), which coincided with the expression and calcium response of P2X7R during the development of leukemia. Then, the ultrastructure of macrophages from BM and spleen were observed by transmission electron microscopy. Normal morphological feature of macrophages with integrated structure, big lysosomes was observed in control samples on day 0 (Fig. 4E). On day 14, apoptotic macrophages were not frequently observed. However, macrophages with cell debris and with cytoplasm focal dissolution or necrosis could be observed (Fig. 4F). On day 35, apoptotic (or necrotic) macrophages were frequently observed (Fig. 4G).

4. Discussion

Macrophages are essential components of innate and adaptive immunity as well as the important components of the tumor microenvironments [27]. Macrophages have remarkable plasticity, and their function and phenotype are controlled by microenvironmental signals [10]. Nucleotides mediated intercellular communication has important impact on cell proliferation and function. However, their impact on macrophages has not been fully understood, especially under pathological conditions [28]. Macrophages are also important regulators in hematopoiesis and were considered as one of niche cells in the hematopoietic microenvironment [11]. In fact, P2XRs are expressed in hematopoietic cells including stem/progenitor cells suggesting the important role of P2XR-mediated cell signaling in hematopoiesis. Furthermore, abnormal expression and dysfunction of P2XRs, especially P2X7R was reported in hematopoietic malignancies [29–32]. In addition, macrophages were implicated to participate in the pathological process of hematopoietic malignancies [33]. However, the full expression spectrum of P2XRs and function of P2X7R in macrophages in leukemia have not been established. Here we explore the significance of nucleotides-mediated intercellular communication by studying the expression of P2XRs and function of P2X7R in macrophages in mouse T-ALL model.

The phenotype of macrophages is strongly affected by microenvironment and the gene expression spectrum in different microenvironment differs dramatically. For example, P2X1R, P2X3R, P2X4R, P2X5R and P2X7R were found in mouse alveolar macrophages [13] while only P2X1R, P2X4R and P2X7R were expressed

in resident peritoneal macrophages [14]. We found that BM and spleen macrophages expressed nearly all P2XRs except P2X5R, which is different from the spectrum of macrophages from other origin. Furthermore, the expression of P2XRs was affected by pathological microenvironment. Up-regulation of P2X4R expression in macrophages was found in autoimmune diseases in nerve system and was suggested as a marker for activated macrophages [34]. Increased expression of P2X7R was detected in macrophages after parasite infection [23]. Here we report that increased expression of P2X7R is observed in both BM and spleen macrophages at late stage of mouse T-ALL. Moreover, up-regulation of P2X1R expression is observed in spleen macrophages but not in BM macrophages. These observations suggest that macrophages play different roles under diverse pathological conditions.

As an ATP-gated cationic channel, exposure to natural ligand ATP or the more potent agonist BzATP renders P2X7R permeable to Na^+, K^+ and Ca^{2+}, while repeated or prolonged application of either agonist induces the formation of a cytolytic pore permeable to larger cations such as positively charged ethidium [35]. The prompt rise in intracellular free calcium concentration upon BzATP activation is a marker for functional P2X7R. In this paper, calcium response was monitored by live cell imaging. A significant increase in fluorescence could be observed upon stimulation with BzATP, which could be blocked by P2X7R specific antagonist KN-62 [36]. These results suggested that the calcium response was P2X7R-mediated and macrophages expressed functional P2X7R. Furthermore, higher fluorescence increase was observed in macrophages from late stage leukemia mice, which was in accordance with the observation that macrophages at late stage of leukemia expressed higher level of P2X7R.

P2X7R-mediated apoptosis is well studied [8] though the mechanism has not been fully understood and data also suggested P2X7R as a survival/growth-promoting receptor related to cell proliferation [7]. We have observed that at late stage of leukemia, the percentage and absolute number of macrophages decreased dramatically in both BM and spleen (data not shown). This phenomenon was due to the increase of apoptosis, since more apoptotic macrophages were detected in BM and spleen by flow cytometry and electron microscopy analysis. We have shown that macrophages at this stage expressed higher level of P2X7R. The massive destruction of tissue construction could be observed in BM and spleen at late stage of leukemia, which might provide high level of extracellular ATP concentration and cause repeated and prolonged activation of P2X7R leading to the formation of cytolytic pores on the membrane of macrophages. Hence, P2X7R-mediated apoptosis should be one of the mechanisms though multiple mechanisms are involved in this process.

In summary, macrophages in mouse BM and spleen showed unique expression pattern of P2XRs. With the development of leukemia, elevated expression of functional P2X7R was detected, which contributed to the massive apoptosis of macrophages at late stage of leukemia. The intercellular communication mediated by nucleotides might orchestrate in the pathological process of leukemia and could be a potential target for the treatment of leukemia.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

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