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Dendritic Cells Show Chemotaxis to Prokineticin 2 : Involvement in the Pathogenesis of Arthritis

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ABSTRACT

Prokineticin (PK) 2 contributes to the immune system and inflammatory diseases, but little is known about the PK2 system in dendritic cells. We examined factors that induce the expression of PK receptor (PKR) 1 and PKR2 and the effects of PK2 in cultured dendritic cells. We maintained bone-marrow derived dendritic cells in culture and stimulated them with lipopolysaccharide and various cytokines. We then evaluated the expression of PKR1 and PKR2. The cells were also stimulated with PK2 and evaluated for chemotaxis. Finally, *In situ* hybridization was performed to detect PKR2 expression in the joints of arthritis model mice. We found that stimulation with lipopolysaccharide, tumornecrosis factor-α, and interleukin (IL)-1β increased PKR2 expression in dendritic cells. Stimulation with IL-6 and transforming growth factor β increased PKR1 expression. Dendritic cells showed chemotaxis to PK2. *In situ* hybridization detected cells positive for CD11c, PKR2, and IL-6 in the synovial tissues of arthritis model mice. These results suggest that various cytokines mediate the PK2 system in dendritic cells and that the role of PK2 is chemotaxis. Cells positive for CD11c, PKR2, and IL-6 might be involved in the pathogenesis of arthritis. Therefore, PK2/PKR2 signaling will become a therapeutic target for rheumatoid arthritis.

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Key words：dendritic cell, prokineticin 2, chemotaxis, arthritis

INTRODUCTION

Prokineticin (PK) 2 is a secreted protein with 5 pairs of disulfide bonds[1]. This protein plays roles in diverse physiological functions, including circadian rhythms, appetite, and pain thresholds[2-5]. In addition, PK2 contributes to immune functions, such as hematopoiesis, chemotaxis, and cytokine production[6-9]. Specifically, PK2 increases neutrophil and monocyte colonies *in vitro*[6] and peripheral blood leukocyte counts in mice[6]. Chemotaxis also occurs in human neutrophils and mouse macrophages[7,8]. In mouse macrophages, PK2 increases interleukin (IL)-1 and IL-12, and decreases IL-10. In lymphocytes, PK2 increases IL-1β production and decreases IL-4 and IL-10 production.

The G protein-coupled receptors for PK2 are PK receptor (PKR) 1 and PKR2[10]. Both PKR1 and PKR2 are highly expressed in lymphocytes and neutrophils, and are also expressed in monocytes, dendritic cells, and natural killer cells[6]. The PK2-PKR1/2 signaling pathway has been implicated, via studies of animal models, in various inflammatory diseases, and suppression of this pathway has been shown to improve pathological conditions[11-17]. In humans, PK2 signaling is reported to be involved in psoriasis and rheumatoid arthritis[18-19]. Signals mediated by PKR1 and PKR2 have not been analyzed in a clearly differentiated manor in animal models or in patients with inflammatory diseases, but PKR1

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expression is predominant in immune cells and in inflammatory tissues, and the PK2/PKR1 signaling pathway is likely involved in inflammatory pathologic conditions[11-15,18]. Although PK2/PKR1 signaling likely suppresses the pathogenesis of arthritis, PK2/PKR2 signaling has, in contrast, been suggested to encourage the pathogenesis of arthritis and mouse models of arthritis. Specifically, in a mouse model of arthritis, we found that PKR2 is highly expressed in arthritic tissues, strongly correlated with the severity of arthritis, and expressed on macrophage-like cells in the pannus[16,17]. In addition, a study of synovial cells from patients with rheumatoid arthritis found that PKR1 expression is decreased and that PKR2 signaling is predominant, resulting in sustained production of IL-6[19].

Identifying cells involved via PK2/PKR2 signaling in the pathogenesis of arthritis might create new therapeutic targets for rheumatoid arthritis and other arthritic diseases. Studies of PK2 have found effects on neutrophils, macrophages, and lymphocytes[6-9], but have shown proinflammatory effects mediated by PK2/PKR1 signaling, which may not explain the pathogenesis mediated by PK2/PKR2 signaling in arthritis. On the other hand, although PKR1 and PKR2 expression has been detected in dendritic cells, little functional analysis has been performed[6,13].

Therefore, in the present study, to determine whether inflammatory stimuli, believed to be involved in the functional differentiation of dendritic cells, induce PKR1 and PKR2 expression and whether PKR2-expressing dendritic cells can be induced under arthritic conditions, we used cultured dendritic cells of mice.

At the same time, we tried to figure out in which phases PKR1 and PKR2 expression are induced. Furthermore, to investigate the effect of PK2 on dendritic cells, we examined whether PK2 shows chemotaxis and induces the production of IL-6, a cytokine involved in the pathogenesis of rheumatoid arthritis. Finally, to investigate whether macrophage-like cells expressing PKR2 in the synovial tissue of a mouse model of arthritis[17]are proinflammatory dendritic cells, we examined whether the dendritic cell marker CD11c co-localizes with PKR2 and IL-6.

METHODS

Animals

We purchased 8 to 10-week-old C57BL/6J mice from Oriental Yeast Co. Ltd. (Tokyo, Japan). All procedures for animal experiments were approved by the Animal Experiment Committee of The Jikei University School of Medicine (approval numbers 2017-35, 2016-36, 2016-095, and 2015-86).

Culture of bone-marrow derived dendritic cells and stimulation with various factors

Bone marrow-derived dendritic cells were induced with a method previously described[20]. Briefly, bone marrow cells were harvested from the femurs and tibias of the 8 to 10-week-old C57BL/6J mice. Recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF ; Peprotech, Inc., Rocky Hill, NJ, USA) at 20 ng/ml was added to 10 ml of RPMI 1640 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and the cells were cultured in this medium in 10-cm culture dishes for 10 days. The day of culture initiation was set as day 0. On days 6 and 8, the 10 ml of medium was aspirated and replaced with 10 ml of a fresh RPMI 1640 medium containing GM-CSF. On day 10, floating cells and weakly adherent cells were used as bone-marrow derived dendritic cells.

The bone marrow-derived dendritic cells were seeded in 24-well plates at 1×10^6 cells/well and subjected to stimulation with the following factors : lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA) at 100 ng/ml, TNF-α (Peprotech) at 10 ng/ml, IL-1β (Peprotech) at 10 ng/ml, interferon (IFN)-γ (Peprotech) at 10 ng/ml, IL-6 (R&D Systems, Inc., Minneapolis, MN, USA) at 10 ng/ml, IL-6 receptor α (IL-6Rα) (R&D Systems) at 100 ng/ml, and TGF-β (R&D Systems) at 5 ng/ml. Each stimulation experiment was performed 3 times (in 3 mice) to confirm reproducibility. No mice were excluded.

Real-time polymerase chain reaction

We extracted messenger (m) RNA from bone marrow derived dendritic cells with an RNeasy Mini Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's protocol. The extraction of mRNA from joint tissues was performed as previously described[17]. All mRNAs were reverse-transcribed with a High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's protocol.

We used an Applied Biosystems StepOne Plus Real-Time PCR System (Thermo Fisher Scientific) for the real-

time polymerase chain reaction. The primers and probes used were from the Taqman gene expression assay : PK2 (Mm00450080_m1), PKR1 (Mm01204733_m1), PKR2 (Mm00769571_m1), IL-6 (Mm00446190_m1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm99999915_g1), and β-actin (Mm00607939_s1). The mRNA expression levels were quantified with the ΔΔCT (delta delta climate threshold) method. As endogenous controls, β-actin was used in the joint tissue experiments and GAPDH was used in the bone marrow-derived dendritic cell experiments.

Western blotting

Bone marrow-derived dendritic cells were lysed in 100 mM Tris-HCl-buffered saline containing 1 mM EDTA, 1 mM EGTA(ethyleneglycol bis-(β-aminoethylether) N,N,N',N'-tetraacetic acid), 2 mM orthovanadate, 1 mM NaF, 2.5 mM sodium pyrophosphate, 1% Triton, 0.1% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.5% deoxycholic acid. The protein concentration in the lysate was measured with a bicinchoninic acid protein assay (Thermo Fisher Scientific) and adjusted to 500 μg/ml. The sample was mixed with 6 μl of 6× sample buffer (0.5 M Tris containing 12% sodium dodecyl sulfate and 6% 2-mercaptoethanol) and incubated at 100°C for 5 minutes. Next, 8 μl of the mixture was subjected to polyacrylamide gel electrophoresis with 10% polyacrylamide gel. Electrophoresis was performed at 120 V for 90 minutes. Subsequent transfer to a nitrocellulose membrane was performed at 15 V for 30 minutes. The transferred membrane was incubated with blocking buffer (Tris-buffered saline containing 0.1% Tween-20 and 5% skim milk), followed by incubation with primary and secondary antibodies diluted in blocking buffer. The primary antibodies used were anti-PKR2 (sc-365696 ; Santa Cruz Biotechnology, Inc., Santa Cruz, TX, USA) and anti-GAPDH (5174 ; Cell Signaling Technology, Beverly, MA, USA) ; the secondary antibodies were horseradish peroxidase-conjugated anti-mouse (7076S ; Cell Signaling Technology) and anti-rabbit (7074S ; Cell Signaling Technology) antibodies, respectively. The signals on the membrane were detected with chemiluminescence with electrochemiluminescence reagents (Bio-Rad Laboratories, Hercules, CA, USA), and images were captured with a ChemiDoc imaging system (Bio-Rad Laboratories).

Chemotaxis

Chemotaxis was evaluated with a 48-well Boyden chamber (Neuro Probe, Inc., Gaithersburg, MD, USA) as previously described[21,22]. Bone marrow-derived dendritic cells were dispersed in magnesium- and calcium-free phosphate-buffered saline (PBS) and the cell suspension was applied to the upper chamber. The PK2 (Peprotech) was diluted in magnesium- and calcium-free PBS to 10^(-7)-10^(-17) mol/l and applied to the lower chamber. Magnesium- and calcium-free PBS was used as a negative control. The pore size of the membrane was 5 μm. The chamber incubated at 37°C for 2 hours. Subsequently, the membrane was fixed with methanol and stained with Diff-Quik (Sysmex Corp., Kobe, Japan) in accordance with the manufacturer's protocol. The stained membrane was enclosed in a slide glass with the mounting medium Permount (Falma, Co., Ltd., Tokyo, Japan), and observed with an optical microscope (Axio Imager A1, Carl Zeiss, Oberkochen, Germany). Three representative fields of view were selected per well and the cell numbers were counted. The mean value was then calculated. The chemotaxis index was defined as the mean number of cells in the well divided by the mean number of cells in the negative control wells in the chamber. All observations were performed in a blinded manner. The experiment was performed 4 times (4 chambers).

Mouse model of arthritis

The 8 to 10-week-old C57BL/6J mice were used to create an anti-collagen antibody-induced model arthritis. Five mice were used for the experiment, considering the incidence of arthritis. No mice were excluded. An antibody cocktail (Chondrex, Inc., Woodinville, WA, USA) was administered at 5 mg/body via the tail vein of the mice (day 0). On day 3, 100 μg/body of LPS (Chondrex) was administered intraperitoneally. The joints were observed and the severity of the arthritis was evaluated. Each limb was scored as follows : 0, normal ; 1, erythematous swelling of 1 joint ; 2, localized erythematous swelling of 2 or more joints ; and 3, swelling of the entire limb. The total score of the 4 limbs was defined as the arthritis score for a mouse (maximum : 12 points).

In situ hybridization

Mice were refluxed with PBS under isoflurane anesthesia, and the joint tissues were harvested. The harvested

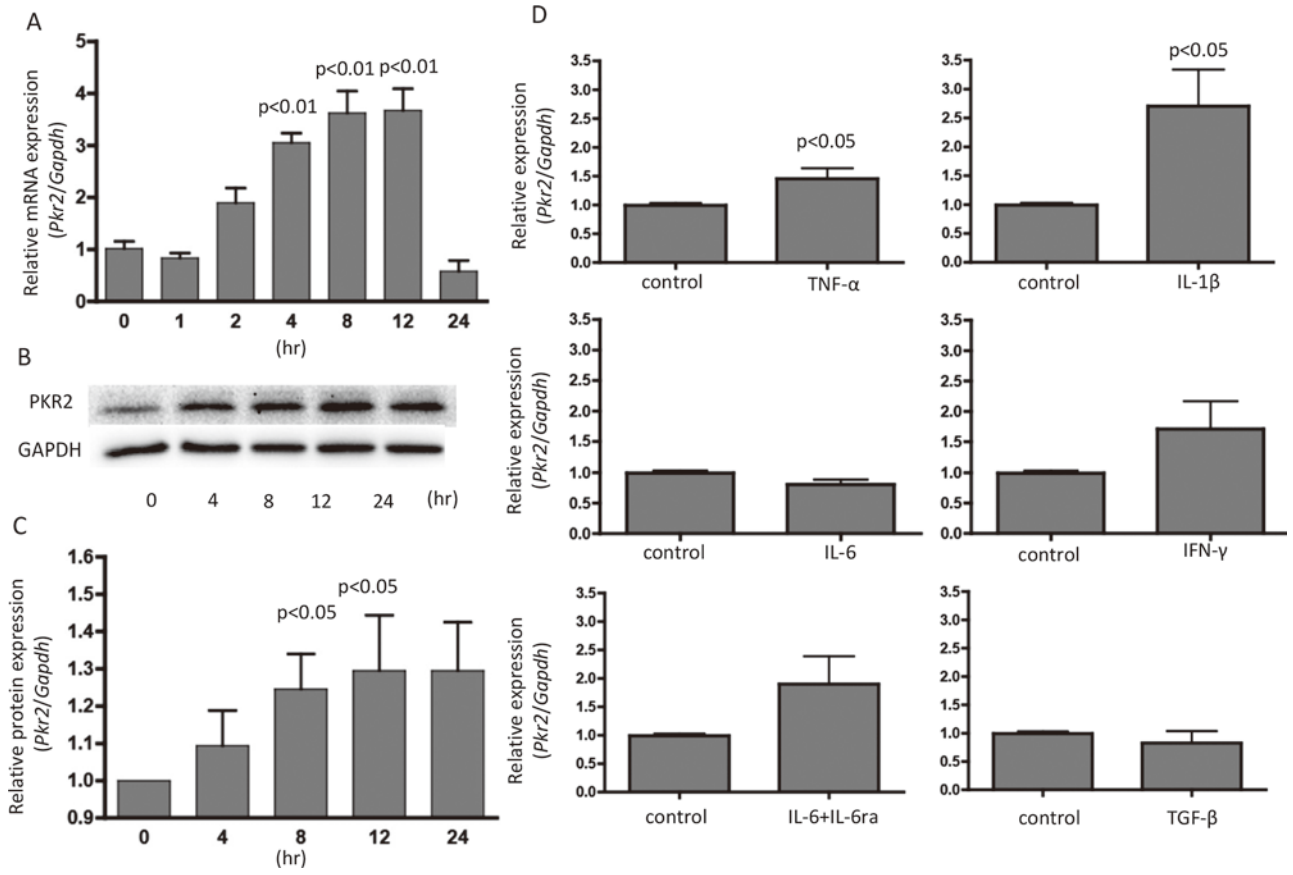

Fig. 1. Prokineticin receptor 2 (PKR2) expression in bone-marrow derived dendritic cells. (A) Time course of PKR2 messenger (m) RNA expression following stimulation with lipopolysaccharide (LPS). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is endogenous control in the bone-marrow derived dendritic cell experiments. One-way analysis of variance (ANOVA), Bonferroni correction. (B) Western blot analysis of the time course of PKR2 protein expression following stimulation with LPS. (C) Analysis of the band intensity in Western blot analysis with densitometry. One-way ANOVA, Bonferroni correction. (D) PKR2 mRNA expression following stimulation with various cytokines. P values by *t*-test. Data are shown as mean ± standard error of the mean. Abbreviations : TNF-α, tumor necrosis factor-α ; IL-6, interleukin 6 ; IL-6ra, IL-6 receptor α ; IL-1β, interleukin 1β ; IFN-γ : interferon γ ; TGF-β, transforming growth factor β

tissues were frozen in an optimal cutting temperature compound with liquid nitrogen, cut into 10-μm-thick sections, and subjected to staining. Staining was performed with an RNAscope Assay System (Advanced Cell Diagnostics, Newark, CA, USA) in accordance with the manufacturer's protocol, using probes for CD11c (catalog no. 311501-C3), PKR2 (catalog no. 527941-C2), and IL-6 (catalog no. 315891). A confocal microscope (LSM880 ; Carl Zeiss) was used for observation and image capture.

Statistical analysis

Statistical analyses were performed with the GraphPad Prism 4 software program (GraphPad Software, Inc., San Diego, CA, USA). One-way analysis of variance was used to analyze time series data and chemotaxis data. A *t*-test was used to compare two groups. The significance level was set at $p<0.05$.

RESULTS

Changes in PKR2 expression in bone-marrow derived dendritic cells following stimulation with various inflammatory factors

Stimulation of bone-marrow-derived dendritic cells with LPS significantly increased PKR2 mRNA expression at 4 to 12 hours (Fig. 1A). The PKR2 protein levels were also elevated at 12 to 24 hours (Fig. 1B, C). Expression of PKR2 was also significantly increased by stimulation with TNF-α and IL-1β (Fig. 1D). Stimulation with IL-6 + IL-6Rα and IFN-γ also increased PKR2, but not to a significant

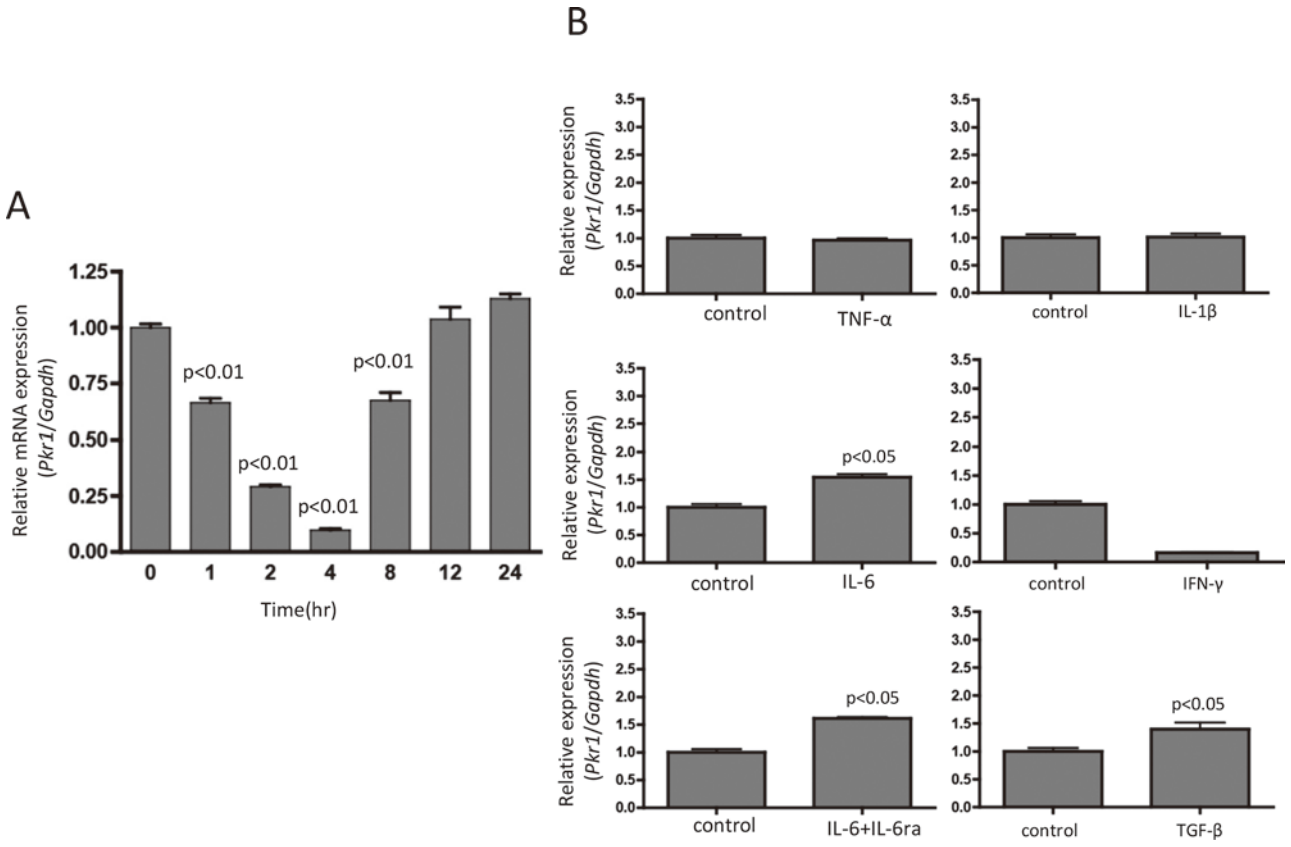

Fig. 2. Prokineticin receptor 1 (PKR1) messenger (m) RNA expression in bone- marrow derived dendritic cells. (A) Time course of prokineticin receptor 2 (PKR2) mRNA expression in dendritic cells following stimulation with lipopolysaccharide. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is endogenous control in the bone-marrow derived dendritic cell experiments. One-way analysis of variance , Bonferroni correction. (B) PKR1 mRNA expression in dendritic cells following stimulation with various cytokines. P values by *t*-test. Data are shown as mean ± standard error of the mean. Abbreviations : LPS, lipopolysaccharide ; GAPDH : glyceraldehyde-3-phosphate dehydrogenase ; TNF-α, tumor necrosis factor α ; IL-6, interleukin 6 ; IL-6ra : IL-6 receptor α ; IL-1β, interleukin 1β ; IFN-γ, interferon γ ; TGF-β, transforming growth factor-β

extent (Fig. 1D).

Changes in PKR1 expression in bone marrow-derived dendritic cells following stimulation with various factors involved n functional differentiation

After dendritic cells had been stimulated with LPS, PKR1 expression decreased, with a maximal effect at 4 hours (Fig. 2A). In contrast, after dendritic cells had been stimulated with IL-6 + IL-6Rα or with TGF-β, PKR1 expression was significantly increased when stimulated with IL-6 + IL-6Rα, and TGF-β (Fig. 2B).

Effect of PK2 on on chemotaxis and the production of cytokines and the effects on IL-6 production of PKR2-inducible factors, TNF-α and IL-1β in the presence or absence of PK2 in bone-marrow–derived dendritic cells

In response to PK2, bone-marrow derived dendritic cells showed chemotaxis (Fig. 3A).

Stimulation of dendritic cells with TNFα and IL-1β increased IL-6 expression (Fig. 3B) ; however, the presence of PK2 did not induce significant changes in IL-6 expression (Fig. 3B).

Localization of CD11c, PKR2, and IL6 in the synovial tissues of joints in an anticollagen-antibody-induced model of arthritis

In an anti-collagen antibody-induced model of arthritis, only PKR2 expression was significantly elevated (Fig. 4A).

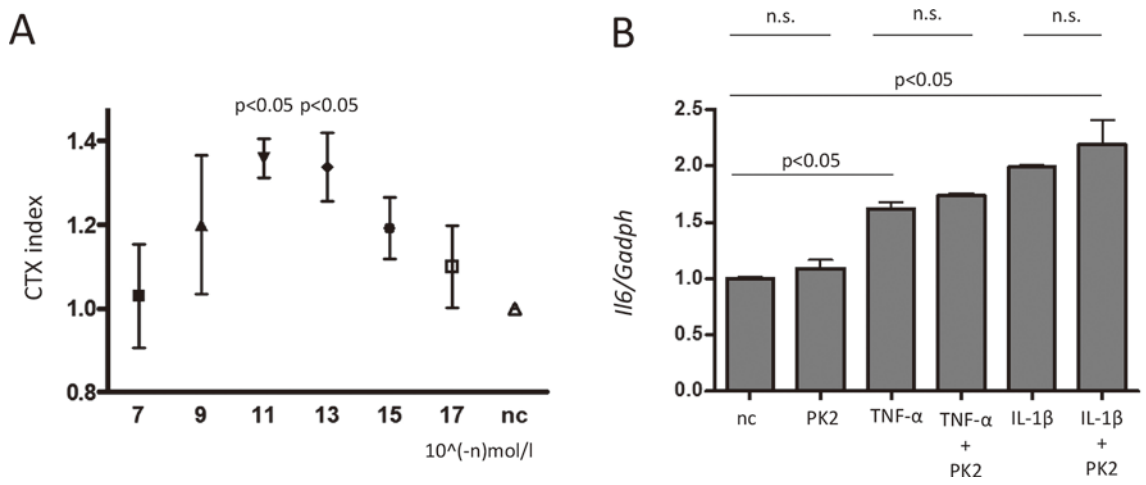

Fig. 3. Effect of prokineticin 2 (PK2) on dendritic cells. (A) Chemotaxis of dendritic cells to PK2. The chemotaxis (CTX) index was defined as the mean number of bone marrow-derived dendritic cells in the well divided by mean number of cells in the negative control wells in the chamber. One-way analysis of variance, Bonferroni correction. (B) Interleukin 6 (IL-6) messenger RNA expression in dendritic cells following stimulation with PK2, tumor necrosis factor α (TNF-α), and interleukin 1β (IL-1β). P values by *t*-test. Data are shown as mean ± standard error of the mean. Abbreviations : GAPDH, glyceraldehyde-3-phosphate dehydrogenase ; n.c., negative control ; n.s., : not significant.

Cells positive for PKR2, CD11c, and IL-6 were observed to be co-localized (Fig. 4B).

Discussion

Dendritic cells are functionally differentiated by various inflammatory stimuli and both induce inflammation[23-25] and suppress inflammation[26,27]. In the present study, PKR2 expression was significantly increased by LPS, TNF-α, and IL-1β (Fig. 1). Stimulation of dendritic cells with LPS increases the expression of major histocompatibility complex (MHC) class II and the proinflammatory cytokines TNF-α and IL-6[23-24]. In addition, TNF-α, like LPS, increases the expression of MHC class II and proinflammatory cytokines in dendritic cells. Furthermore, IL-1β promotes inflammatory cytokine production in dendritic cells[25]. The conditions under which PKR2 was induced in the present study were all inflammatory stimuli that induce inflammation-inducing dendritic cells (Fig. 1), suggesting that PKR2 induction occurs in inflammation-inducing dendritic cells. In fact, in the present study we showed that TNF-α and IL-1β, which are PKR2-inducing stimuli, induced IL-6 expression in dendritic cells (Fig. 3) ; this finding suggests that IL-6, a representative cytokine involved in the pathogenesis of arthritis, is induced under conditions in which PKR2 is induced in dendritic cells. Therefore, we have hypothesized that dendritic cells are involved in PK2/PKR2 signaling[16,17,19], which is important in the pathogenesis of arthritis.

In the present study, the expression of PKR1 by dendritic cells was decreased after they had been stimulated with LPS and increased after having been stimulated with IL-6 + IL-6Rα, or with TGF-β (Fig. 2). In previous studies, IL-6 has been found to suppress MHC class II expression in dendritic cells and to decrease their ability to activate T cells[26] and TGF-β has been found to induce permissogenic dendritic cells[27]. Thus, the conditions under which PKR1 was induced in the present study were all inflammatory stimuli that induce inflammation-suppressing dendritic cells (Fig. 2) ; therefore, PKR1 might be induced in inflammation-suppressing dendritic cells.

Thus, PKR1 and PKR2 may be induced in different inflammatory phases, and from the viewpoint of functional differentiation of dendritic cells, we believe that suppression of PK2/PKR1 signaling and dominance of PK2/PKR2 signaling[19] are important for the pathogenesis of arthritis.

The present study found that dendritic cells were chemotactic to PK2 (Fig. 3A). In previous studies, the role of PK2 was mainly chemotactic in neutrophils and macrophages[6-7], and the results for dendritic cells of the present study were consistent with earlier studies. These results suggest that the role of PK2/PKR2 signaling in the pathogenesis of arthritis is to localize proinflammatory dendritic

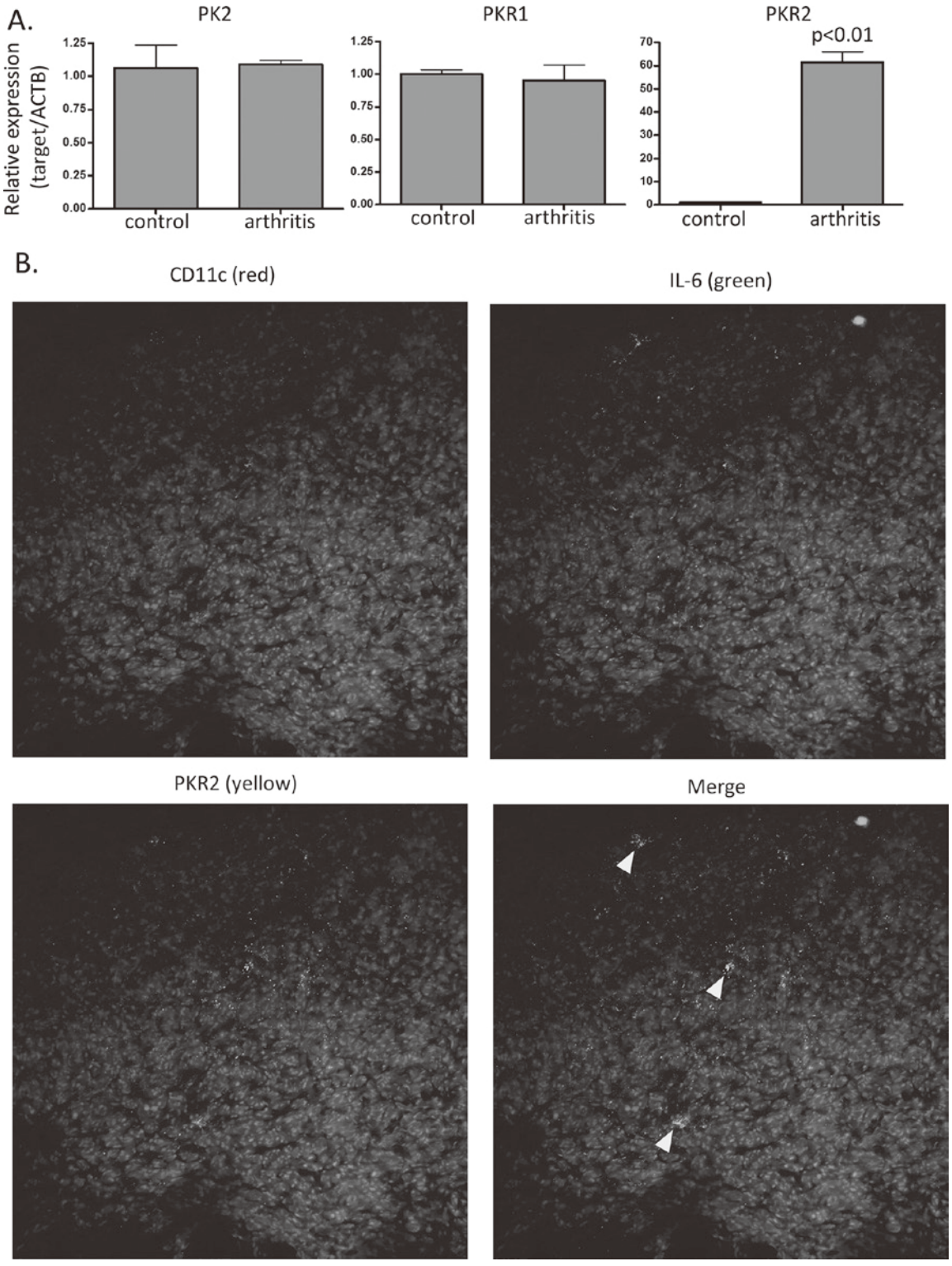

Fig. 4. Expression of prokineticin receptor 2 (PKR2) and localization of CD11c, PKR2, and interleukin 6 (IL-6) in the synovial tissues of anti-collagen antibody-induced arthritis model mice. (A) Relative expression of prokineticin 2 (PK2), prokineticin receptor 1 (PKR1), and PKR2 in joints. P values by *t*-test. Data are shown as mean ± standard error of the mean. (B) *In situ* hybridization of synovium tissues from the arthritis model mice for CD11c (red), PKR2 (yellow), IL-6 (green), and 4',6-diamidino-2-phenylindole (DAPI) (gray). The right lower panel shows a merged picture, with arrows indicating cells positive for CD11c, PKR2, and IL6 cells. Abbreviations : LPS : lipopolysaccharide ; ACTB : actin-beta.

cells to synovial tissues by chemotaxis. In fact, PKR2 is highly expressed in arthritic tissues of mice[16,17], suggesting that one of the factors contributing to this increased expression is the localization of proinflammatory dendritic cells to the pannus.

In a mouse models of arthritis, dendritic cells have been detected in the pannus and shown to be involved in the pathogenesis of arthritis[24,31-33]. These previous studies have shown that suppressing the ability of dendritic cells to produce cytokines such as IFN-γ, IL-6, IL-1β, and TNF-α decreases the severity of arthritis. These findings suggest that the presence of dendritic cells producing inflammatory cytokines is important for the pathogenesis of arthritis. In the present study, we found that dendritic cells both positive for PKR2 or for- IL-6 in the pannus of arthritis model mice (Fig. 4B). This result supports the possibility that inflammation-induced dendritic cells localize to the pannus via PK2/PKR2 signaling.

The present study had several possible limitations. The first is that the specific mechanism of PKR signaling changes by various inflammatory stimuli is unknown because intracellular signaling has not been inhibited. From this point of view, whether the changes in expression observed in this study were primary or secondary to inflammatory stimuli is unclear. This is an issue for further investigation. A second limitation is that we had not examined whether PK2 is actually involved in the localization of dendritic cells to the pannus *in vivo*. Therefore, in future studies, we would like to investigate PK2 and PKR2 antagonists by intra-articular administration.

CONCLUSIONS

The expression of PKR2 in dendritic cells might be induced by cytokines involved in rheumatoid arthritis. A function of PK2/PKR2 signaling in dendritic cells is chemotaxis, which might be involved in the localization of inflammation-induced dendritic cells to the pannus and might contribute to the pathogenesis of rheumatoid arthritis by PK2/PKR2 signaling. Therefore, we believe that our findings provide an important foundation for the future use of PK2/PKR2 signaling as a novel therapeutic target for rheumatoid arthritis.

Authors have no conflict of interest.

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