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ATP and its metabolite adenosine cooperatively upregulate the antigen-presenting molecules on dendritic cells leading to IFN-γ production by T cells

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Dendritic cells (DCs) present foreign antigens to T cells *via* the major histocompatibility complex (MHC), thereby inducing acquired immune responses. ATP accumulates at sites of inflammation or in tumor tissues, which triggers local inflammatory responses. However, it remains to be clarified how ATP modulates the functions of DCs. In this study, we investigated the effects of extracellular ATP on mouse bone marrow–derived dendritic cells (BMDCs) as well as the potential for subsequent T cell activation. We found that high concentrations of ATP (1 mM) upregulated the cell surface expression levels of MHC-I, MHC-II, and co-stimulatory molecules CD80 and CD86 but not those of co-inhibitory molecules PD-L1 and PD-L2 in BMDCs. Increased surface expression of MHC-I, MHC-II, CD80, and CD86 was inhibited by a pan-P2 receptor antagonist. In addition, the upregulation of MHC-I and MHC-II expression was inhibited by an adenosine P1 receptor antagonist and by inhibitors of CD39 and CD73, which metabolize ATP to adenosine. These results suggest that adenosine is required for the ATP-induced upregulation of MHC-I and MHC-II. In the mixed leukocyte reaction assay, ATP-stimulated BMDCs activated CD4 and CD8T cells and induced interferon-γ (IFN-γ) production by these T cells. Collectively, these results suggest that high concentrations of extracellular ATP upregulate the expression of antigen-presenting and co-stimulatory molecules but not that of co-inhibitory molecules in BMDCs. Cooperative stimulation of ATP and its metabolite adenosine was required for the upregulation of MHC-I and MHC-II. These ATP-stimulated BMDCs induced the activation of IFN-γ-producing T cells upon antigen presentation.

Dendritic cells (DCs) recognize and take up foreign antigens such as bacteria, viruses, and tumor cells and produce antigen-derived peptides to present to T cells *via* the major histocompatibility complex (MHC); this leads to the activation of antigen-specific acquired immune responses (1, 2). There are two types of MHC molecules: class I (MHC-I) and class II (MHC-II). MHC-I presents antigens derived from cytoplasmic proteins to CD8T cells. DCs can also present foreign antigens by MHC-I through the cross-presentation mechanism. Activated CD8T cells, also known as cytotoxic T lymphocytes (CTLs), directly kill virus-infected cells and tumor cells (3, 4). By contrast, MHC-II is specifically expressed on antigen-presenting cells and presents extracellular antigens to CD4T cells. Activated CD4T cells have several functions, such as inducing the activation of macrophages and promoting antibody production by B cells (5, 6). In addition to MHC molecules, co-stimulatory molecules such as CD80 and CD86 on the cell surface are required to activate T cells. In contrast, co-inhibitory molecules such as programmed PD-L1 and PD-L2 suppress T cell activation (7).

ATP, an energy transfer molecule, exists abundantly inside cells but is rarely found in the intercellular space in normal tissues. Currently, extracellular ATP has been recognized as a signaling molecule that induces signals *via* specific receptors, resulting in a variety of effects (8, 9). Tissue damage triggers local ATP release as a danger signal that spreads to the surrounding cells (10). In addition, several cells, such as macrophages and neutrophils, release ATP extracellularly in response to stimuli, affecting themselves or surrounding cells (10). ATP is released into the extracellular space from the cytosol through pannexin or connexin hemichannels or released from vesicles *via* exocytosis (9, 11)

ATP induces signals *via* P2 purinergic receptors, including P2X receptors, which are ligand-gated ion channel receptors (12), and P2Y receptors, which are G protein-coupled receptors (13). Extracellular ATP is known to act on several kinds of immune cells, such as macrophages, neutrophils, and DCs, to promote cell migration or induce an inflammatory response (9). As a result, extracellular ATP has been involved in inflammatory diseases such as neuroinflammatory diseases and rheumatoid arthritis (14, 15). ATP also plays a role in anticancer immunity. Tumor growth is promoted in P2X7-deficient mice *via* the suppression of immune cell chemotaxis, including the inhibition of DC recruitment to the tumor site (16, 17). ATP released from dead tumor cells activates immune cells and augments the anti-tumor immune response (18, 19). These findings indicate that ATP is critical for the activation of inflammation and tumor immunity.

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Extracellular ATP is metabolized to adenosine by ecto-nucleases—mostly CD39 and CD73—that remove the phosphate group, converting ATP to AMP and then to adenosine (14). This adenosine exerts its physiological effects by acting on P1 adenosine receptors (A1, A2a, A2b, and A3), which are G protein-coupled receptors (20). In contrast to ATP, adenosine is known for its ability to reduce inflammation and suppress immunological responses (15, 21). Therefore, it is believed that ATP and adenosine are crucial for controlling immunological responses.

Several studies have reported the effects of high concentrations of ATP. Since the EC_{50} of P2X7 for ATP is above 0.1 mM (22), relatively high concentrations of ATP are required to activate the P2X7. High concentrations of ATP (>1.5 mM) are required to induce IL-1β production *via* inflammasome activation in neutrophils (23). High concentrations of ATP (>1 mM) promote the killing of bacteria taken up *via* phagocytosis by macrophages (24, 25). ATP has different effects on CD4T cells in a concentration-dependent manner: at a low concentration (250 nM), ATP induces the proliferation of activated CD4T cells, whereas at a high concentration (1 mM), it enhances the function of regulatory T cells by acting on the P2X7 receptor (26). Using recently developed probes, high concentrations of extracellular ATP (∼1 mM) have been observed even under physiological conditions (27). High levels of ATP have been observed in tumor tissues and areas of inflammation; the local ATP concentration can exceed 1 mM (18, 28–30). Therefore, high concentrations of ATP may have a variety of impacts under normal and pathological circumstances.

DCs are activated by various external stimuli, and the type of stimulus determines the characteristics of the subsequently activated T cells. Toll-like receptor (TLR)-mediated signals are well-known activation stimuli of DCs. Gram-negative bacteria-derived lipopolysaccharide (LPS), a TLR4 ligand and a well-defined DC activator, induces the upregulation of antigen-presenting molecules such as MHC-I and MHC-II and co-stimulatory molecules such as CD80 and CD86 on the cell surface and induces cytokine production (31, 32).

Effects of ATP on DC activation have also been reported (33). ATP stimulation upregulates the expression of CD80 and CD86 in human monocyte-derived DCs (34) and murine bone marrow–derived dendritic cells (BMDCs) (35). Although these previous studies demonstrated that ATP stimulation influences the antigen-presenting function of DCs, it is not completely understood how ATP impacts the expression of cell surface molecules such as antigen-presenting molecules, co-stimulatory molecules, and co-inhibitory molecules. The receptors by which ATP impacts DCs are also unknown. In addition, these previous studies mainly used relatively lower concentrations of ATP (∼10 μM), and the effects of high concentrations of ATP remain to be clarified. In this study, we analyzed the characteristics and the mechanism of ATP-induced activation of DCs and the type of T cell activation induced by them.

Results

Effect of extracellular ATP stimulation on the cell surface expression of antigen presentation-related molecules in BMDCs

We examined the effect of extracellular ATP stimulation on the functions of DCs using BMDCs differentiated by a granulocyte-macrophage colony-stimulating factor (GM-CSF). Since high concentrations of ATP induce cell death in some kinds of cells (26, 36), we analyzed the viability of mouse BMDCs after ATP stimulation. ATP stimulation at concentrations of 0.1, 0.3, and 1 mM for 24 h did not affect the viability of immature BMDCs, and cell death was observed upon stimulation with 3 mM ATP (Fig. S1*A*). We also examined the effect of ATP on the viability of previously LPS-stimulated BMDCs (mature BMDCs). ATP stimulation caused cell death in mature BMDCs at concentrations of 1 and 3 mM (Fig. S1*B*).

We then examined the effect of various concentrations of ATP on the cell surface MHC-II and CD86 expression on BMDCs (immature BMDCs). There was no significant change in MHC-II and CD86 expression in the presence of 0.1 mM ATP, whereas 0.3 and 1 mM of ATP induced increases in the surface expression of MHC-II and CD86 in a dose-dependent manner (Fig. 1*A*). Based on these results, we further examined the effects of 1 mM ATP stimulation on the expression of antigen presentation-related molecules on BMDCs in comparison with the effect of LPS stimulation. ATP stimulation significantly increased the expression of the antigen presentation molecules, MHC-I and MHC-II, and co-stimulatory molecules, CD80 and CD86 (Fig. 1*B*). However, the levels of these proteins induced by ATP stimulation were lower than those induced by LPS (Fig. 1*B*). Additionally, LPS induced an increase in the cell surface expression of the co-inhibitory molecules PD-L1 and PD-L2, whereas ATP stimulation did not have this effect (Fig. 1*B*).

While BMDCs differentiated with GM-CSF have characteristics of the DCs induced in inflammatory conditions, BMDCs differentiated with FMS-like tyrosine kinase 3 ligand (Flt3L) have characteristics of tissue-resident steady-state DCs (37). Therefore, we also examined the effect of ATP in Flt3L-induced BMDCs. Similar to the results of the GM-CSF-induced BMDCs, ATP stimulation induced the upregulation of MHC-II and CD86 expression. The effect of ATP on PD-L1 expression was low compared to that induced by LPS stimulation in Flt3L-induced BMDCs (Fig. 1*C*).

Effect of extracellular ATP stimulation on the cytokine production by BMDCs

Since cytokine production by DCs affects the activation status of T cells after antigen presentation, we examined cytokine production from ATP- and LPS-stimulated BMDCs. We analyzed the production of IL-1β and IL-6, which are inflammatory cytokines; IL-12, which induces the differentiation of T cells into Th1 cells; and IL-10, which has an immunosuppressive effect. When BMDCs were stimulated with LPS, a

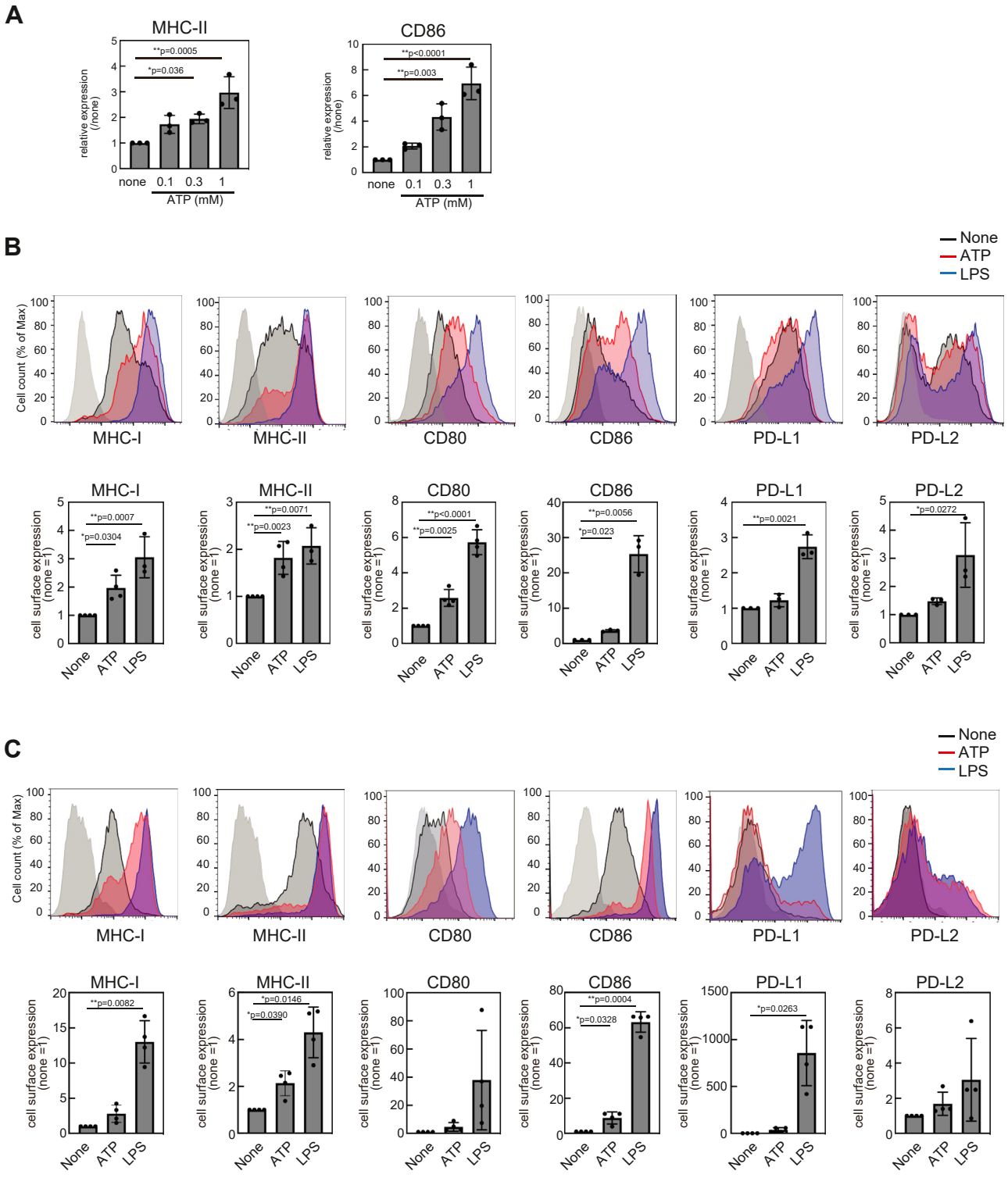

Figure 1. Effects of ATP stimulation on the cell surface molecules in BMDCs. *A*, GM-CSF-induced BMDCs were incubated with ATP (0, 0.1, 0.3,1 mM) for 24 h at 37 °C. The cell surface expression of MHC-II, CD86 was analyzed by flow cytometry. The relative means of fluorescence intensity were presented (None = 1). *B*, GM-CSF-induced BMDCs or (*C*) Flt3L-induced BMDCs were incubated with ATP (1 mM) or LPS (100 ng/ml) for 24 h at 37 °C. The cell surface expression of MHC-I, MHC-II, CD80, CD86, PD-L1, and PD-L2 was analyzed by flow cytometry (*light gray fill*, control IgG). The graphs presented under the histogram pattern of flow cytometry are the relative means of fluorescence intensity (None = 1). Experiments (n = 3 or 4) were independently performed, and the average value ± SD was presented. Statistical differences between groups were determined by Dunnett's multiple comparisons tests. The comparisons were made with None groups. *Asterisks* depict a significant difference; *$p < 0.05$, **$p < 0.01$. *p* values are indicated in each figure panel. BMDC, bone marrow-derived dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; MHC, major histocompatibility complex.

time-dependent increase in the production of IL-1β, IL-6, IL-12, and IL-10 was observed (Fig. 2, *A*–*D*). In contrast, the production of these cytokines was not observed, or very little was produced, in ATP-stimulated BMDCs (Fig. 2, *A*–*D*).

Role of P2X receptors in the activation of ATP-stimulated BMDCs

Next, we examined the receptors involved in the ATP-induced activation of BMDCs. Treatment with the pan-P2 receptor antagonist, PPADS, suppressed the ATP-induced upregulation of MHC-I, MHC-II, CD80, and CD86 (Fig. 3*A*). Quantitative RT-PCR analysis showed that the expression of ATP receptors in the P2X (P2X1-P2X7) and P2Y (P2Y2, and P2Y4) families in BMDCs were not affected by ATP stimulation (Fig. S2). The mRNA expression of *P2X6* and *P2Y4* was not detected in BMDCs (Fig. S2). Since P2X4 and P2X7 have been reported to have several functions in immune cells, we examined the effects of P2X4 and P2X7 receptor antagonists on the ATP-induced upregulation of MHC-I, MHC-II, CD80, and CD86. P2X4 receptor antagonist PSB12062 did not suppress the ATP-induced cell surface levels of these molecules but enhanced the expression of CD86 (Fig. 3*B*). The P2X7 receptor antagonist A740003 suppressed the ATP-induced cell surface MHC-I and MHC-II expression but did not affect the expression of CD80 and CD86 (Fig. 3*B*). These results suggest that the expression of MHC-I and MHC-II, but not that of CD80 or CD86, is induced *via* P2X7 in ATP-stimulated BMDCs.

Role of adenosine in the activation of ATP-stimulated BMDCs

We investigated the possible effects of adenosine, an ATP metabolite, on the ATP-induced expression of antigen presentation-related proteins. We observed that BMDCs expressed CD39 and CD73 on their cell surface (Fig. 4*A*), which are responsible for metabolizing ATP to adenosine. Treatment with the pan-adenosine receptor antagonist, CGS15943, suppressed the ATP-induced upregulation of MHC-I and MHC-II (Fig. 4*B*), whereas this treatment had no effect on the ATP-induced upregulation of CD80 and CD86 (Fig. 4*B*). Treatment with the CD39 inhibitor POM-1 and the CD73 inhibitor AMPCP also suppressed the ATP-induced upregulation of MHC-I and MHC-II (Fig. 4*C*). Moreover, 1 mM adenosine treatment upregulated the cell surface expression levels of both MHC-I and MHC-II on BMDCs (Fig. 4*D*). These results suggest that ATP is metabolized to adenosine by CD39 and CD73, and the generated adenosine is required for the upregulation of MHC-I and MHC-II expression. Since the ATP-induced upregulation of MHC-I and MHC-II expression was inhibited by P2X7 antagonists (Fig. 3*B*), this suggested that both P2X7 receptor-mediated stimulation and adenosine receptor-mediated stimulation were required to induce the upregulation of MHC-I and MHC-II by ATP stimulation. Since adenosine alone upregulated MHC-I and MHC-II expression (Fig. 4*D*), it was thought that a P2X7 receptor antagonist would not suppress ATP-induced upregulation of MHC-I and MHC-II expression because adenosine, which was generated from ATP, would

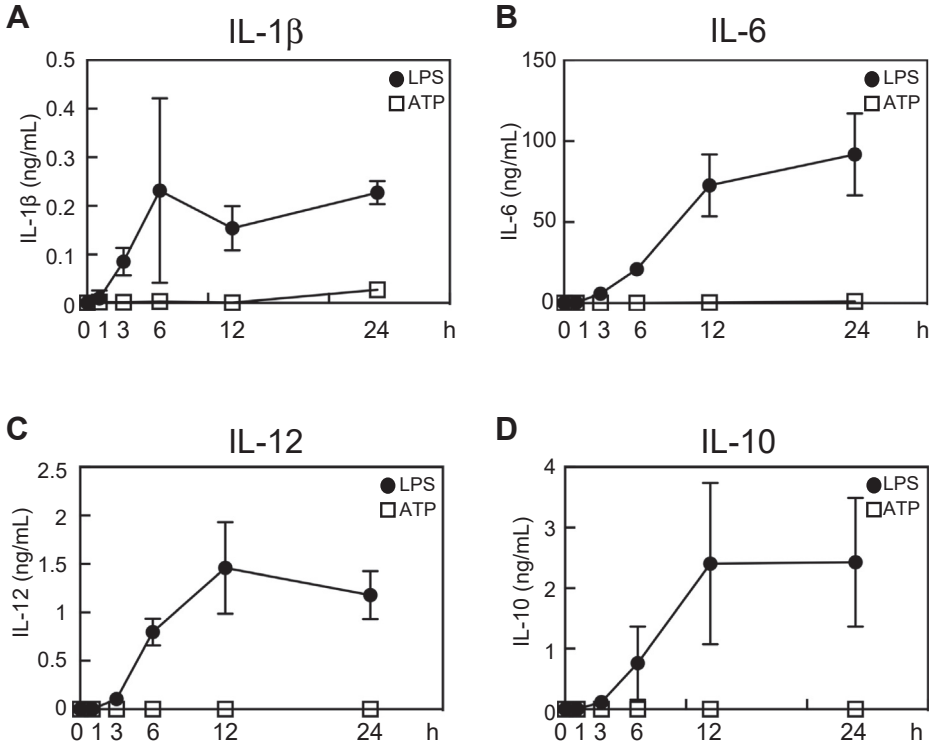

Figure 2. Cytokine production by the ATP-stimulated BMDCs. GM-CSF-induced BMDCs were stimulated with ATP (1 mM) or LPS (100 ng/ml) for the periods indicated at 37 °C. The amount of IL-1β (*A*), IL-6 (*B*), IL-12 (*C*), and IL-10 (*D*) contained in the cell culture supernatants was measured by ELISA. Experiments (n = 3) were independently performed and the average value ± SD was presented. BMDC, bone marrow-derived dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor.

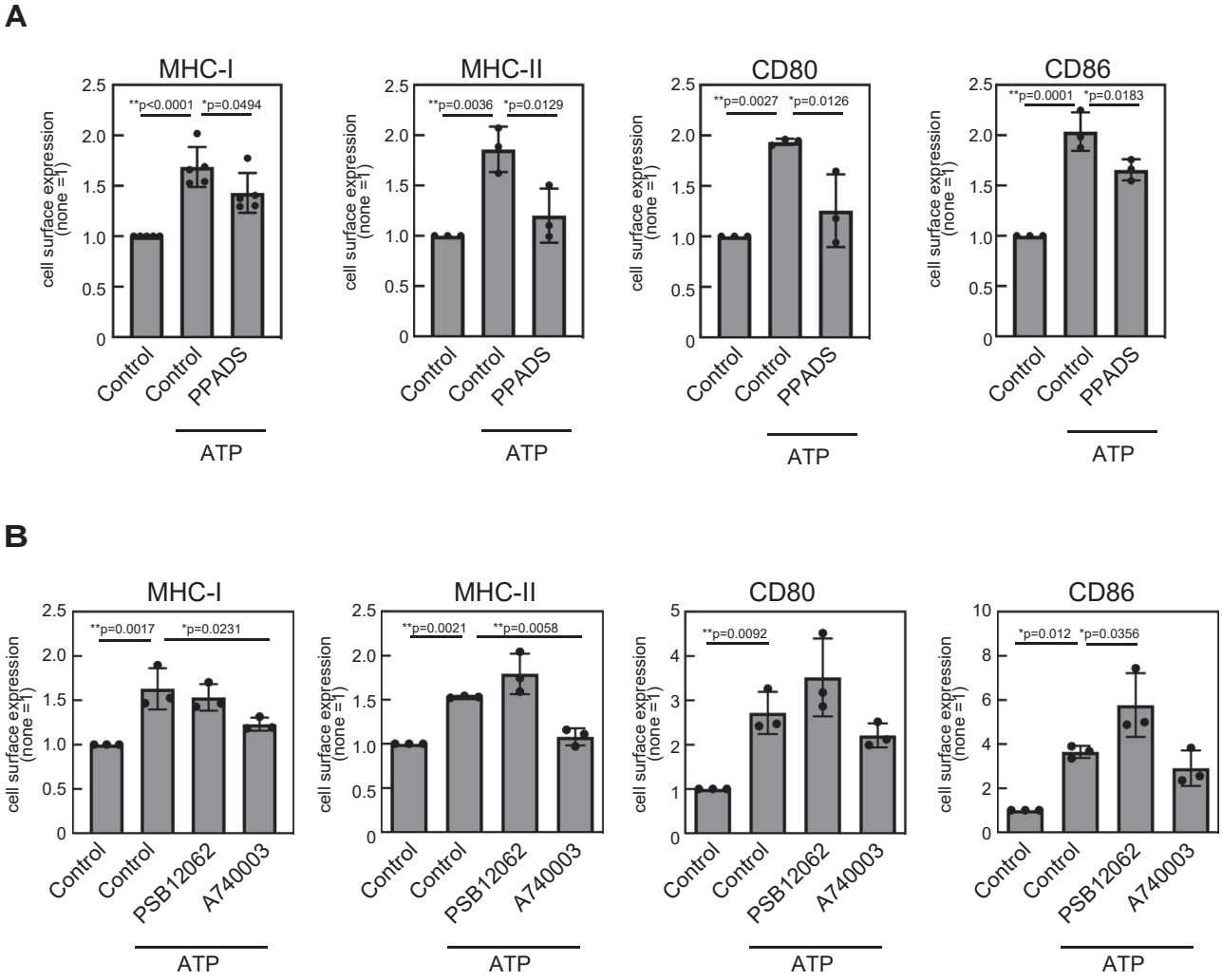

Figure 3. Effects of P2 receptor antagonists on the cell surface molecules in BMDCs. *A*, GM-CSF-induced BMDCs were pretreated with PPADS (30 μM) for 30 min at 37 °C, and then the cells were stimulated with ATP (1 mM) for 24 h at 37 °C. The cell surface expression levels of MHC-I, MHC-II, CD80, and CD86 were analyzed by flow cytometry. *B*, GM-CSF-induced BMDCs were pretreated with P2X4 antagonist PSB12062 (5 μM) or P2X7 antagonists A740003 (30 μM) for 30 min, and then the cells were stimulated with ATP (1 mM) for 24 h at 37 °C. The cell surface expression levels of MHC-I, MHC-II, CD80, and CD86 were analyzed by flow cytometry. Controls contain 0.1% DMSO as a vehicle of inhibitors. Experiments (n = 3–5) were independently performed and the average value ± SD was presented. Statistical differences between groups were determined by Dunnett's multiple comparisons test. The comparisons were made with Control (ATP(+)) groups. *Asterisks* depict a significant difference; *$p < 0.05$, **$p < 0.01$. *p*-values are indicated in each figure panel. BMDC, bone marrow-derived dendritic cell; DMSO, dimethyl sulfoxide; GM-CSF, granulocyte-macrophage colony-stimulating factor; MHC, major histocompatibility complex; PPADS, pyridoxalphosphate-6-azophenyl-2′,*A*′-disulfonic acid.

induce upregulation of them. To explain this seeming contradiction, we hypothesized a coordinated action of relatively low concentrations of ATP and adenosine. We analyzed the effects of ATP and adenosine using relatively low concentrations. Stimulation with ATP and adenosine alone did not induce MHC-II expression at a concentration of 0.1 mM, but simultaneous stimulation with 0.1 mM of ATP and 0.1 mM of adenosine significantly upregulated MHC-I and MHC-II expression (Fig. 4*E*).

Effect of extracellular ATP stimulation on the T cell activation ability of BMDCs

We examined the antigen presentation ability of ATP-stimulated BMDCs by analyzing the mixed leukocyte reaction (MLR). In this assay, the alloreactive CD4T cells and CD8T cells from the lymph node are activated by BMDCs in an MHC-I- and MHC-II-dependent manner, respectively (38). The amounts of IL-2 and interferon-gamma (IFN-γ) released in the coculture supernatants were analyzed. IL-2 production was increased in the supernatant cocultured with the LPS-stimulated BMDCs at ratios of 1:10 and 1:20, whereas a significant enhancement of IL-2 production was not observed in the supernatant cocultured with the ATP-stimulated BMDCs compared to that with unstimulated BMDCs (Fig. 5*A*). At all ratios examined, IFN-γ production was increased in the supernatant cocultured with the ATP-stimulated BMDCs compared to that with the unstimulated BMDCs (Fig. 5*A*). Higher IFN-γ production was observed in the supernatant cocultured with the LPS-stimulated BMDCs at 1:20, but not at ratios of 1:5 and 1:10, compared to that with the unstimulated BMDCs (Fig. 5*A*). At the 1:5 and 1:10 ratios, IFN-γ production

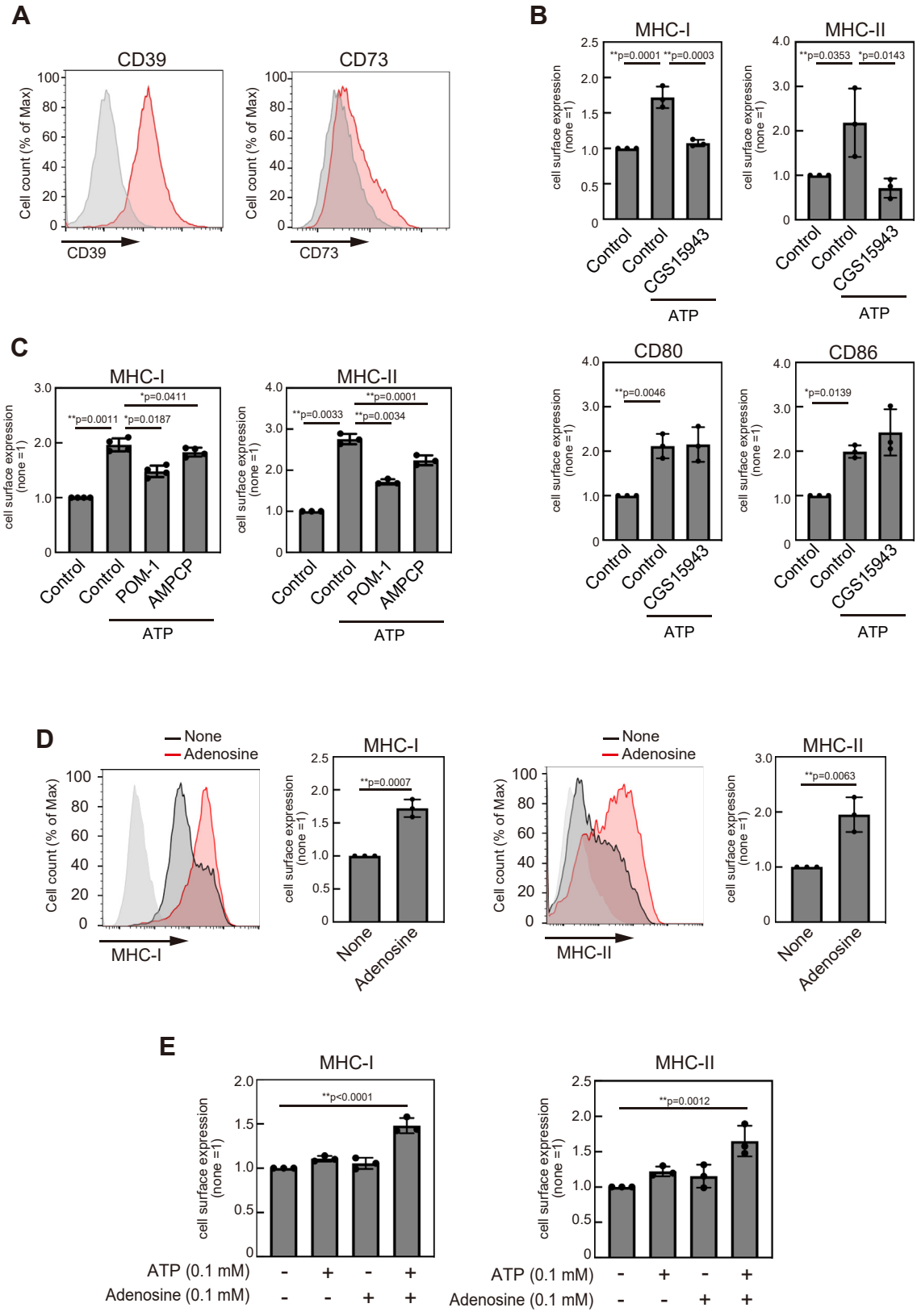

Figure 4. Role of adenosine on the cell surface molecules in BMDCs. *A*, the cell surface expression levels of CD39 and CD73 on GM-CSF-induced BMDCs were analyzed by flow cytometry (*Gray fill*, control IgG). *B*, GM-CSF-induced BMDCs were pretreated with CGS15943 (10 μM) for 30 min, and then the cells were stimulated with ATP (1 mM) for 24 h at 37 °C. The cell surface expression levels of MHC-I, MHC-II, CD80, and CD86 were analyzed by flow cytometry. *C*, GM-CSF-induced BMDCs were pretreated with CD39 inhibitor (POM-1) (10 μM) or CD73 inhibitor (AMPCP) (10 μM) for 30 min, and then the cells were stimulated with ATP (1 mM) for 24 h at 37 °C. The cell surface expression levels of MHC-I and MHC-II were analyzed by flow cytometry. Controls contain 0.1% DMSO as a vehicle of inhibitors. *D*, GM-CSF-induced BMDCs were incubated with adenosine (1 mM) for 24 h at 37 °C. The cell surface expression levels of MHC-I and MHC-II were analyzed by flow cytometry (*gray fill*, control IgG). The graphs to the right of the histogram pattern of flow cytometry are the relative

was much higher in the supernatant cocultured with the ATP-stimulated BMDCs than that with the LPS-stimulated BMDCs (Fig. 5*A*). Using the 1:5 ratio condition, we investigated the effects of a P2 receptor antagonist, PPADS, and a P1 receptor antagonist, CGS15943, which inhibited the ATP-induced upregulation of cell surface antigen presentation-related molecules (Figs. 3*A* and 4*B*). IFN-γ production induced by ATP-stimulated BMDCs was suppressed by both PPADS and CGS15943 (Fig. 5*B*). These results suggest that ATP- and adenosine-induced changes in BMDCs, which likely enhanced the expression of antigen presentation molecules, augmented IFN-γ production in the coculture supernatant of the MLR assay.

We then examined whether these effects of ATP also occurred in other types of DCs. The MLR assay results showed that Flt3L-induced BMDCs had enhanced IFN-γ production after being cocultured with T cells, similar to the results of GM-CSF-induced BMDCs (Fig. 5*C*). We also examined the effect of ATP on mouse spleen-resident DCs in the MLR assay. The viability of spleen DCs was quite low after 1 mM ATP stimulation but was not affected by 0.3 mM ATP treatment; therefore, spleen DCs stimulated with 0.3 mM ATP were used for the MLR assay. Enhanced IFN-γ production from T cells was also observed when ATP-stimulated spleen DCs were used. Unlike BMDCs, enhancement of IFN-γ production from T cells was also observed when LPS-stimulated spleen DCs were used at the 1:5 ratio (Fig. 5*D*).

Analysis of IFN-γ-producing cells induced by antigen presentation

IFN-γ is well known to be produced by activated CD4T ($CD4^+$, $CD3^+$ cells), CD8T ($CD8^+$, $CD3^+$ cells), and NK/NKT cells ($DX5^+$ cells). We investigated IFN-γ-expressing cells by analyzing the intracellular IFN-γ by flow cytometry in the coculture of BMDCs with lymph node cells at a 1:5 ratio for 24 h. IFN-γ-producing cells were observed in both CD4 and CD8T cells after coculturing with BMDCs that were either unstimulated, stimulated with ATP, or stimulated with LPS; however, the percentage of IFN-γ^+ cells in CD8T cells was lower than that in CD4T cells (Fig. 6*A*). The ratio of NK/NKT cells in the lymph node cells was low (less than 1.0%), and IFN-γ production from NK/NKT cells was hardly observed (Fig. 6*A*). Compared to unstimulated or LPS-stimulated BMDCs, activation by ATP-stimulated BMDCs increased the percentage of IFN-γ^+ cells in both CD4 and CD8T cells (Fig. 6*B*). We next investigated the activation of CD4 and CD8T cells by ATP-stimulated BMDCs by analyzing the cell surface expression of the activation marker CD69 in the coculture of BMDCs with lymph node cells at a 1:5 ratio for 24 h. Coculture with ATP-stimulated BMDCs enhanced the expression of CD69 in both CD4 and CD8T cells compared to that in cells cocultured with unstimulated BMDCs (Fig. 6*C*). In contrast, BMDCs stimulated with LPS did not activate CD4 or CD8T cells (Fig. 6*C*). These results indicate that ATP stimulation of BMDCs enhances the activation of CD4 and CD8T cells upon antigen presentation.

Discussion

In this study, we examined the effect of extracellular ATP on the cell surface expression of antigen presentation-related molecules and the antigen presentation function of DCs. Previous reports have shown that ATP induces the activation of DCs, but the precise action and its mechanism remained unknown. We found that high concentrations of extracellular ATP upregulated the expression of antigen-presenting molecules (MHC-I and MHC-II) and co-stimulatory molecules (CD80 and CD86) but not that of co-inhibitory molecules (PD-L1 and PD-L2). These ATP-stimulated DCs induced the activation of IFN-γ producing T cells upon antigen presentation. Additionally, we found that adenosine, which was metabolized from ATP, was required for the ATP-induced upregulation of MHC-I and MHC-II expression. Our results indicate that when stimulated with high concentrations of ATP, ATP and the metabolite adenosine are cooperatively induced the upregulation of MHC-I and MHC-II, resulting in the activation of DCs to induce IFN-γ production in T cells (Fig. 7).

Several kinds of stimulations modulate the functions of DCs. TLR stimulation is well known to enhance the antigen presentation ability of DCs by regulating both cytokine production and the cell surface expression of antigen presentation-related proteins (31, 32). In this study, as previously reported, LPS stimulation strongly induced the upregulation of the antigen-presenting molecules, MHC-I, MHC-II, CD80, CD86, PD-L1, and PD-L2. We found that ATP stimulation induced upregulation of MHC-I, MHC-II, CD80, and CD86 expression; however, ATP did not induce an increase in PD-L1 or PD-L2 expression in BMDCs. Activation of DCs is known to induce the production of several cytokines. The production of IL-6, IL-10, and IL-12 was strongly induced upon LPS stimulation, whereas ATP stimulation induced little or no production of these cytokines. These results indicate that the activation pattern induced by ATP was different from that induced by LPS stimulation.

Using the MLR assay, we demonstrated that ATP-stimulated BMDCs induced higher levels of IFN-γ production from T cells than those of unstimulated or LPS-stimulated BMDCs cocultured at a 1:5 ratio. When lymph node cells were cocultured with LPS-stimulated BMDCs, IFN-γ production was enhanced at a ratio of 1:20, but not at ratios of 1:10 and 1:5. Since LPS stimulation promotes the expression of the inhibitory factors, PD-L1 and PD-L2, as well as the production

means of fluorescence intensity (None = 1). *E*, GM-CSF-induced BMDCs were incubated with (+) or without (–) ATP (0.1 mM) or adenosine (0.1 mM) for 24 h at 37 °C. The cell surface expression levels of MHC-I and MHC-II were analyzed by flow cytometry. The expression level was analyzed by means of fluorescence intensity. Experiments (n = 3–4) were independently performed and the average value ± SD was presented. Statistical differences between groups were determined by Dunnett's multiple comparisons test (*B*, *C* and *E*) or Student's *t* test (*D*). The comparisons were made with Control (ATP(+)) groups (*B* and *C*) or ATP(–), Adenosine(–) groups (*E*). *Asterisks* depict a significant difference; $*p < 0.05$, $**p < 0.01$. *p*-values are indicated in each figure panel. BMDC, bone marrow-derived dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; MHC, major histocompatibility complex.

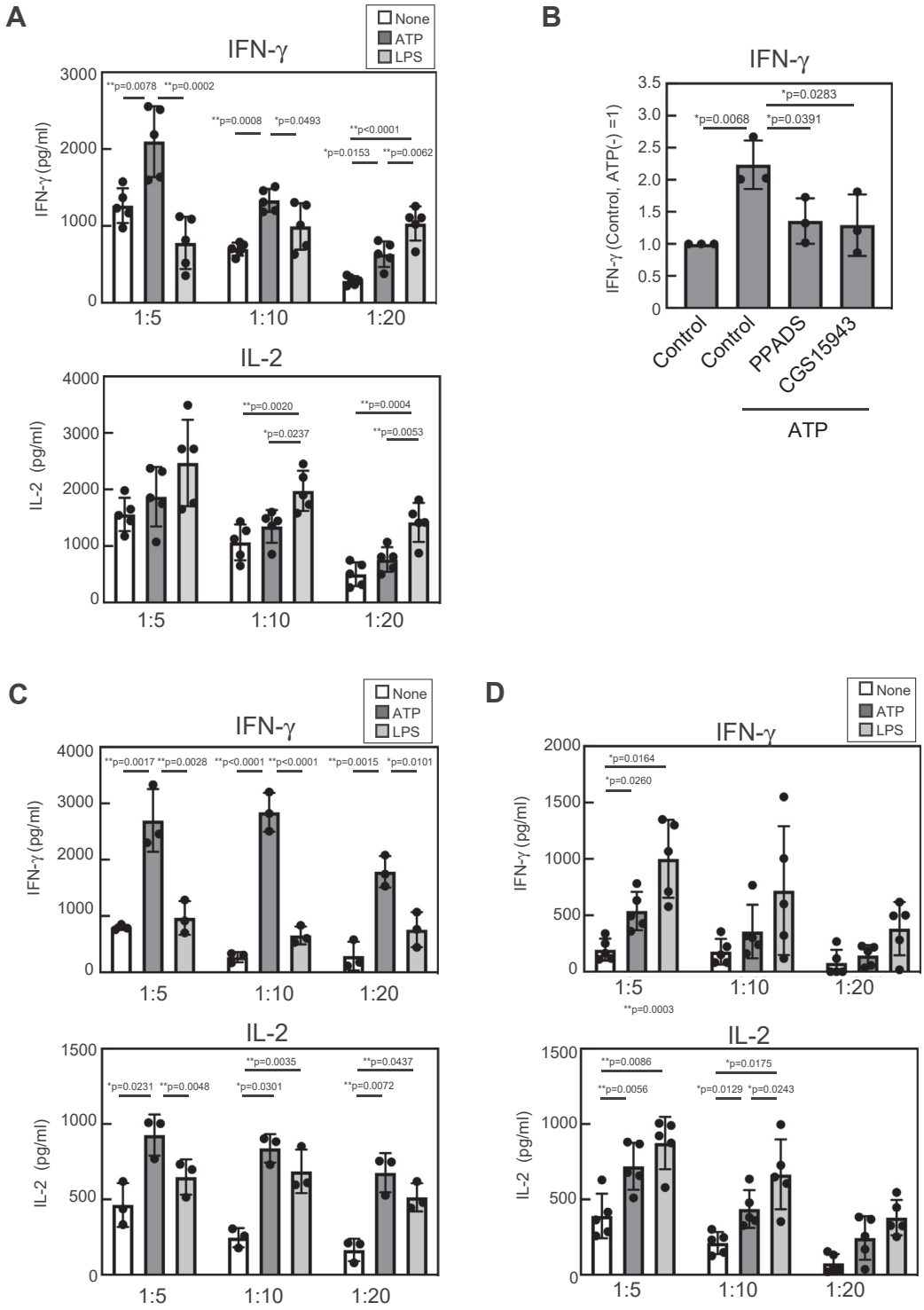

Figure 5. IFN-γ and IL-2 production from T cells cocultured with ATP-stimulated DCs. *A*, GM-CSF-induced BMDCs were stimulated with ATP (1 mM) or LPS (100 ng/ml) for 24 h at 37 °C. For the MLR assay, the BMDCs and lymph node cells were cocultured at ratios of BMDCs: lymph node cells = 1: 5, 1:10, 1:20 for 24 h at 37 °C. The amounts of IFN-γ and IL-2 in the culture supernatants were measured by ELISA. *B*, GM-CSF-induced BMDCs were pretreated with PPADS (30 μM) or CGS15943 (10 μM) for 30 min, and then the cells were stimulated with ATP (1 mM) for 24 h at 37 °C. The BMDCs and lymph node cells were cocultured at a ratio of BMDCs:lymph node cells = 1:5 for 24 h at 37 °C. The amounts of IFN-γ in the culture supernatants were measured by ELISA. Controls contain 0.1% DMSO as a vehicle of inhibitors. Values were presented as the relative amount of IFN-γ production (Control (ATP(−)) = 1). *C*, Flt3L-induced BMDCs were stimulated with ATP (1 mM) or LPS (100 ng/ml) for 24 h at 37 °C. *D*, spleen DCs were stimulated with ATP (0.3 mM) or LPS (100 ng/ml) for 24 h at 37 °C. MLR assay was performed using the same method above. Experiments (n = 3–5) were independently performed and the average value ± SD was presented. Statistical differences between groups were determined by Tukey's multiple comparison test (*A*, *C* and *D*) or Dunnett's multiple comparisons test (The comparisons were made with Control (ATP(+)) groups) (*B*). *Asterisks* depict a significant difference; $*p < 0.05$, $**p < 0.01$. *p*-values are indicated in each figure panel. BMDC, bone marrow-derived dendritic cell; DMSO, dimethyl sulfoxide; Flt3L, FMS-like tyrosine kinase 3 ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-gamma; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction.

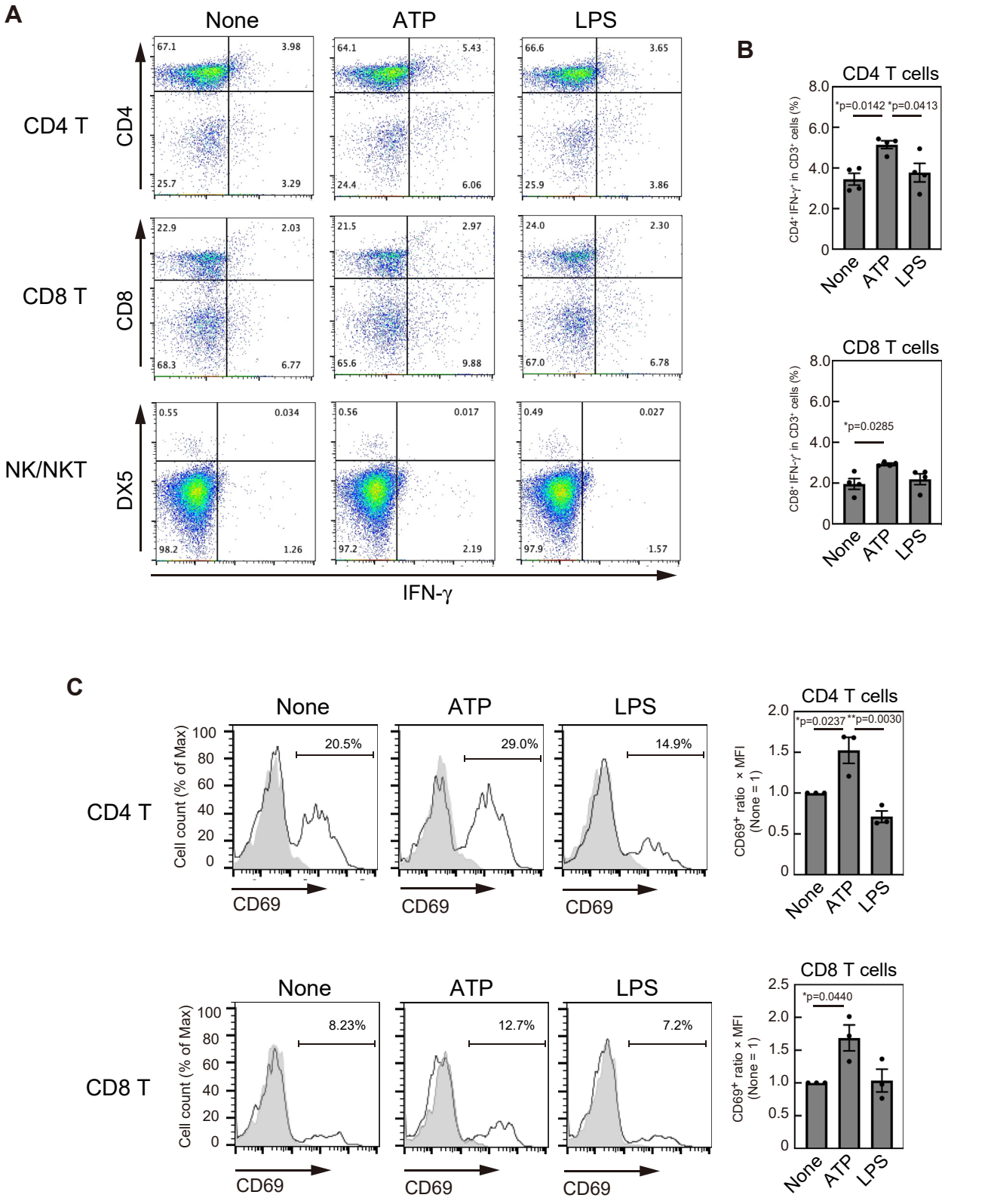

Figure 6. Induction of T cell activation by the ATP-stimulated BMDCs. *A*, GM-CSF-induced BMDCs were stimulated with ATP (1 mM) or LPS (100 ng/ml) for 24 h at 37 °C. For the MLR assay, the BMDCs and lymph node cells were cocultured at a 1:5 ratio for 24 h at 37 °C. Intracellular expression of IFN-γ in the cells was analyzed with flow cytometry. CD4T cells were determined by $CD3\varepsilon^+/CD4^+$ cells, CD8T cells were determined by $CD3\varepsilon^+/CD8^+$ cells, and NK and NKT cells were determined by $DX5^+$ cells. *B*, the percentages of $CD4^{+}$/IFN-γ^+ or $CD4^{+}$/IFN-γ^+ in $CD3\varepsilon^+$ cells are shown on the graphs. *C*, GM-CSF-induced BMDCs were stimulated with ATP (1 mM) or LPS (100 ng/ml) for 24 h at 37 °C. The BMDCs and lymph node cells were cocultured at a 1:5 ratio for 24 h at 37 °C. The cell surface CD69 expression levels in CD4T and CD8T cells were analyzed with flow cytometry (*gray fill*, control IgG). The ratios of $CD69^+$ cells in CD4T or CD8T cells multiplied by the fluorescence intensity of CD69 are shown in the *right graph*. The values were expressed as a relative expression level (None = 1). Experiments (n = 3–4) were independently performed and the average value ± SD was presented. Statistical differences between groups were

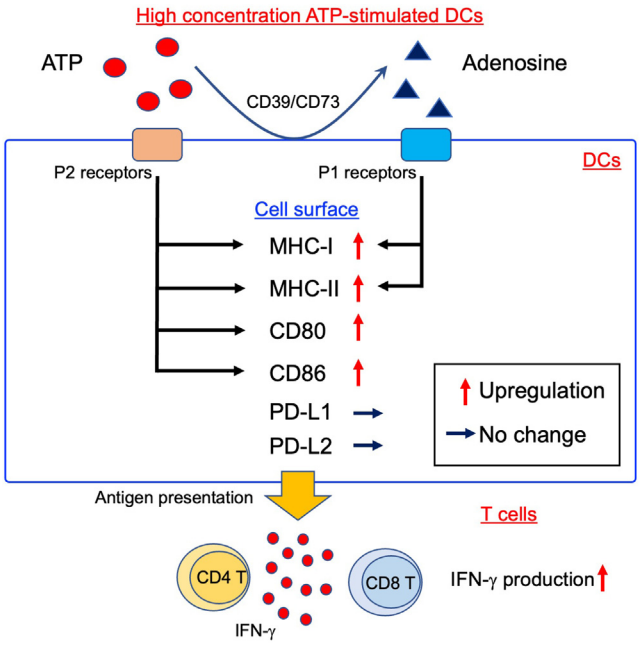

Figure 7. Schematic overview of ATP-induced BMDC activation. High concentrations of extracellular ATP upregulated the cell surface expression of MHC-I, MHC-II, CD80, and CD86 but not of PD-L1 and PD-L2 on BMDCs. ATP and adenosine, which were produced by CD39 and CD73 from ATP, cooperatively induced upregulation of the cell surface MHC-I and MHC-II. These ATP-stimulated BMDCs activate both CD4 and CD8T cells and promote IFN-γ production from these T cells. BMDC, bone marrow-derived dendritic cell; IFN-γ, interferon-gamma; MHC, major histocompatibility complex.

of IL-10, activation and IFN-γ production from the T cells may be suppressed with a relatively high proportion of BMDCs in the coculture. In contrast to the effects of LPS stimulation, ATP stimulation did not induce the expression of these inhibitory factors; thus, ATP-stimulated BMDCs may effectively enhance the T cell activation of DCs even with a high proportion of BMDCs in the coculture. A similar effect was observed in Flt3L-induced BMDCs. Spleen DCs stimulated with 0.3 mM ATP showed induction of IFN-γ production from T cells, although LPS-stimulated spleen DCs also induced a high level of IFN-γ production from T cells. These differences may be related to the characteristics of the population of DCs in the spleen, which constitutes a mixture of several types of DCs. Furthermore, the maturation states of DCs may be varied. In spleen DCs, a decrease in viability was observed with the stimulation of 1 mM ATP. ATP has been reported to induce cell death in some types of cells (36, 39). We observed that 1 mM ATP stimulation resulted in decreased viability in mature BMDCs. Spleen DCs may contain populations that are likely more mature and therefore more susceptible to cell death induced by ATP.

Activated Th1 CD4T cells, CD8T cells, NK cells, and NKT cells are known to be capable of producing IFN-γ. We observed that the CD4 and CD8T cells mainly expressed IFN-γ in the MLR assay. The ratios of the IFN-γ^+ cells in the CD4 and CD8T cells were increased after being cocultured with ATP-stimulated BMDCs. We also found that the CD69 expression level in CD4 and CD8T cells increased after being cocultured with ATP-stimulated BMDCs. These results suggest that ATP-stimulated BMDCs activate CD4 and CD8T cells through the MHC-T cell receptor interaction to induce IFN-γ production. Since the percentage of IFN-γ^+ cells in CD8T cells was smaller than that in CD4T cells, the majority of IFN-γ production may have been derived from CD4T cells. IL-12 is well known to induce naive CD4T cell differentiation in IFN-γ producing Th1 cells (40). In our study, although ATP did not induce IL-12 production, ATP-stimulated BMDCs induced IFN-γ production from CD4T cells. Induction of Th1 independent of IL-12 has also been reported (41). Thus, ATP-stimulated BMDCs may induce IFN-γ-production mediated by IL-12-independent Th1 cell differentiation.

In this study, PPADS, a pan-P2 receptor antagonist, inhibited the ATP-induced upregulation of MHC-I, MHC-II, CD80, and CD86. We examined the role of P2X4 and P2X7 receptors in the ATP-induced upregulation of MHC-I, MHC-II, CD80, and CD86. We found that the ATP-induced upregulation of cell surface MHC-I and MHC-II was inhibited by the P2X7 antagonist, whereas the upregulation of CD80 and CD86 was not affected. ATP may act on receptors other than P2X7 and P2X4 to induce the expression of CD80 and CD86 in BMDCs. We observed that BMDCs expressed the P2 receptors, P2X2, P2X5, and P2Y2, in addition to P2X4 and P2X7. Several studies have analyzed the functions of these P2 receptors. P2X2 receptors are mainly expressed in neurons and are involved in neurotransmission (42). The function of P2X5 has not been well studied. P2Y2 receptors enhanced the chemotaxis of eosinophils and DCs in a mouse model of airway inflammation (43). ATP activates human monocyte-derived DCs *via* the P2Y11 receptor (44). However, mice lack the *P2Y11* (45). These P2 receptors could be involved in the ATP-induced upregulation of CD80 and CD86 in BMDCs.

We examined the involvement of adenosine, an ATP metabolite. We found that the adenosine receptor antagonist CGS15943 inhibited the ATP-induced expression of MHC-I and MHC-II but did not affect the expression of CD80 and CD86. These results indicate that both P2X7 and adenosine receptor-mediated signaling are required for the upregulation of MHC-I and MHC-II expression upon ATP stimulation. BMDCs expressed CD39 and CD73, and inhibitors against these molecules suppressed the action of ATP. These results suggest that adenosine generated from ATP contributes to the effects of ATP on MHC-I and MHC-II upregulation. The weak effect of the CD73 inhibitor may be related to the contribution of another ecto-alkaline phosphatase that converts ATP to adenosine (46). There are four known adenosine receptors: A1, A2a, A2b, and A3, which have been reported to have different effects on DCs (33). Adenosine and A2 receptor agonists were

determined by Tukey's multiple comparison test. *Asterisks* depict a significant difference; *$p < 0.05$, **$p < 0.01$. *p*-values are indicated in each figure panel. BMDC, bone marrow-derived dendritic cell; DMSO, dimethyl sulfoxide; Flt3L, FMS-like tyrosine kinase 3 ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-gamma; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MLR, mixed leukocyte reaction.

reported to induce the upregulation of MHC-I and MHC-II in human monocyte-derived DCs. However, these adenosine-stimulated DCs showed attenuated T cell activation in the MLR assay, despite the induction of antigen-presenting molecule expression (47). Adenosine has anti-inflammatory effects and suppresses the function of several immune cells (16, 21). Adenosine is also considered to be one of the causes of immunosuppression in tumors (48). In our study, we found that ATP and adenosine generated from ATP cooperatively stimulate BMDCs, inducing T cell activation. Adenosine may act in an inhibitory manner when stimulated alone but may lead to DC activation when stimulated simultaneously with ATP.

We observed that the upregulation of MHC-I and MHC-II induced by 1 mM ATP was inhibited by both P2X7 receptor and adenosine receptor antagonists. However, the P2X7 inhibitor should have no effect if the upregulation of MHC-I and MHC-II was induced by adenosine alone because we observed that 1 mM adenosine alone upregulated MHC-I and MHC-II expression. Therefore, we hypothesized that there is an enhancing effect of ATP and adenosine signals. We observed that stimulation with adenosine 0.1 mM or ATP 0.1 mM alone did not induce upregulation of cell surface MHC-I and MHC-II. However, the expression of MHC-I and MHC-II on cell surfaces did increase when these two were stimulated simultaneously. This finding suggests that relatively low concentrations (0.1 mM) of ATP and adenosine cooperate to upregulate MHC-I and MHC-II expression. After 1 mM ATP stimulation, ATP and adenosine, which is generated from ATP, may cooperatively induce the upregulation of MHC-I and MHC-II. Since the generated adenosine is degraded by extracellular metabolic enzymes such as adenosine deaminase or transported into the cell by transporters (49), the concentration of adenosine generated after 1 mM ATP stimulation may not be high enough to upregulate MHC expression by itself. There have been reports of responses requiring both ATP and adenosine. For instance, ATP and adenosine are required for ATP-induced neutrophil migration (50, 51). There might be an interaction between adenosine and ATP receptor signaling. Nevertheless, further studies of the mechanisms of action of ATP in regulating the expression of antigen presentation-related molecules are necessary.

The extracellular ATP concentration has been reported to be elevated in the tumor microenvironment (TME) (28), likely produced by macrophages (18) or dead tumor cells (52). Since IFN-γ production by CD4 and CD8T cells is important for triggering the immune reaction to eliminate tumor cells (53), ATP accumulation might stimulate DCs and trigger the development of IFN-γ-producing T cells in the TME, thereby enhancing immune responses against tumor cells. Extracellular ATP stimulation is reported to induce activation of the NLRP3 inflammasome and caspase-1 in TLR-stimulated DCs, macrophages, and neutrophils that accumulate in the TME, resulting in the production of IL-1β. This produced IL-1β induces inflammation involved in anti-tumor activity through the activation of T cells (23, 54). In our study, since IL-1β was not produced by ATP stimulation, ATP-induced enhancement of antigen presentation-related molecules might occur independent of inflammasome activation. Some types of DCs have been reported to suppress T cell activation by inducing inhibitory factors. Several tumor-producing factors are known to induce suppressive DCs (55). To develop novel anti-tumor immune therapeutics, an adjuvant that appropriately activates DCs and induces efficient antigen presentation is required (56). Although LPS-stimulated BMDCs did not induce IFN-γ production from T cells when there was a high proportion of BMDCs in the MLR assay, ATP-stimulated BMDCs induced IFN-γ production from T cells even when the ratio of DCs was increased. ATP may be a useful adjuvant for inducing strong anti-tumor immunity. Further analysis is necessary to elucidate the mechanisms of action of ATP as well as the difference between ATP stimulation and LPS stimulation on DCs.

In conclusion, our results demonstrated that high concentrations of extracellular ATP enhance the expression of antigen-presenting and co-stimulatory molecules but not that of co-inhibitory molecules. The upregulation of MHC-I and MHC-II expression was mediated by ATP and its metabolite adenosine. We further demonstrated that ATP-stimulated DCs induced the activation of IFN-γ-producing T cells upon antigen presentation. The mechanism of ATP-induced DC activation may be a novel therapeutic target for modulating DC-mediated immune response.

Experimental procedures

Materials

The following reagents were obtained the sources indicated: ATP, adenosine, LPS (Serotype O55:B5) and PBS12062 from Sigma-Aldrich; pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), A740003, CGS15943, POM-1, adenosine 5′-(α, β-methylene)diphosphate (AMPCP) from Cayman Chemical; murine recombinant GM-CSF, APC-labeled anti-mouse CD11c antibody (N418), FITC-labeled anti-mouse MHC-II ($I\text{-}A^k$) antibody (10-3.6), FITC-labeled anti-mouse MHC-I ($H2\text{-}K^k$) antibody (36-7-5), FITC-labeled anti-mouse CD80 antibody (16-10A1), FITC-labeled anti-mouse CD86 antibody (GL-1), PE-labeled anti-mouse PD-L1 antibody (10F.9G2), PE-labeled anti-mouse PD-L2 antibody (TY25), FITC-labeled anti-mouse CD3ε antibody (145-2C11), APC- and PerCP/Cyanine5.5-labeled anti-mouse CD4 antibody (GK1.5), APC- and PE/Cyanine7-labeled anti-mouse CD8 antibody (53-6.7), PE-labeled anti-mouse CD69 antibody (H1.2F3), PE-labeled anti-mouse IFN-γ antibody (W18272D), APC-labeled CD49b antibody (DX5), ELISA kit for mouse IFN-γ and mouse IL-1β from BioLegend; ELISA kit for mouse IL-2, mouse IL-10, mouse IL-12 p70 and mouse IL-6 from Thermo Fisher Scientific. All other chemicals were

commercial products of reagent grade. ATP, adenosine, and LPS were dissolved in the culture medium. Inhibitors were dissolved in dimethyl sulfoxide (DMSO) to prepare concentrated stock solutions.

Mice

Male B10.BR mice (H-2^k) and male BALB/c mice (H-2^d) were obtained from Japan SLC (Hamamatsu, Japan), and all mice were kept in an animal facility at Okayama University. This study was approved by the Committee on Animal Experiments of Okayama University (OKU-2018087, OKU-2021754).

Preparation of BMDCs and spleen DCs

GM-CSF-induced BMDCs were prepared as previously reported (57). Briefly, Bone marrow cells were collected from the tibia and femur of mice. The cells were cultured in RPMI-1640 medium containing 10% FBS and 50 μM β-mercaptoethanol in the presence of 10 ng/ml mouse GM-CSF at 37 °C in 5% CO_2 for 7 days. To prepare Flt3L-induced BMDCs, the bone marrow cells were cultured for 10 days in RPMI-1640 medium containing 10% FBS, 50 μM β-mercaptoethanol in the presence of a one-fifth volume of culture supernatant of B16-Flt3L cells (obtained from Dr Paul Roche, National Institutes of Health) (58). For the preparation of spleen DCs, Spleens were treated with collagenase D (1 mg/ml) and DNase I (1 unit/ml) for 30 min at 37 °C. $CD11c^+$ cells in the spleen cells were isolated by using MojoSort mouse CD11c nanobeads and MojoSort Magnet (BioLegend).

Flow cytometry

BMDCs were harvested and washed with FACS staining medium (FACS-SM, PBS(−) containing 2% FBS). The cells were incubated with fluorescent dye-conjugated antibodies in FACS-SM on ice for 30 min. The cells were subsequently washed three times. The cells were analyzed with a flow cytometer (Gallios: Beckman Coulter).

Mixed lymphocyte reaction

Lymph nodes were collected from BALB/c mice, and single-cell suspension was prepared by crushing the lymph nodes and removing debris with a cell strainer (40 μm). The lymph node cells used in this experiment comprised approximately 75% T cells ($CD3^+$ cells) and 25% B cells ($B220^+$ cells). NK/NKT cells ($DX5^+$ cells) accounted for less than 1% of the total cells. Among the T cells ($CD3^+$ cells), approximately 70% were CD4T cells ($CD4^+$ cells), and 30% were CD8T cells ($CD8^+$ cells). BMDCs or spleen DCs from B10.BR mice were stimulated with ATP or LPS for 24 h at 37 °C. The cells were washed with culture medium, and then BMDCs and lymph node cells were cocultured at ratios of 1:5, 1:10, and 1:20 for 24 h at 37 °C. The culture supernatants were harvested and the amounts of IL-2 and IFN-γ were analyzed by ELISA. To analyze the T cell activation, the cells were harvested, and the cell surface expression levels of CD3ε, CD4 or CD8, and CD69 were analyzed with a flow cytometer.

Measurement of intracellular IFN-γ expression by flow cytometry

BMDCs and lymph node cells were cocultured for 24 h at 37 °C. To inhibit cytokine release, brefeldin A (10 μg/ml) was added during the last 6 h prior. The cells were harvested, and the cell surface CD11c, CD3ε, CD4 or CD8, and DX5 were stained with the fluorescent-labeled antibodies. The cells were fixed with fixation buffer (BioLegend) and then permeabilized with permeabilization buffer (BioLegend) following the manufacturer's instructions. The cells were then stained with a PE-labeled anti-IFN-γ antibody. The cells were analyzed with a flow cytometer (Gallios: Beckman Coulter).

Measurement of cytokine production

The amounts of cytokines, IL-6, IL-10, IL-12, IL-1β, IFN-γ, and IL-2, in culture supernatants were measured using ELISA kits according to the manufacturer's instructions.

Statistical analysis

Statistical significance for comparisons between the two groups was determined using the Student's t test. Comparisons among multiple groups were performed by one-way ANOVA with Dunnett's multiple comparison test or Tukey's multiple comparison test. $^*p < 0.05$ and $^{**}p < 0.01$ were considered statistically significant.

Data availability

All the data contained within the manuscript.

Supporting information—This article contains supporting information.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: AMPCP, adenosine 5'-(α, β-methylene)diphosphate; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; DMSO, dimethyl sulfoxide; Flt3L, FMS-like tyrosine kinase 3 ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-gamma; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MLR,

mixed leukocyte reaction; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; TME, tumor microenvironment.

References

1. Mellman, I., and Steinman, R. M. (2001) Dendritic cells: specialized and regulated antigen processing machines. *Cell* **106**, 255–258
2. Trombetta, E. S., and Mellman, I. (2005) Cell biology of antigen processing in vitro and in vivo. *Annu. Rev. Immunol.* **23**, 975–1028
3. Cresswell, P., Ackerman, A. L., Giodini, A., Peaper, D. R., and Wearsch, P. A. (2005) Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunol. Rev.* **207**, 145–157
4. Joffre, O. P., Segura, E., Savina, A., and Amigorena, S. (2012) Cross-presentation by dendritic cells. *Nat. Rev. Immunol.* **12**, 557–569
5. Zhu, J., and Paul, W. E. (2008) CD4T cells: fates, functions, and faults. *Blood* **112**, 1557–1569
6. Roche, P. A., and Furuta, K. (2015) The ins and outs of MHC class II-mediated antigen processing and presentation. *Nat. Rev. Immunol.* **15**, 203–216
7. Sharpe, A. H. (2009) Mechanisms of costimulation. *Immunol. Rev.* **229**, 5–11
8. Eltzschig, H. K., Sitkovsky, M. V., and Robson, S. C. (2012) Purinergic signaling during inflammation. *N. Engl. J. Med.* **367**, 2322–2333
9. Idzko, M., Ferrari, D., and Eltzschig, H. K. (2014) Nucleotide signalling during inflammation. *Nature* **509**, 310–317
10. Murao, A., Aziz, M., Wang, H., Brenner, M., and Wang, P. (2021) Release mechanisms of major DAMPs. *Apoptosis* **26**, 152–162
11. Harada, Y., Kato, Y., Miyaji, T., Omote, H., Moriyama, Y., and Hiasa, M. (2018) Vesicular nucleotide transporter mediates ATP release and migration in neutrophils. *J. Biol. Chem.* **293**, 3770–3779
12. Kaczmarek-Hajek, K., Lorinczi, E., Hausmann, R., and Nicke, A. (2012) Molecular and functional properties of P2X receptors–recent progress and persisting challenges. *Purinergic Signal.* **8**, 375–417
13. Abbracchio, M. P., Burnstock, G., Boeynaems, J. M., Barnard, E. A., Boyer, J. L., Kennedy, C., *et al.* (2006) International union of pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol. Rev.* **58**, 281–341
14. Savio, L. E. B., de Andrade Mello, P., da Silva, C. G., and Coutinho-Silva, R. (2018) The P2X7 receptor in inflammatory diseases: angel or demon? *Front. Pharmacol.* **9**, 52
15. Antonioli, L., Blandizzi, C., Pacher, P., and Hasko, G. (2013) Immunity, inflammation and cancer: a leading role for adenosine. *Nat. Rev. Cancer* **13**, 842–857
16. Adinolfi, E., Capece, M., Franceschini, A., Falzoni, S., Giuliani, A. L., Rotondo, A., *et al.* (2015) Accelerated tumor progression in mice lacking the ATP receptor P2X7. *Cancer Res.* **75**, 635–644
17. De Marchi, E., Orioli, E., Pegoraro, A., Sangaletti, S., Portararo, P., Curti, A., *et al.* (2019) The P2X7 receptor modulates immune cells infiltration, ectonucleotidases expression and extracellular ATP levels in the tumor microenvironment. *Oncogene* **38**, 3636–3650
18. Kroemer, G., Galluzzi, L., Kepp, O., and Zitvogel, L. (2013) Immunogenic cell death in cancer therapy. *Annu. Rev. Immunol.* **31**, 51–72
19. Zhou, J., Wang, G., Chen, Y., Wang, H., Hua, Y., and Cai, Z. (2019) Immunogenic cell death in cancer therapy: present and emerging inducers. *J. Cell. Mol. Med.* **23**, 4854–4865
20. Antonioli, L., Pacher, P., Vizi, E. S., and Hasko, G. (2013) CD39 and CD73 in immunity and inflammation. *Trends Mol. Med.* **19**, 355–367
21. da Silva, J. L. G., Passos, D. F., Bernardes, V. M., and Leal, D. B. R. (2019) ATP and adenosine: role in the immunopathogenesis of rheumatoid arthritis. *Immunol. Lett.* **214**, 55–64
22. Jarvis, M. F., and Khakh, B. S. (2009) ATP-gated P2X cation-channels. *Neuropharmacology* **56**, 208–215
23. Karmakar, M., Katsnelson, M. A., Dubyak, G. R., and Pearlman, E. (2016) Neutrophil P2X7 receptors mediate NLRP3 inflammasome-dependent IL-1beta secretion in response to ATP. *Nat. Commun.* **7**, 10555
24. Kusner, D. J., and Adams, J. (2000) ATP-induced killing of virulent Mycobacterium tuberculosis within human macrophages requires phospholipase D. *J. Immunol.* **164**, 379–388
25. Fairbairn, I. P., Stober, C. B., Kumararatne, D. S., and Lammas, D. A. (2001) ATP-mediated killing of intracellular mycobacteria by macrophages is a P2X(7)-dependent process inducing bacterial death by phagosome-lysosome fusion. *J. Immunol.* **167**, 3300–3307
26. Trabanelli, S., Ocadlikova, D., Gulinelli, S., Curti, A., Salvestrini, V., Vieira, R. P., *et al.* (2012) Extracellular ATP exerts opposite effects on activated and regulatory CD4+ T cells via purinergic P2 receptor activation. *J. Immunol.* **189**, 1303–1310
27. Morciano, G., Sarti, A. C., Marchi, S., Missiroli, S., Falzoni, S., Raffaghello, L., *et al.* (2017) Use of luciferase probes to measure ATP in living cells and animals. *Nat. Protoc.* **12**, 1542–1562
28. Pellegatti, P., Raffaghello, L., Bianchi, G., Piccardi, F., Pistoia, V., and Di Virgilio, F. (2008) Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. *PLoS One* **3**, e2599
29. Amores-Iniesta, J., Barbera-Cremades, M., Martinez, C. M., Pons, J. A., Revilla-Nuin, B., Martinez-Alarcon, L., *et al.* (2017) Extracellular ATP activates the NLRP3 inflammasome and is an early danger signal of skin allograft rejection. *Cell Rep.* **21**, 3414–3426
30. Di Virgilio, F., Sarti, A. C., Falzoni, S., De Marchi, E., and Adinolfi, E. (2018) Extracellular ATP and P2 purinergic signalling in the tumour microenvironment. *Nat. Rev. Cancer* **18**, 601–618
31. Iwasaki, A., and Medzhitov, R. (2004) Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* **5**, 987–995
32. Duan, T., Du, Y., Xing, C., Wang, H. Y., and Wang, R. F. (2022) Toll-like receptor signaling and its role in cell-mediated immunity. *Front. Immunol.* **13**, 812774
33. Silva-Vilches, C., Ring, S., and Mahnke, K. (2018) ATP and its metabolite adenosine as regulators of dendritic cell activity. *Front. Immunol.* **9**, 2581
34. la Sala, A., Ferrari, D., Corinti, S., Cavani, A., Di Virgilio, F., and Girolomoni, G. (2001) Extracellular ATP induces a distorted maturation of dendritic cells and inhibits their capacity to initiate Th1 responses. *J. Immunol.* **166**, 1611–1617
35. Wilhelm, K., Ganesan, J., Muller, T., Durr, C., Grimm, M., Beilhack, A., *et al.* (2010) Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R. *Nat. Med.* **16**, 1434–1438
36. Surprenant, A., Rassendren, F., Kawashima, E., North, R. A., and Buell, G. (1996) The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). *Science* **272**, 735–738
37. Xu, Y., Zhan, Y., Lew, A. M., Naik, S. H., and Kershaw, M. H. (2007) Differential development of murine dendritic cells by GM-CSF versus Flt3 ligand has implications for inflammation and trafficking. *J. Immunol.* **179**, 7577–7584
38. Felix, N. J., and Allen, P. M. (2007) Specificity of T-cell alloreactivity. *Nat. Rev. Immunol.* **7**, 942–953
39. Di Virgilio, F., Chiozzi, P., Ferrari, D., Falzoni, S., Sanz, J. M., Morelli, A., *et al.* (2001) Nucleotide receptors: an emerging family of regulatory molecules in blood cells. *Blood* **97**, 587–600
40. Moser, M., and Murphy, K. M. (2000) Dendritic cell regulation of TH1-TH2 development. *Nat. Immunol.* **1**, 199–205
41. Skokos, D., and Nussenzweig, M. C. (2007) CD8- DCs induce IL-12-independent Th1 differentiation through Delta 4 Notch-like ligand in response to bacterial LPS. *J. Exp. Med.* **204**, 1525–1531
42. Richler, E., Shigetomi, E., and Khakh, B. S. (2011) Neuronal P2X2 receptors are mobile ATP sensors that explore the plasma membrane when activated. *J. Neurosci.* **31**, 16716–16730
43. Muller, T., Robaye, B., Vieira, R. P., Ferrari, D., Grimm, M., Jakob, T., *et al.* (2010) The purinergic receptor P2Y2 receptor mediates chemotaxis of dendritic cells and eosinophils in allergic lung inflammation. *Allergy* **65**, 1545–1553
44. Wilkin, F., Duhant, X., Bruyns, C., Suarez-Huerta, N., Boeynaems, J. M., and Robaye, B. (2001) The P2Y11 receptor mediates the ATP-induced maturation of human monocyte-derived dendritic cells. *J. Immunol.* **166**, 7172–7177
45. Dreisig, K., and Kornum, B. R. (2016) A critical look at the function of the P2Y11 receptor. *Purinergic Signal.* **12**, 427–437

46. Ohkubo, S., Kimura, J., and Matsuoka, I. (2000) Ecto-alkaline phosphatase in NG108-15 cells : a key enzyme mediating P1 antagonist-sensitive ATP response. *Br. J. Pharmacol.* **131**, 1667–1672
47. Panther, E., Corinti, S., Idzko, M., Herouy, Y., Napp, M., la Sala, A., *et al.* (2003) Adenosine affects expression of membrane molecules, cytokine and chemokine release, and the T-cell stimulatory capacity of human dendritic cells. *Blood* **101**, 3985–3990
48. Ohta, A. (2016) A metabolic immune checkpoint: adenosine in tumor microenvironment. *Front. Immunol.* **7**, 109
49. Boison, D., and Yegutkin, G. G. (2019) Adenosine metabolism: emerging concepts for cancer therapy. *Cancer Cell* **36**, 582–596
50. Corriden, R., Chen, Y., Inoue, Y., Beldi, G., Robson, S. C., Insel, P. A., *et al.* (2008) Ecto-nucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1/CD39) regulates neutrophil chemotaxis by hydrolyzing released ATP to adenosine. *J. Biol. Chem.* **283**, 28480–28486
51. Chen, Y., Corriden, R., Inoue, Y., Yip, L., Hashiguchi, N., Zinkernagel, A., *et al.* (2006) ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science* **314**, 1792–1795
52. Krysko, D. V., Garg, A. D., Kaczmarek, A., Krysko, O., Agostinis, P., and Vandenabeele, P. (2012) Immunogenic cell death and DAMPs in cancer therapy. *Nat. Rev. Cancer* **12**, 860–875
53. Shankaran, V., Ikeda, H., Bruce, A. T., White, J. M., Swanson, P. E., Old, L. J., *et al.* (2001) IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* **410**, 1107–1111
54. Sakaki, H., Fujiwaki, T., Tsukimoto, M., Kawano, A., Harada, H., and Kojima, S. (2013) P2X4 receptor regulates P2X7 receptor-dependent IL-1beta and IL-18 release in mouse bone marrow-derived dendritic cells. *Biochem. Biophys. Res. Commun.* **432**, 406–411
55. Bandola-Simon, J., and Roche, P. A. (2019) Dysfunction of antigen processing and presentation by dendritic cells in cancer. *Mol. Immunol.* **113**, 31–37
56. Vermaelen, K. (2019) Vaccine strategies to improve anti-cancer cellular immune responses. *Front. Immunol.* **10**, 8
57. Masaki, K., Hiraki, Y., Onishi, H., Satoh, Y., Roche, P. A., Tanaka, S., *et al.* (2020) Ligation of MHC class II induces PKC-dependent clathrin-mediated endocytosis of MHC class II. *Cells* **9**, 1810
58. Mach, N., Gillessen, S., Wilson, S. B., Sheehan, C., Mihm, M., and Dranoff, G. (2000) Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Res.* **60**, 3239–3246