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ORIGINAL ARTICLE

EXPRESSION AND FUNCTION OF THE P2Y14 RECEPTOR IN MURINE PERITONEAL MACROPHAGES

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Abstract The P2Y14 receptor is activated by UDP-glucose (UDPG), which is a well-known glycosyl donor that participates in the biosynthesis of carbohydrates, and is widely expressed in immune cells. During inflammation and mechanical stress, damaged cells release nucleotides, including ATP and UDPG, as danger signals that act as P2Y receptor agonists. These nucleotide-induced signals participate in the regulation of immune responses. In this study, to investigate P2Y14 expression further, we performed flow cytometric analysis using an anti-P2Y14 monoclonal antibody. The results indicated that P2Y14 is expressed in murine immune cells, including T cells, B cells, monocytes, granulocytes, and CD11b^{high} macrophages. Interestingly, the expression levels of P2Y14 differed between immature and mature monocytes, and in CD11b^{high} macrophages, P2Y14 was gradually downregulated as peritonitis was terminated. The expression of CD11b is reduced by the efferocytosis of apoptotic neutrophils during peritonitis, and the induced CD11b^{low} macrophages, which emerge in the resolution of peritonitis, play an important role in the termination of inflammation. Consistent with this observation, we revealed that administration of UDPG to mice with induced peritonitis increased the number of CD11b^{low} macrophages. As P2Y14 is expressed in CD11b^{high} macrophages but not in CD11b^{low} macrophages, UDPG may participate in the conversion to the CD11b^{low} phenotype. These data suggest a novel regulatory pathway of the inflammatory response via P2Y14 expressed on macrophages.

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Key words: P2Y receptor; UDP-glucose; macrophage; inflammation; immune suppression.

原　著

マウス腹腔マクロファージにおける P2Y14 受容体の発現と機能

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抄録　P2Y14 のリガンドである UDPG は炎症時に細胞内から放出され，免疫反応を制御することが知られている．本研究では，マウス免疫細胞における P2Y14 発現を詳細に解析する為，モノクローナル抗体を用いたフローサイトメトリーを行った．その結果，P2Y14はT細胞，B 細胞，単球，顆粒球，F4/80$^+$ CD11b^{high}マクロファージに発現し，腹膜炎が収束するにつれてマクロファージ上の P2Y14 発現は低下した．炎症収束期において，腹腔マクロファージは炎症促進的に働く F4/80$^+$ CD11b^{high}から，炎症抑制的に働く F4/80$^+$ CD11b^{low}へと変化することが知られている．腹膜炎モデルマウスへの UDPG 腹腔内投与により，F4/80$^+$ CD11b^{low}マクロファージの増加が認められたことからも，UDPG がマクロファージに発現する P2Y14 を介して炎症の収束に関与することが示唆される．

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キーワード：P2Y 受容体；UDP-glucose；マクロファージ；炎症；免疫抑制．

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Introduction

P2Y receptors are 7-transmembrane G protein-coupled receptors[1,2]. To date, 8 P2Y subclasses have been identified (P2Y 1, 2, 4, 6, 11, 12, 13, and 14), and these receptors are activated by extracellular nucleotides, such as ATP, ADP, UTP, UDP, and UDP- sugars[3]. UDP-glucose (UDPG) functions as a glycosyl donor in the biosynthesis of carbohydrates and as an agonist for P2Y14[4]. During inflammation, damaged cells release extracellular signaling nucleotides, such as ATP, UTP, and UDPG, as danger signals[5,6], and this is a key mechanism for immune responses. However, numerous inflammatory-related molecules can damage host tissue[7]; therefore, negative regulation of immune responses is required to terminate acute inflammation.

P2Y14 is expressed in various cells and tissues, including epithelial cells[8], T cells[9], plasmacytoid dendritic cells[10], hematopoietic stem cells[11], and the female reproductive tract[12], and it regulates immune responses by stimulating the secretion of cytokines and controlling cell activation[13]. Furthermore, a previous study using microarray analysis showed that P2Y14 mRNA was highly expressed in peritoneal macrophages and the mRNA level was upregulated by LPS[11]. However, the function of P2Y14 on macrophages remains unclear.

Macrophages, which are divided into M1 and M2 subsets, play an important role in host defense and induce different immune responses[14,15]. LPS activates M1 macrophages, which then produce TNF, IL-6, IL-12, and iNOS. By contrast, M2 macrophages participate in antiparasitic activity, tissue remodeling, angiogenesis, and tumor progression[16]. Moreover, a new subset of macrophages expressing a low level of CD11b has been identified in late stages of murine peritonitis[17,18]. $CD11b^{high}$ macrophages switch to $CD11b^{low}$ macrophages due to the uptake of apoptotic neutrophils. The characteristics of $CD11b^{low}$ macrophages are distinct from those of M1/M2 macrophages; $CD11b^{low}$ macrophages produce low levels of inflammatory cytokines in response to LPS but produce a higher level of TGF-β than $CD11b^{high}$ macrophages[17]. Thus, $CD11b^{low}$ macrophages are characterized as suppressors of acute inflammation.

In this study, we examined the expression of P2Y14 in peritoneal macrophages and other immune cells by using an anti-P2Y14 monoclonal antibody (MoAb). Of interest, the expression levels of P2Y14 changed during monocyte maturation, which raises the possibility that monocyte differentiation is regulated by P2Y14. P2Y14 expression was also detected on macrophages, and we report the effect of UDPG on peritoneal macrophages and propose a new function of P2Y14 in macrophages.

Materials and Methods

Mice

Female C57BL/6 mice (7 weeks old) were purchased from CLEA Japan. Mice were maintained under pathogen-free conditions in laminar flow isolation hoods in a barrier facility. The experiment was approved by the Animal Research Committee of Hirosaki University and performed in accordance with the Guidelines for Animal Experimentation, Hirosaki University, Japan.

Reagents

Anti-mouse FITC-conjugated CD3e, CD45R/B220, CD11b, and Ly-6G and Ly-6C (Gr-1); biotinylated anti-Gr-1; streptavidin-phycoerythrin (PE); and streptavidin-PE-Cy5 were purchased from BD Biosciences. UDPG and zymosan A were purchased from Sigma-Aldrich. Lipofectamine 2000 was obtained from

Invitrogen. PE-anti-F4/80 was purchased from Biolegend. FcR Blocking Reagent and Anti-Biotin MicroBeads were obtained from Miltenyi Biotec. Rat anti-mouse FcγRII/III (24G2) was purified from hybridoma culture supernatant in our laboratory. Anti-mouse P2Y14 MoAb was produced by Sekisui Chemical Co., Ltd., and biotin labeling to the antibody was performed in our laboratory.

Transfection

The human embryonic kidney cell line HEK293 was transfected transiently with the pIRES-EGFP mammalian expression vector (Clontech) containing full-length murine P2Y14 cDNA using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions and grown in a 25 cm^2 tissue culture flask for 2 days. Then, the transfectants were harvested, stained with the produced biotin-labeled anti-P2Y14 MoAb followed by streptavidin-PE, and analyzed by flow cytometry.

Zymosan-induced murine peritonitis

Murine peritonitis was induced by intraperitoneal (i.p.) injection of 1 mg zymosan A in 1 ml sterile PBS. At different time points, peritoneal exudates were collected into 4 ml PBS. In some experiments, 500 μl UDPG (100 μM) was injected i.p. 48 h after the injection of zymosan, and the peritoneal cells were recovered 66 h after peritonitis initiation.

Depletion of Gr-1$^+$ cells

Because peritoneal cells contain neutrophils that express Gr-1 and CD11b, the Gr-1$^+$-depleted cell fraction was isolated from mice with induced peritonitis and stained with the anti-P2Y14 antibody. Firstly, cells were magnetically labeled with FcR Blocking Reagent, biotin-conjugated anti-Gr-1, and Anti-Biotin MicroBeads. Then, the Gr-1- labeled cells were placed in the magnetic field and isolated into Gr-1$^+$ and Gr-1$^-$ cell fractions by using the EasySep magnetic sorting system (Stem Cell Technologies) in accordance with the manufacturer's instructions. The isolated Gr-1$^-$ cells were triple-stained with FITC-conjugated anti-CD11b, PE-conjugated anti-F4/80, and biotinylated anti-P2Y14 antibodies, followed by staining with streptavidin-PE-Cy5.

Flow cytometry

Murine peripheral blood cells, spleen cells, and bone marrow cells were also double-stained with FITC-conjugated anti-CD3e, anti-CD45R/B220, anti-CD11b, anti-Gr-1, and biotinylated-anti-P2Y14, followed by staining with streptavidin-PE. Peritoneal macrophages were triple-stained with FITC-conjugated anti-CD11b, PE-conjugated anti-F4/80, and biotin-conjugated anti-Gr-1, followed by staining with streptavidin-PE-Cy5. P2Y14 staining was performed using Gr-1$^+$ -depleted peritoneal cells as described above. Nonspecific reaction by Fc receptors was blocked by the addition of 24G2 MoAb. Stained cells after washing were examined by flow cytometric analysis on an FC500 instrument (Beckman Coulter).

Results

Expression of P2Y14 in murine immune cells

Previous studies have demonstrated that P2Y14 mRNA was highly expressed in macrophages and other immune cells[9, 19]. For further investigation of P2Y14 expression in murine immune cells, we performed flow cytometric analysis using anti-P2Y14 MoAb. First, the specificity of the produced anti-P2Y14 MoAb was confirmed by the staining of mouse P2Y14-expressing HEK293 transfectants (Figure 1). The MoAb exhibited strong reactivity with P2Y14 expressed on HEK293 cells in the GFP-positive gate. Conversely, no reactivity was

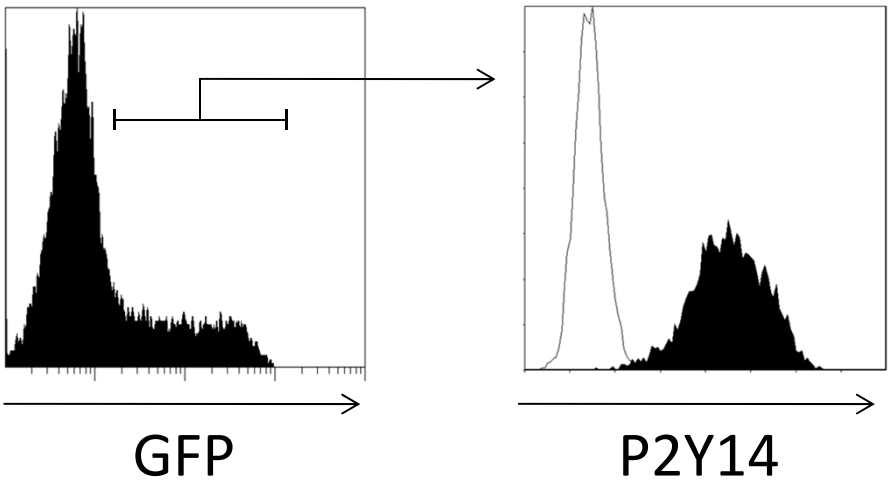

Figure 1 Reactivity of anti-P2Y14 MoAb determined by flow cytometry.
HEK293 cells were transfected transiently with a pIRES-EGFP mammalian expression vector containing full-length murine P2Y14 cDNA by lipofection. Then, the transfectants were used to confirm the specificity of the produced biotin-labeled anti-P2Y14 MoAb by using streptavidin-PE staining and flow cytometry.

observed in untransfected HEK293 cells (data not shown).

Peripheral blood, spleen, and bone marrow cells were stained with appropriate antibodies (anti-CD3e, anti-CD45R/B220, anti-CD11b, and anti-Gr-1), and then each sample was double-stained with anti-P2Y14 MoAb. The results in peripheral blood cells indicated that P2Y14 was highly expressed in B cells and moderately expressed in monocytes, but T cells expressed low levels of P2Y14; no expression was detected in granulocytes (Figure 2, upper panels). Furthermore, in spleen and bone marrow cells, the levels of P2Y14 in T cells and B cells were comparable to those in peripheral T cells and B cells; however, inconsistent P2Y14 expression was observed in monocytes and granulocytes (Figure 2, middle and lower panels). Interestingly, P2Y14 expression on peripheral monocytes was higher than that on immature monocytes in bone marrow. By contrast, P2Y14 expression was detected in spleen- and bone marrow-derived granulocytes but not in peripheral granulocytes. These data suggest that P2Y14 is constitutively expressed in B cells; however, the expression levels of P2Y14 in monocytes and granulocytes change during differentiation.

Expression of P2Y14 and CD11b in peritoneal macrophages

Our flow cytometric analysis indicated that the expression of P2Y14 was upregulated in mature monocytes; therefore, we next examined whether P2Y14 was expressed on peritoneal macrophages. The peritoneal macrophages were recovered from mice with induced peritonitis, and then the cells were depleted of Gr-1$^+$ cells and triple-stained with anti-CD11b, anti-F4/80, and anti-P2Y14. At 66 h after peritonitis initiation by zymosan, macrophages (Figure 3A, middle panel; F4/80$^+$ Gr-1$^-$ population) were divided into 2 populations according to the expression level of CD11b: CD11b^{high} and CD11b^{low} macrophages (Figure 3A, right panel). The CD11b^{low} macrophages, a new macrophage phenotype that emerges during the resolution of inflammation, are reported to participate in the termination of inflammatory responses[17]. The number of CD11b^{low} macrophages gradually

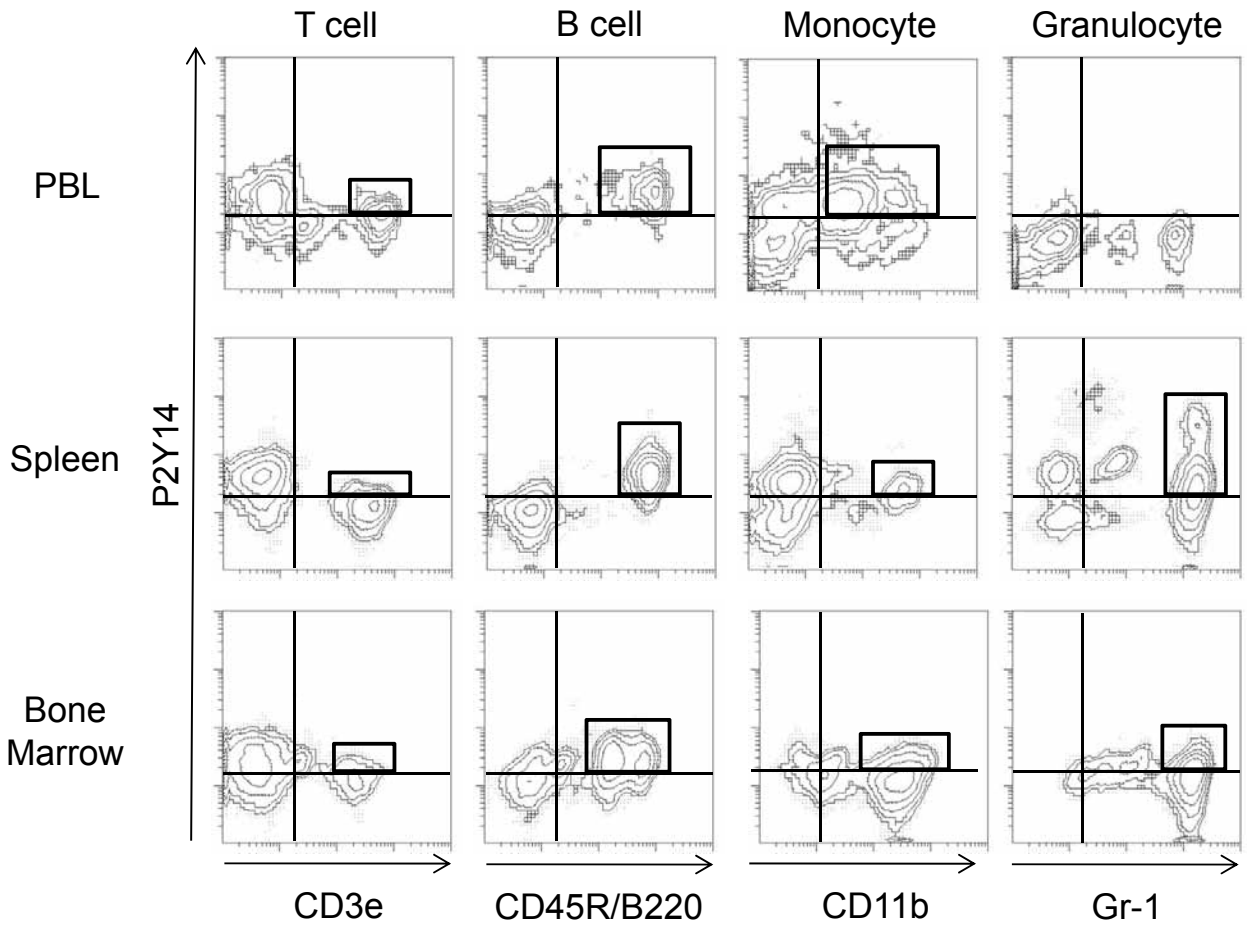

Figure 2 Expression of P2Y14 in peripheral blood, spleen, and bone marrow cells.
Flow cytometric analysis was performed using anti-P2Y14 MoAb. Murine immune cells were stained with FITC-conjugated antibodies against CD3e (T cells), CD45R/B220 (B cells), CD11b (monocytes), and Gr-1 (granulocytes), and then each sample was double-stained with biotinylated anti-P2Y14 MoAb followed by streptavidin-PE. P2Y14 expression was detected in many of the immune cell types, particularly in B cells and monocytes. These cells were collected from peripheral blood (upper panels), spleen (middle panels), and bone marrow (lower panels).

increased from 24 h to 66 h after peritonitis initiation[18]. Time course changes of P2Y14 expression in peritoneal macrophages were examined at 24, 48, and 66 h after zymosan injection. P2Y14 was moderately expressed in $CD11b^{high}$ macrophages at 24 h (21.2 %) and 48 h (20.4 %) but low level of P2Y14 was observed at 66 h (8.0 %) (Figure 3B). Of note, the expression level gradually decreased from 24 to 66 h as $CD11b^{low}$ macrophages increased in number, suggesting that P2Y14 may be related to the conversion of macrophage phenotype from $CD11b^{high}$ to $CD11b^{low}$.

Effect of UDPG on peritoneal macrophage differentiation

P2Y14 was detected on $CD11b^{high}$ macrophages after peritonitis initiation; therefore, we analyzed the effect of UDPG on peritoneal $CD11b^{high}$ macrophages. UDPG, which is a P2Y14 ligand, was injected i.p. at 48 h after the injection of zymosan, and the peritoneal cells were collected at 66 h after peritonitis initiation. The recovered cells were stained for F4/80, Gr-1, and CD11b, and then the percentages of $F4/80^{+}$, $Gr\text{-}1^{-}$, and $CD11b^{high}$ /$CD11b^{low}$ cells

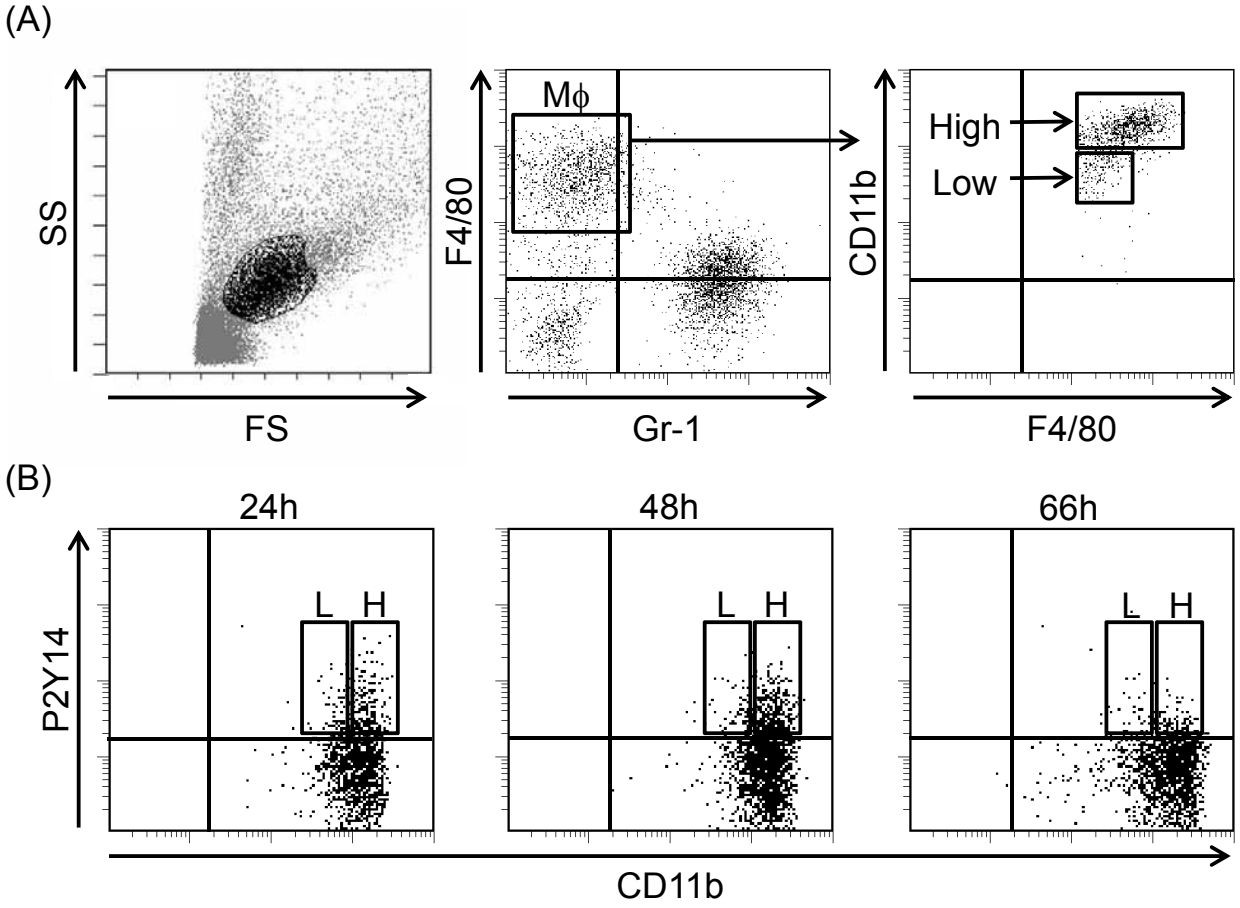

Figure 3 Identification of peritoneal macrophages and expression of P2Y14 on $CD11b^{low}$ and $CD11b^{high}$ macrophages.
Peritoneal macrophages were induced by zymosan injection as described in the Materials and Methods. (A) Flow cytometric analysis of $F4/80^{+}$ $Gr\text{-}1^{-}$ macrophages (middle panel) in peritoneal cells from mice with induced peritonitis at 66 h after peritonitis initiation revealed 2 populations with differential expression levels of CD11b: $CD11b^{high}$ and $CD11b^{low}$ (right panel). (B) Expression of P2Y14 and CD11b in Gr-1-depleted peritoneal cells was analyzed in gated $F4/80^{+}$ cells at the indicated time points after zymosan injection. The gates for $CD11b^{high}$ and $CD11b^{low}$ are indicated.

were calculated from the total number of macrophages. The data indicated that UDPG-injected mice (Figure 4, lower panel, Nos.5 and 6) exhibited a larger $CD11b^{low}$ macrophage population than the PBS-injected control mice, although the difference did not reach statistical significance (Figure 4, upper panel, Nos. 1-3). Although these effects were not observed in mouse No. 4, UDPG appears capable of inducting phenotype conversion in peritoneal macrophages, suggesting a potential role of UDPG in the regulation of inflammatory responses.

Discussion

P2Y14 is activated by UDPG, which is an extracellular signaling molecule released by damaged cells[5]. In this study, we investigated the expression of P2Y14 on murine immune cells and the function of P2Y14 in peritoneal macrophages. Our analysis of P2Y14 expression by using a P2Y14 MoAb revealed that low levels of P2Y14 were expressed in T cells and high or moderate levels were expressed in B

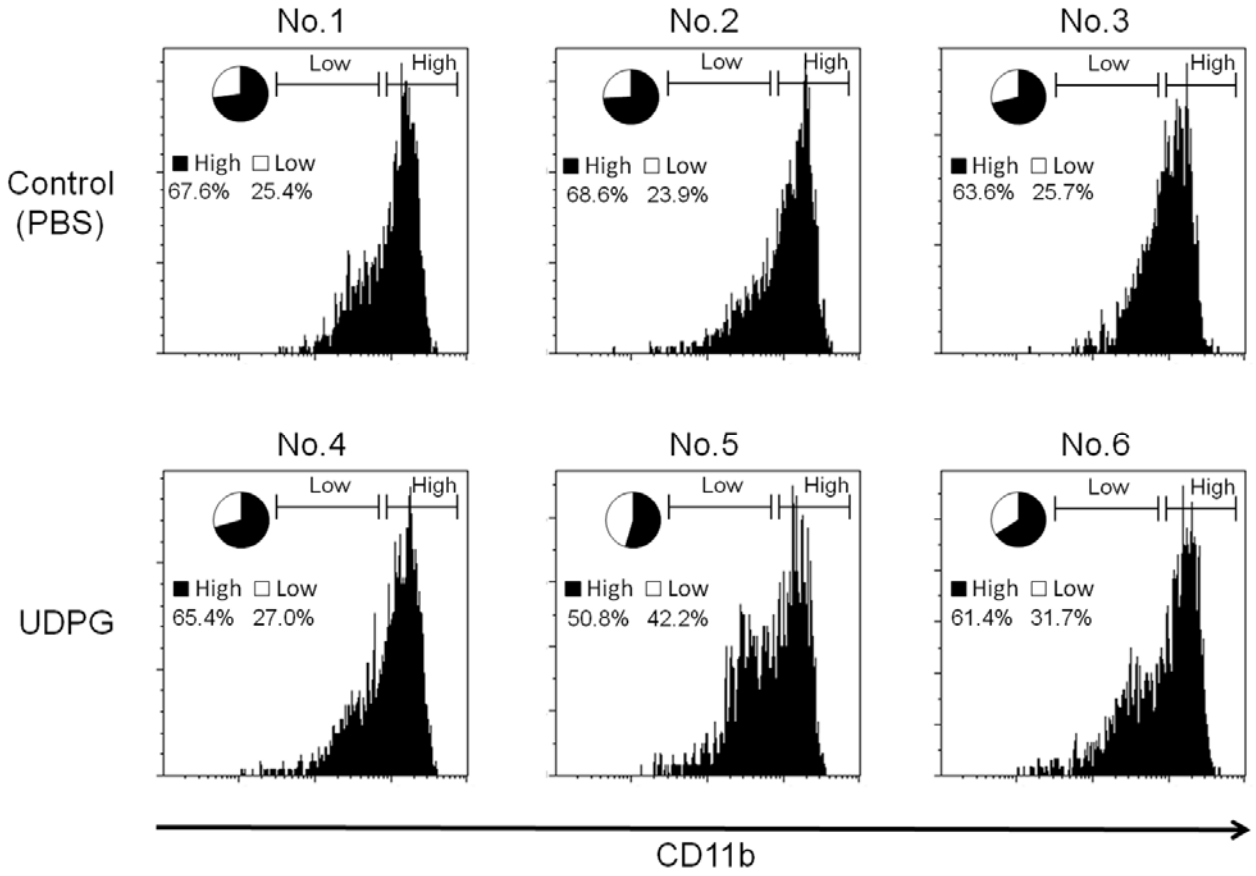

Figure 4 Effect of UDPG on macrophage subsets after peritonitis induction.
Mice with induced peritonitis were injected i.p. with UDPG or PBS at 48 h after peritonitis initiation. At 66 h after zymosan injection, peritoneal cells were harvested, and the expression levels of CD11b and distribution of $CD11b^{high}$ and $CD11b^{low}$ macrophages were analyzed by flow cytometry. The $F4/80^{+}$ $Gr\text{-}1^{-}$ population was gated as described for Figure 3A. The size of the $CD11b^{low}$ population was increased by UDPG injection in mice No. 5 and No. 6. The data are representative of the results for individual PBS- or UDPG-injected mice.

cells and peripheral blood, spleen, and bone marrow monocytes; however, P2Y14 was only found on spleen- and bone marrow-derived granulocytes but not in those from peripheral blood. By contrast, higher P2Y14 expression has been detected in murine spleen-derived T cells than in spleen-derived B cells by RT-PCR analysis, and P2Y14 on the T cells was functional[9]. Our data also revealed that P2Y14 was not expressed in the thymus (data not shown). These results suggest that although P2Y14 expression is low, signaling from P2Y14 is induced in T cells. Interestingly, P2Y14 was expressed at higher levels in peripheral monocytes than in monocytes derived from bone marrow, which indicated that P2Y14 might be involved in the regulation of monocyte differentiation.

Zymosan-induced peritoneal macrophages, which were regarded as peripheral monocyte-differentiated cells, also expressed P2Y14; however, the expression was observed in $CD11b^{high}$ macrophages but not in $CD11b^{low}$ macrophages. Recently, $CD11b^{low}$ macrophages were identified and characterized[17]. $CD11b^{low}$ macrophages are reported to contribute to the resolution of acute inflammation by secreting low levels of inflammatory cytokines

and producing high levels of TGF-β. During peritonitis, CD11b^{high} macrophages are converted to CD11b^{low} macrophages via the engulfment of apoptotic neutrophils, and CD11b^{low} macrophages appear during the resolution of peritonitis. Our results indicated that the expression of P2Y14 was detected only in CD11b^{high} macrophages; in addition, the expression level decreased as peritonitis was terminated. Previously, P2Y14 in peritoneal macrophages was demonstrated to be upregulated by LPS[19]. Taken together, our results suggest that inflammatory responses are controlled by P2Y14 in CD11b^{high} macrophages.

P2Y receptor agonists, including ATP, ADP, UTP, and UDP, have been demonstrated to increase phagocytic activity and chemotaxis in phagocytes[20-23], but the effect of UDPG on phagocytosis is unknown. P2Y14 expression in macrophages has been demonstrated using microarray analysis[19]; however, the function of P2Y14 in macrophages is unclear. Thus, to assess the effect of UDPG on macrophages, we analyzed the macrophage phenotypes after UDPG injection in mice with induced peritonitis. The number of CD11b^{low} macrophages increased, suggesting that this increase is attributable to P2Y14 expression on CD11b^{high} macrophages. CD11b^{low} macrophages, which emerge in a late stage of peritonitis and are different from M1 or M2 macrophages, are known as "pro-resolving" macrophages[17], and saturated efferocytosis induces the downregulation of CD11b. Inflammatory responses during peritonitis are modulated by pro-resolving lipid mediators, including resolvin E1/D1, dexamethasone, and protectin D1, which regulate neutrophil infiltration and macrophage activation to terminate inflammation[17, 24, 25]. The increase in the number of CD11b^{low} macrophages induced by UDPG injection may be attributable to the downregulation of CD11b signaling in CD11b^{high} macrophages, upregulation of neutrophil efferocytosis in CD11b^{high} macrophages, or enhancement of the production of anti-inflammatory lipid mediators.

In conclusion, the present study demonstrated that P2Y14 was expressed in monocytes, peritoneal macrophages, T cells, B cells, and spleen/bone marrow-derived granulocytes. Moreover, UDPG affected the expression level of CD11b on macrophages during peritonitis, and CD11b expression was gradually downregulated as inflammation was terminated. UDPG is a P2Y14 agonist as well as an inflammatory mediator derived from damaged cells; therefore, our data indicate that P2Y14 participates in the termination of inflammatory responses by regulating macrophage differentiation.

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